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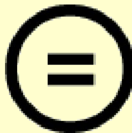
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이학박사 학위논문

# Studies on the stability control mechanism of the MRE11-RAD50-NBS1 complex in response to DNA damage

## DNA 손상에 대한 반응에서 MRE11-RAD50-NBS1 복합체의 안정성 조절 기작에 관한 연구

2023년 02월

서울대학교 대학원
협동과정 유전공학전공
김 현 섭

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지도 교수 이 준 규

이 논문을 이학박사 학위논문으로 제출함

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서울대학교 대학원

협동과정 유전공학전공

김 현 섭

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| 위 원 | _______________ (인) |
| 위 원 | _______________ (인) |

# Studies on the stability control mechanism of the MRE11-RAD50-NBS1 complex in response to DNA damage

A dissertation submitted in partial fulfillment
of the requirements for the degree of

DOCTOR OF PHILOSOPHY

to the faculty of

Interdisciplinary Graduate Program in Genetic Engineering

at

SEOUL NATIONAL UNIVERSITY

by

Hyunsup Kim

Date Approved

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# ABSTRACT

## Studies on the stability control mechanism of the MRE11-RAD50-NBS1 complex in response to DNA damage

Hyunsup Kim
Interdisciplinary Graduate Program in Genetic Engineering
Seoul National University

Proper cellular response to DNA double-strand breaks (DSBs), which are the most cytotoxic DNA lesions, is critical for maintaining the integrity of genome. The MRE11-RAD50-NBS1 (MRN) complex plays essential roles in the DSB response and is a target of various modifications and controls. However, the mechanism by which the MRN complex stability is regulated in the DSB response remains unknown. In this study, I show that RECQL4, whose mutations are associated with Rothmund-Thomson Syndrome (RTS), is required for DNA DSB response, and its helicase activity

are required for stable maintenance of the MRN complex on DSB sites during a DSB response. The MRN complex is prematurely disassembled from DSB sites in a manner dependent upon $SCF^{SKP2}$ dependent lysine 48-linked ubiquitination of NBS1 in RECQL4-defective cells. This early disassembly of the MRN complex can be prevented by expressing a deubiquitinase, USP28, which sufficiently restored homologous recombination repair and ATM activation abilities in RTS and RECQL4-depleted cells. These results suggest that the essential role of RECQL4 in DSB response is the stable maintenance of the MRN complex on DSB sites, and that defects in DSB response in RTS cells can be recovered by controlling the stability of the MRN complex.

To further investigate the role and control of this ubiquitination during the DSB response in cells with intact RECQL4, I screen several deubiquitinases and identify USP2 as a new deubiquitinase that acts at DSB sites to counteract NBS1 ubiquitination. USP2 is recruited to DSB sites in a manner dependent on ATM, a major checkpoint kinase against DSBs, and stably interacts with NBS1 and RECQL4 in immunoprecipitation experiments. Phosphorylation of two critical residues in the N-terminus of USP2 by ATM is required for its recruitment to DSBs and its interaction with RECQL4. While inactivation of USP28 or USP2 alone does not substantially influence the DSB response, inactivation of both deubiquitinases results in premature disassembly of the MRN

complex from DSB sites and defects in ATM activation and homologous recombination repair abilities. These results suggest that deubiquitinases counteracting NBS1 ubiquitination are essential for the stable maintenance of the MRN complex and proper cellular response to DSBs.

**Keyword:** DNA double-strand break, RECQL4, the MRE11-RAD50-NBS1 complex, USP28, USP2, Rothmund-Thomson syndrome

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| | |
|---|---|
| ATM | Ataxia Telangiectasia mutated |
| BS | Bloom syndrome |
| CtIP | CtBP−interacting protein |
| CCLE | cancer cell line encyclopedia |
| DAPI | 4′,6−diamidino−2−phenylindole |
| DNA−PK | DNA−dependent protein kinase |
| DSB | double strand break |
| EDTA | ethylenediaminetetraacetic acid |
| EGFP | enhanced green fluorescent protein |
| FLAG | DYKDDDDK amino acid tag |
| HEK293T | human embryonic kidney 293T |
| HR | homologous recombination |
| IP | immunoprecipitation |
| kDa | kilo Dalton |
| LB | Lamin B1 |
| MRN | MRE11−RAD50−NBS1 |
| NHEJ | non−homologous end joining |
| NCS | neocarzinostatin |

| | |
|---|---|
| WS | Werner syndrome |
| R4 | RECQL4 |
| RPA | replication protein A |
| RTS | Rothmund–Thomson syndrome |
| SDS | Sodium dodecyl sulfate |
| siRNA | small interfering RNA |
| SKP2 | S phase kinase associated protein 2 |
| USP | ubiquitin specific proteases |
| VCP | valosin–containing protein |
| WST–1 | water–soluble tetrazolium 1 |
| WT | wild–type |
| $\gamma$H2AX | phosphorylated H2AX |

# 1. Introduction

## 1.1. Research background

### 1.1.1. DNA double-strand break response in mammalian cells

Proper cellular response to DNA double-strand breaks (DSBs), the most cytotoxic type of DNA damage, is critical for maintaining the integrity of genome to avoid instability that may result in genetic diseases, carcinogenesis, and cell death (Pastink, Eeken, and Lohman 2001; Jensen and Rothenberg 2020). To maintain genome integrity against DSBs, cells have evolved to activate checkpoint signaling for cell cycle control and repair DSBs via two major repair pathways; non-homologous end joining (NHEJ) and homologous recombination (HR) (Chapman, Taylor, and Boulton 2012). NHEJ, an error-prone repair process, occurs throughout the cell cycle, whereas HR is an error-free repair pathway that functions in the S/G2 phase (Mao et al. 2008). In these cellular responses to DSBs, various proteins are recruited to DSB sites to act as sensors, signal transducers, or effectors, which are coordinated by phosphatidylinositol 3-kinases, such as ataxia telangiectasia-mutated (ATM) and DNA-dependent protein kinase (DNA-PK) (Davis, Chen, and Chen 2014; Shiloh 2003).

In the current model of DSB response, the MRE11-RAD50-NBS1 (MRN) complex plays key roles as a sensor and a signal

transducer by rapidly recognizing and locating DSBs (Lamarche, Orazio, and Weitzman 2010). After binding to DSB sites, the MRN complex recruits and activates ATM, a central regulator of the DSB response signaling pathway (Uziel et al. 2003; You et al. 2005). Activated ATM phosphorylates many substrates, including histone H2AX and MDC1 (Liu et al. 2012; Burma et al. 2001), which in turn recruit more MRN complexes and ATM to DSB sites and consequently amplify the MRN-ATM signaling (Chapman and Jackson 2008; Lou et al. 2006). Other checkpoint mediators, such as 53BP1 and BRCA1, key factors for DSB repair pathway choice, are also recruited to MDC1 and contribute to ATM activation and further repair events (Daley and Sung 2014).

### 1.1.2. DNA end resection in homologous recombination repair

The MRN complex also acts as an effector in the HR repair process by participating in end resection (Cejka 2015). This activity is promoted by the binding of CtBP-interacting protein (CtIP), a cofactor of the MRE11 endonuclease (Sartori et al. 2007). After binding of CtIP to the MRN complex, short-range resection of the DSB ends is initiated (Anand et al. 2016). Subsequently, an extensive 3′ -end protruding single-stranded DNA is generated by the action of EXOI and DNA2 (Nimonkar et al. 2011). This 3’ - overhang is coated by single-strand DNA binding protein, RPA, followed by replacement of RAD51 for homology search and DNA

strand invasion for HR (Liu et al. 2010).

### 1.1.3. Modification of the MRN complex in DSB response

As the MRN complex plays key roles in the DSB response from damage sensing to repair, it is one of the major control targets of various modifications such as phosphorylation and ubiquitination. ATM phosphorylates all three components of the MRN complex, which is critical for checkpoint activation and DSB repair (Lavin et al. 2015). Phosphorylation of MRE11 by polo-like kinase 1 inhibits the recruitment of the MRN complex to DSBs (Li et al. 2017). CDK phosphorylation of NBS1 influences the choice of repair pathway for dysfunctional telomeres (Rai et al. 2017). The ubiquitination-dependent control of the MRN complex has also been reported in many studies. Lysine (K) 6-linked ubiquitination of NBS1 proteins by the ring finger protein RNF8 promotes optimal binding to DSBs (Lu et al. 2012). K63-linked ubiquitination of NBS1 by $SCF^{SKP2}$ E3 ubiquitin ligase and Pellino1 facilitates ATM activation and binding of the MRN complex to DSB sites, respectively (Wu et al. 2012; Ha et al. 2019).

### 1.1.4. RECQL4 and other RECQ family helicases in DSB response

RECQL4, identified in a human genome search for RECQ helicases (Kitao et al. 1998), and found to be associated with the type II Rothmund-Thomson syndrome (RTS) (Kitao et al. 1999), is one of

five RECQ family helicases, which are well conserved from prokaryote to eukaryote. Humans have five RECQ helicases: RECQL1, WRN, BLM, RECQL4, and RECQL5 (Croteau et al. 2014). These helicases play various roles in maintaining genome integrity and their mutations are associated with genetic diseases. Defects in BLM, WRN, RECQL4 are associated with Bloom (BS), Werner (WS), and RTS, all of which present genomic instability and cancer disposition (Bernstein, Gangloff, and Rothstein 2010). Patients with WS typically have an aging appearance and early onset of age-related disorders after adolescence (Yokote et al. 2017). RTS presents pleotropic phenotypes, including poikiloderma and cancer predisposition (Larizza, Roversi, and Volpi 2010), and BS commonly features short stature, sun-sensitive skin, growth deficiency and immune abnormalities (Cunniff, Bassetti, and Ellis 2017).

WRN, BLM, and RECQL4 are all involved in the DNA DSB repair process in various ways and their helicase activity is crucial for several processes. WRN stimulates NHEJ by its helicase and exonuclease activities (Shamanna et al. 2016). BLM plays both pro- and anti-recombination roles by stimulating the end-resection activity of DNA2 and by the displacement of RAD51 from resected DNA intermediates (Patel et al. 2017).

RECQL4 also has a core helicase domain, but unlike most of RECQ helicases, RECQL4 does not have helicase-and-RNase D C-terminal and RECQ C-terminal domains (Bernstein, Gangloff, and

Rothstein 2010). Despite the lack of resemblance between C−terminal domain of RECQL4 and the RECQ C−terminal domain, C−terminal domain of RECQL4 is critical for its helicase activity (Kaiser, Sauer, and Kisker 2017). RECQL4 has been shown to be recruited very rapidly to DSB sites (Aleksandrov et al. 2018), and its domain of amino acids 363–492, which is adjacent to the helicase domain and shares no homology with the recruitment domains of WRN and BLM, is essential for binding to DSB sites (Singh et al. 2010). The helicase activity is reportedly required for both HR repair (Abe et al. 2011; Lu et al. 2016) and activation of ATM (Park et al. 2019) in response to DNA DSBs. RECQL4 has also been postulated as a participant in 5′ −end resection by MRE11 and CtIP during HR repair (Lu et al. 2016)

## 1.2. Purpose of research

Previous observations of the association between repair factors and RECQL4 suggested that RECQL4 acts as a key element of the DSB repair process. To figure out the mechanism through which it performs essential roles in the DSB response, I examined DSB response in RTS and RECQL4-depleted cells and found that the MRN complex is prematurely disassembled from DSB sites, which leaded to HR repair defects and decreased cell survival in DSB condition. I identified this premature disassembly is caused by $SCF^{SKP2}$-dependent K-48 ubiquitination of NBS1. Further, I found that preventing its early disassembly from DSBs by overexpression of a deubiquitinase, USP28, is sufficient to restore DSB response in RECQL4-defective cells. These results showed that the essential role of RECQL4 in DSB response is the stable maintenance of the MRN complex, and defects in DSB response in RECQL4-defective cells can be recovered by counteracting the ubiquitination of NBS1 through expression of USP28.

Although DSB repair defects in RECQL4-defective cells are recovered by USP28 overexpression, the cellular role of NBS ubiquitination and deubiquitination is still obscure because premature disassembly of the MRN complex during the DSB response was observed only in RECQL4-defective cells, and depletion of USP28 did not markedly influence the DSB response,

except in a few cell lines such as H460 (Zhang et al. 2006; Knobel et al. 2014). These observations, which appear to be contradictory, may imply the presence of redundant deubiquitinases acting on the MRN complex during DSB response.

So, I explored other potential deubiquitinases, the overexpression of which could restore the stability of the MRN complex in RECQL4−depleted cells. By screening several deubiquitinases that were shown to be associated with DNA damage responses, cell cycle, or carcinogenesis, I identified USP2 as a new deubiquitinase that acts at DSB sites to counteract NBS1 ubiquitination by $SCF^{SKP2}$ E3 ligase. Overexpression of USP2 restored not only the stability of the MRN complex at DSB sites but also HR repair and ATM activation abilities in RECQL4−depleted cells. Live−cell imaging of cells expressing EGFP−fused USP2 after laser micro−irradiation, mapping of domains responsible for recruitment to DSB sites, and analyses of phospho−deficient and phospho−mimetic mutants of USP2 showed that USP2 is recruited to DSB sites in an ATM−dependent manner, and phosphorylation of two critical residues in the N−terminus of USP2 is essential for its recruitment to DSBs and interaction with RECQL4. By inactivation of USP2 and USP28, I also showed that the action of deubiquitinases counteracting NBS1 ubiquitination is essential for the stable maintenance of the MRN complex on DSB sites and proper cellular response to DSBs.

# 2. Materials and Methods

## 2.1. Cell culture and reagents

U2OS and HEK293T cells were cultured Dulbecco′ s modified Eagle′ s medium (DMEM, Welgene, Korea), supplemented with 10% FBS (Welgene, Korea) and antibiotics (Welgene, Korea). RTS skin primary fibroblasts cells (AG17524 and AG18371) were obtained from Coriell cell repositories (NJ, USA) and cultured in alpha minimum essential medium Eagle (MEM, Welgene, Korea), supplemented with 15% FBS and antibiotics. TRI-DR-U2OS cells (Khurana et al. 2014) were cultured in Dulbecco′ s modified Eagle′ s medium, supplemented with 10% tetracycline-free FBS (Takara) and antibiotics. All the cells were maintained at 37 °C in a humidified incubator containing 5% $CO_2$.

Transfection of plasmids was performed with polyfect (QIAGEN) or Lipofectamine 3000 (Invitrogen). For depletion of proteins, siRNAs were transfected with an electroporator (Invitrogen) and incubated for 48 h. All siRNAs were synthesized by ST Pharm (Daejeon, Korea) or Bioneer (Daejeon, Korea). The sequences of the sense strand of siRNAs used in this study were as follows:

GL, 5′-AACGUACGCGGAAUACUUCGA-3′ (targeting firefly luciferase)

RECQL4, 5′-GACUGAGGACCUGGGCAAA-3′

SKP2, 5′- GAUAGUGUCAUGCUAAAGAAU-3′

CDC34, 5′-GGAAGUGGAAAGAGAGAGCAA-3′

UBC5, 5′-GAGAAUGGACUCAGAAAUA-3′

UBC13, 5′-CCAGAUGAUCCAUUAGCAA-3′

USP28, 5′- CUGCAUUCACCUUAUCAUU-3′

For generating DNA DSBs, cells were treated with NCS (N9162, Sigma-Aldrich) at a concentration of 200 ng/mL for 15 min, unless indicated otherwise. The proteasome inhibitor, MG132, was purchased from Apexbio (A2585). The ATM inhibitor, KU-55933 (SML1109), was from Sigma-Aldrich. The DNA-PK inhibitor, NU7441 (Axon 1463) was from Axon Medchem (Axon2678). The USP2 inhibitor, ML364, was from Axon Medchem (2678). The PARP inhibitor, olaparib, was from Sellekchem. The PARG inhibitor, PDD00017273, was from Sigma-Aldrich.

## 2.2. Plasmids preparation

For the expression of mutant RECQL4 defective in helicase activity, cDNAs encoding RECQL4 with amino acid substitution in Walker A motif (K508G) or Walker B motif (D605A and E606A) were generated by PCR and subcloned into pcDNA3.1(-) plasmid.

For wild-type or various mutant USP2 protein expression, wild-type USP2 cDNA, N-terminal region cDNA (1-160 aa for CD1, 1-258 aa for CD2), and C terminal region (350-605 aa for ND1, 250-605 aa for ND2) were amplified by polymerase chain reaction and

inserted into the pcDNA3.1 (−) plasmids containing various epitope tags. For alanine or glutamic acid substitution of the ATM phosphorylation sites of USP2(S2, S96, T137, S142), a site-directed mutagenesis kit from Enzynomics (EZ004S) was used according to the manufacturer's instructions.

### 2.3. Immunofluorescence staining

For immunostaining of MRE11, NBS1, RAD50, RAD51, and USP28, cells were grown on coverslips, treated with 200 ng/ml NCS for 15 min and incubated in fresh medium for the indicated time. Cells were then pretreated with a cold buffer containing non-ionic detergent (10 mM PIPES pH 7.0, 100 mM NaCl, 300 mM sucrose, 3 mM $MgCl_2$, 0.5% Triton X-100) on ice for 5 min and fixed with 4% paraformaldehyde in PBS for 10 min at 25 ℃. Fixed cells were washed with PBS containing 0.25% Triton X-100 on ice for 5-10 min, and then incubated in a blocking buffer (5% BSA and 0.25% Triton X-100 in PBS) for 30 min at 25 ℃. The indicated proteins were labeled with the respective primary and secondary antibodies in the blocking buffer for 1 h at 25 ℃. The nuclei of cells were stained with 0.1 μg/mL 4′-6′-diamidino-2-phenylindole (DAPI) in PBS for 3 min or in mounting solution (VECTASHIELD, H-1200, Vector, USA) in the dark. Fluorescent images were obtained by fluorescence microscopy (Nikon TE2000-U or Zeiss LSM880), and cells containing 20 or more foci were counted as

foci−positive cells for quantitation.

### 2.4. Laser micro−irradiation and real−time imaging of fluorescent proteins

For laser micro−irradiation, U2OS cells grown in glass−bottomed dishes (SPL, Korea, 101350) were treated with 5 μg/mL Hoechst 33342, 10 min prior to micro−irradiation. An LSM880 laser confocal microscope system with a temperature−controlled $CO_2$ chamber (Zeiss, Germany) was used. Fixed wavelength of laser (405 nm) at a scan speed of 8.19−32.77 μs/pixel with 1−5 iterations, and Plan−Apochromat 63X oil objective lens were used. Defined regions of interest were irradiated with 20−100% laser output. Time−lapse images were captured and fluorescence intensities of irradiated areas relative to those of non−irradiated areas within the nucleus were obtained using the ZEISS ZEN 2.3 SP1 software (Zeiss, Germany).

### 2.5. Immunoprecipitation and immunoblotting

For immunoprecipitation, cells were lysed in a buffer containing 40 mM Tris−HCl, pH 7.5, 100 mM NaCl, 2.5 mM $MgCl_2$, 1 mM dithiothreitol, 5% glycerol, 0.2% NP−40, 20 mM NaF, 0.1 mM sodium orthovanadate, and protease inhibitors. After sonication, benzonase (90 units/mL) was added and the reaction was incubated at 4 °C for 1 h. Cell lysates were cleared by centrifugation at

18,000 × *g* for 10 min and used for immunoprecipitation. To prepare whole cell extracts for immunoblotting, cells were lysed in a buffer containing 40 mM Tris-HCl, pH 7.5, 150 mM NaCl, 1% NP-40, 1 mM ethylenediaminetetraacetic acid (EDTA), 0.25% sodium deoxycholate, 20 mM NaF, 0.1 mM sodium orthovanadate, and protease inhibitors. The lysate was sheared by sonication and the concentrations of proteins were measured by Bradford assay. Approximately 30 μg of protein was subjected to sodium dodecyl sulfate-polyacrylamide gel electrophoresis.

### 2.6. Ubiquitination assay

Ubiquitination assay for NBS1 ubiquitination was performed as described by Choo and Zhang (Choo and Zhang 2009) with slight modification. HEK293T cells were transfected with expression vectors for HA-tagged ubiquitin and indicated proteins and incubated for 24 h. Thereafter, the cells were pretreated with MG132 (40 μM) for 1 h, following NCS treatment (200 ng/mL). Protein extracts were prepared by boiling the cells at 95 °C in cell lysis buffer (20 mM Tris-HCl, pH 8.0, 150 mM NaCl, 2% SDS, 10 mM N-ethylmaleimide and protease inhibitors) for 10 min, and then shearing by sonication. The extracts were diluted with 9-times the volume of dilution buffer (10 mM Tris-HCl, pH 8.0, 150 mM NaCl, 2 mM EDTA, 1% Triton X-100) and incubated at 4 ˚ C for 30 min on the rotation wheel. The cell extracts were cleared by

centrifugation at 18,000 × *g* for 30 min, and the supernatant was used for immunoprecipitation. Beads were washed twice with immunoprecipitation buffer and three times with wash buffer (10 mM Tris-HCl, pH 8.0, 1 M NaCl, 1 mM EDTA, 1% NP-40).

### 2.7. Protein stability assay

U2OS cells were transfected with the indicated siRNAs and incubated for 48 h. Before harvest, the cells were pretreated with 50 μg/mL cycloheximide (01810, Sigma) for 1 h and then treated with NCS (200 ng/mL). The cells were harvested and lysed at the indicated times, and the levels of MRE11 and NBS1 proteins were quantified with a chemiluminescence imaging system (ATTO Ez-CaptureII, CS analyzer) after immunoblotting.

### 2.8. Homologous recombination repair assay

The HR reporter cell line TRI-DR-U2OS carrying doxycycline-inducible I-*Sce*1 was obtained from Dr. Philipp Oberdoerffer (NCI/NIH). Cells were treated with 10 μg/ml doxycycline for 48 h for I-*Sce*1 induction, and GPF-positive cells were analyzed using a flow cytometer (BD, Accuri).

### 2.9. WST-1 assay for cell viability

The assay was carried out as described in the manufacturer instruction (EZ-Cytox, DoGenBio). In brief, AG18371 cells

transfected with RECQL4, wild-type USP28, or C171A mutant USP28 were cultured in 96-well plates and exposed to different concentration of bleomycin (sc-200134a, Santa Cruz) or cisplatin (NB2251, Novus) for 48 h. U2OS cells were transfected with the indicated siRNAs and cultured in 96 well plates for 48 h and exposed to different concentration of bleomycin in the presence or absence of ML364 for 24 h. After incubation, cells were treated with 10% WST-1 reagent for 1 h, and absorbance was measured using Epoch2 Microplate Spectrophotometer (BioTek).

### 2.10. Statistics analysis

Statistical significance between groups was determined by two-tailed Student' s *t*-test using GraphPad Prism5 software. Data are presented as mean ± SD (standard deviation) or SEM (standard error of the means). All statistical tests performed are indicated in the Figure legends.

### 2.11. Antibodies

Anti-RECQL4 antibody was prepared by Abfrontier (Korea) by immunizing rabbits with recombinant N-terminus (amino acid residues 1-241) RECQL4.

Other antibodies used in this study were summarized in table 1.

| Antibody | WB | IP | ICC | Catalog No. |
|---|---|---|---|---|
| FLAG | 1:3000 | – | – | F1804, Sigma |
| GAPDH | 1:5000 | – | – | sc25778, Santa Cruz |
| GFP | 1:500 | 1:100 | – | sc–9996, Santa Cruz |
| HA | 1:2000 | 1:200 | – | AE008, ABclonal |
| K48 | 1:1000 | – | – | ab140601, Abcam |
| Lamin B1 | 1:5000 | – | – | ab16048, Abcam |
| MRE11 | 1:3000 | – | 1:500 | GTX30294, GeneTex |
| NBS1 | 1:1000 | – | 1:100 | A7703, ABclonal |
| pATM (Ser–1981) | 1:1000 | – | 1:200 | #4526, Cell Signaling |
| RAD50 | 1:200 | – | 1:50 | sc–74460, Santa Cruz |
| RAD51 | – | – | 1:100 | GTX100469, GeneTex |
| RECQL4 | 1:1000 | – | – | Prepared in Abfrontier |
| SKP2 | 1:200 | – | – | sc–7164, Santa Cruz |
| USP2 | 1:1000 | – | – | A10399, ABclonal |
| USP28 | 1:1000 | – | 1:100 | A9292, ABclonal |
| γH2AX | 1:10000 | – | 1:500 | A300–081A, Bethyl |
| γH2AX | – | – | 1:100 | 05–636, EMD Millipore |
| Alexa Fluor 488 anti–mouse IgG | – | – | 1:1000 | A11001, ThermoFisher |
| Alexa Fluor 488 anti–rabbit IgG | – | – | 1:1000 | A11008, ThermoFisher |
| Alexa Fluor 594 anti–mouse IgG | – | – | 1:1000 | A11005, ThermoFisher |
| Alexa Fluor 594 anti–rabbit IgG | – | – | 1:1000 | A11012, ThermoFisher |

Table 1. Antibodies used in this study.

# 3. Results

## 3.1. Stable maintenance of the MRN complex is sufficient to restore the DNA DSB response in cells lacking RECQL4 helicase activity.

### 3.1.1. The MRN complex is prematurely disassembled from DSB sites in RTS and RECQL4-depleted cells.

Because both HR repair and ATM activation were reported to be impaired in RECQL4-defective cells (Abe et al. 2011; Lu et al. 2016; Park et al. 2019), I reasoned that factors commonly involved in both these processes may be affected by the defects in RECQL4. By analyzing proteins at DSB sites using immunostaining after treating cells with neocarzinostatin (NCS), a DSB-inducing reagent, I found that MRN foci formed in RECQL4-depleted U2OS cells, as in mock-depleted cells, but they started to rapidly disappear during the post-incubation period (Figure 1, A and B). Foci of all three MRN component proteins were reduced, but NBS1 foci decreased most rapidly, and the proportion of NBS1 foci-positive cells was less than 20% after incubation for 40 min (Figure 1B). In contrast, total cellular levels of the MRN component proteins did not change significantly (Figure 1C), suggesting that depletion of RECQL4 may affect the MRN complex assembled on DSB sites only, and results in premature disassembly of the MRN complex from these sites.

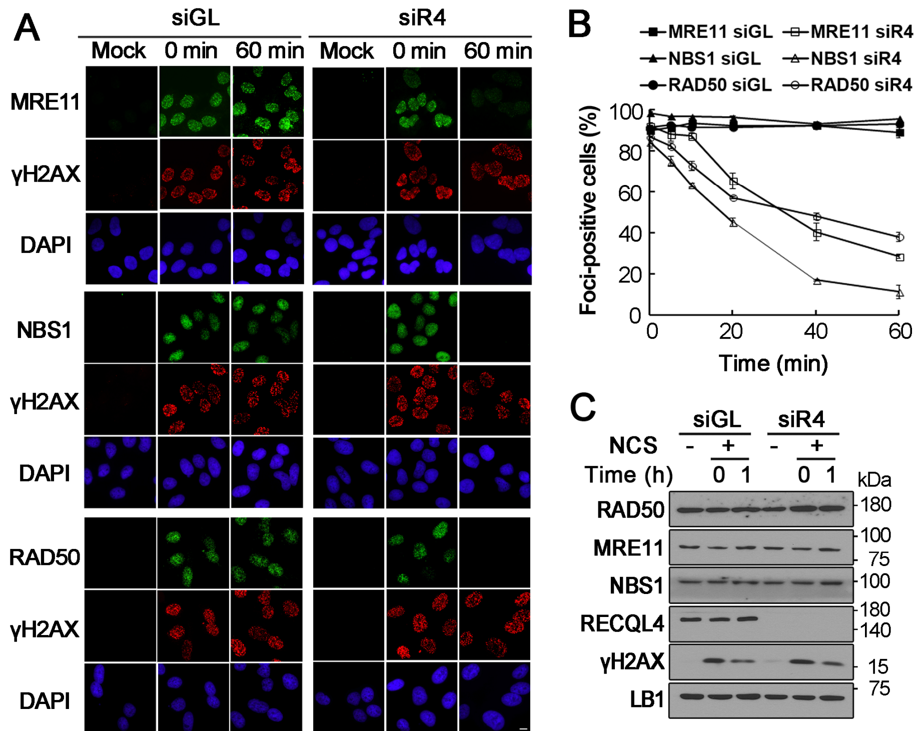



Figure 1. The MRN complex is prematurely disassembled from DSB sites in RECQL4-depleted U2OS cells.

(A and B) Immunostaining of the MRN component proteins. U2OS cells transfected with RECQL4 or GL2 (siGL) siRNAs were treated with NCS for 15 min and incubated in a fresh medium for the indicated times. Representative images of MRE11, NBS1, and RAD50 immunostaining (A) and graphs for the percentages of foci-positive cells (B) are shown. Data in graphs are means ± SD; n = 3. Scale bar: 10 μm.

(C) Western blots of U2OS cells prepared as in (A) and (B).

There are several reports that the binding of MRE11 protein to DSB sites depends on complex formation with RAD50 and NBS1 (Kissling et al. 2022; Desai-Mehta, Cerosaletti, and Concannon 2001; Kim et al. 2021), suggesting that MRE11 is a good marker for tracing the MRN complex at DSB sites. As shown in Figure 2, early dissociation of the MRN complex from DSB sites was also observed in RECQL4-depleted cells subjected to laser micro-irradiation. The level of EGFP-fused MRE11 bound to micro-irradiation sites reached a peak at around 100 s after micro-irradiation, and then decreased in RECQL4-depleted cells, whereas this level did not change significantly in mock-depleted cells (Figure 2). Premature disassembly of the MRN complex from DSB sites, which was prevented by expression of RECQL4, was also observed in NCS-treated fibroblast cells collected from RTS patients (Figure 3).

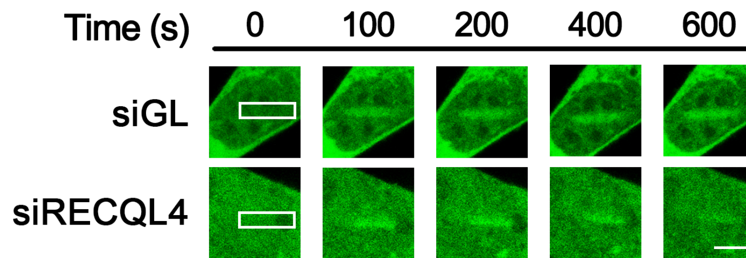



Figure 2. EGFP−fused MRE11 is prematurely dissociated from DSB sites in RECQL4−depleted cells.

EGFP−MRE11 binding to micro−irradiation sites in RECQL4−(siRECQL4) or mock−depleted (siGL) U2OS cells. Cells were transfected with indicated siRNA and incubated for 24 h, followed by EGFP−MRE11 transfection and incubation for additional 24 h. Scale bar: 10 μm.

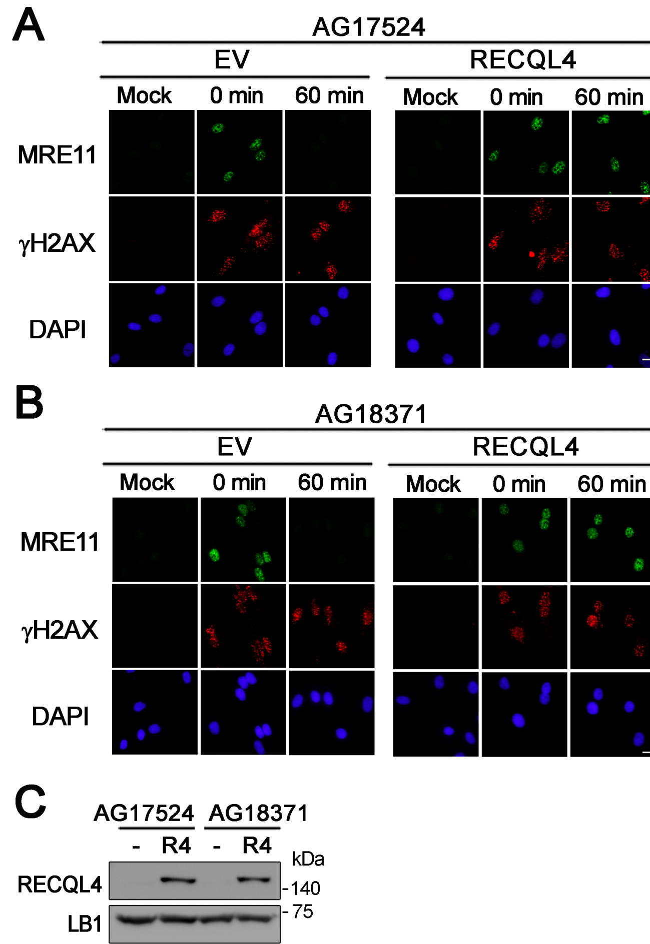



Figure 3. Instability of the MRN complex in RTS cells is recovered by expression of RECQL4.

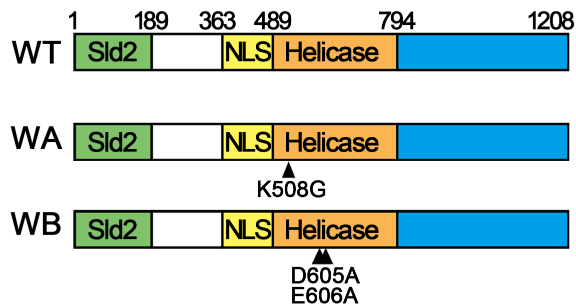
(A and B) Immunostaining of MRE11 in RTS cells (AG17524 or AG18371) transfected with empty (EV) or RECQL4 expression vectors. Cells were treated with NCS for 15 min and incubated in a fresh medium for the indicated times. Scale bar: 10 μm.

(C) Western blots of RTS cells prepared as in Figure 3A and B

### 3.1.2. The helicase activity of RECQL4 is required for stable maintenance of the MRN complex on DSB sites during DSB response.

To test whether the helicase activity of RECQL4 contributes to the stable maintenance of the MRN complex during DSB response, I expressed RECQL4 mutant proteins defective in ATP binding (K508G in Walker A motif) or ATP hydrolysis (D605A and E606A in Walker B motif) in RECQL4-depleted cells (Figure 4A). Subsequently, I examined MRE11 foci after NCS treatment. Early disappearance of MRE11 foci was prevented by the expression of wild type RECQL4 proteins, whereas MRE11 foci still disappeared during incubation in cells expressing mutant RECQL4 proteins defective in DNA helicase activity (Figure 4, B and C). As the N-terminus of RECQL4 is essential for DNA replication, depletion of RECQL4 might indirectly affect the stability of MRN complexes on DSB sites by altering the cell cycle. However, premature disassembly of the MRN complex was observed in both cyclin A-positive and -negative cells (Kim et al. 2021), which ruled out the possibility that depletion of RECQL4 might indirectly affect the MRN stability by altering the cell cycle. Furthermore, expression of Walker A or Walker B mutant RECQL4 proteins, which contain intact N-termini, thereby supporting DNA replication, failed to restore the stability of the MRN complex on DSB sites (Figure 4). Collectively, these results suggest that the MRN complex formed on

DSB sites are prematurely disassembled in RECQL4−defective cells due to the lack of RECQL4 helicase activity.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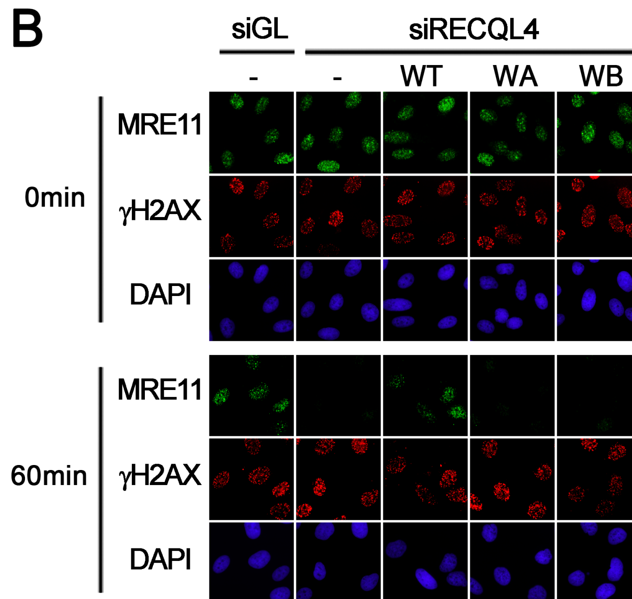
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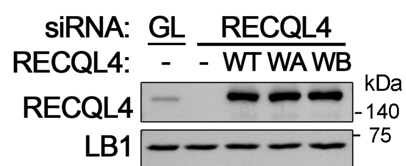
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**Figure 4. Stable maintenance of the MRN complex on DSB sites requires helicase activity of RECQL4.**

(A) Diagrams showing the structure of wild-type (WT) Walker A mutant (WA), and Walker B mutant (WB) of RECQL4. Different domains of RECQL4 were distinguished by colors. The numbers on the top of the diagram indicate the position of amino acid residues in the WT protein.

(B) Immunostaining of RECQL4-depleted U2OS cells transfected with wild type (WT), Walker A (WA), or Walker B (WB) mutant RECQL4. Cells were treated with NCS, and then incubated in fresh medium for the indicated times.

(C) The western blot showing depletion of endogenous RECQL4 and expression of wild type and mutant RECQL4 proteins.

### 3.1.3. $SCF^{SKP2}$−dependent ubiquitination is responsible for premature disassembly of the MRN complex from DSB sites.

Inhibitors of ATM or DNA−dependent protein kinase did not prevent premature disassembly of the MRN complex (Kim et al. 2021). To obtain an insight into how the MRN complex is prematurely disassembled from DSB sites, I examined whether proteasome is required for this process. As shown in Figure 5, MRN foci were stably maintained in RECQL4−depleted cells treated with a proteasome inhibitor, MG132, suggesting that disassembly of the MRN complex from DSB sites may be an active process requiring proteasomal activity. Therefore, premature disassembly of the MRN complex from DSB sites may be responsible for the known defects in DSB response found in RECQL4−defective cells.

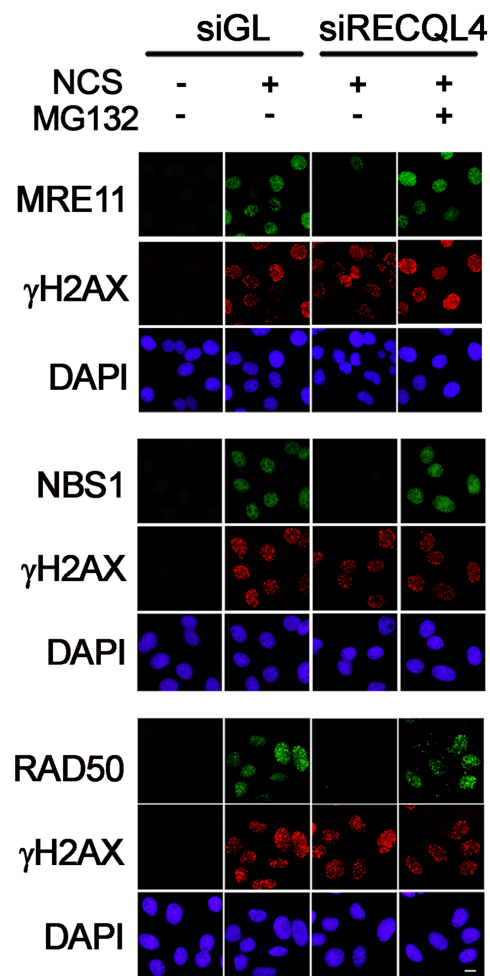



Figure 5. Premature disassembly of the MRN complex is recovered by inhibition of proteasome.

Immunostaining of MRE11, NBS1, or RAD50 in mock- (siGL) or RECQL4-depleted U2OS cells treated with NCS and released for 1 h in the absence (-) or presence (+) of MG132 (50 μg/mL). Scale bar: 10 μm.

Given that among the three MRN proteins, NBS1 appeared to be removed from DSB sites most rapidly (Figure 1B), I explored the possibility that NBS1 may be a target for ubiquitination. Previously, NBS1 was shown to be ubiquitinated by $SCF^{SKP2}$ E3 ligase (Wu et al. 2012). Therefore, I first examined whether $SCF^{SKP2}$ E3 ligase is involved in the instability of the MRN complex in RECQL4−depleted cells. Depletion of SKP2 or CDC34, an E2 ubiquitin−conjugating enzyme supporting lysine (K) 48−linked ubiquitination (Petroski and Deshaies 2005), clearly increased the stability of the MRN complex on DSB sites, as judged by the appearance of MRE11 foci in RECQL4−depleted cells, whereas depletion of UBC5 or UBC13, two other E2 ubiquitin−conjugating enzymes supporting K48− or K63−linked ubiquitination, did not (Figure 6). These results suggest that $SCF^{SKP2}$ E3 ligase, using CDC34 as an E2, may be responsible for the instability of the MRN complex observed in RECQL4−defective cells.

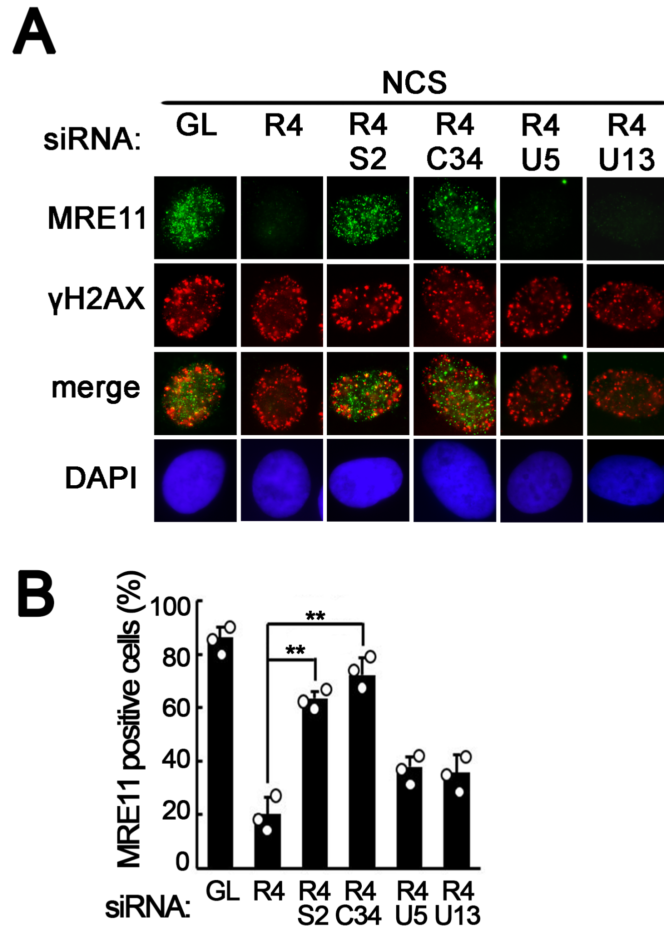



Figure 6. SCF$^{SKP2}$-dependent ubiquitination is responsible for premature disassembly of the MRN complex.

(A and B) Immunostaining of MRE11 in U2OS cells transfected with indicated siRNA and treated with NCS and released for 1 h. Representative images of MRE11 immunostaining (A) and graphs for the percentages of foci-positive cells (B) are shown. R4: RECQL4; S2: SKP2; C34: CDC34; U5: UBC5; U13: UBC13. Data in graphs are means ± SD; n = 3. ** $P < 0.01$

### 3.1.4. K48–linked ubiquitination of NBS1 by $SCF^{SKP2}$ E3 ligase increases in cells with DSBs.

As $SCF^{SKP2}$ E3 ligase, using CDC34 as an E2, supports K48–linked ubiquitination which ultimately results in proteasome–dependent degradation, I examined whether degradation of the MRN component proteins occurs in RECQL4–depleted cells during DSB response. I did not detect a significant decrease in the total protein level of any MRN component proteins in RECQL4–depleted cells after NCS treatment (Figure 1C). However, the stability of the NBS1 protein, which was observed in cells treated with cycloheximide, decreased in RECQL4–depleted cells in a DSB–dependent manner, whereas the stability of the MRE11, another MRN component protein, did not change significantly (Figure 7). Furthermore, K48–linked ubiquitination of NBS1 was significantly increased in both mock– and RECQL4–depleted cells after NCS treatment, and depletion of SKP2 almost completely eliminated this ubiquitination (Figure 8).

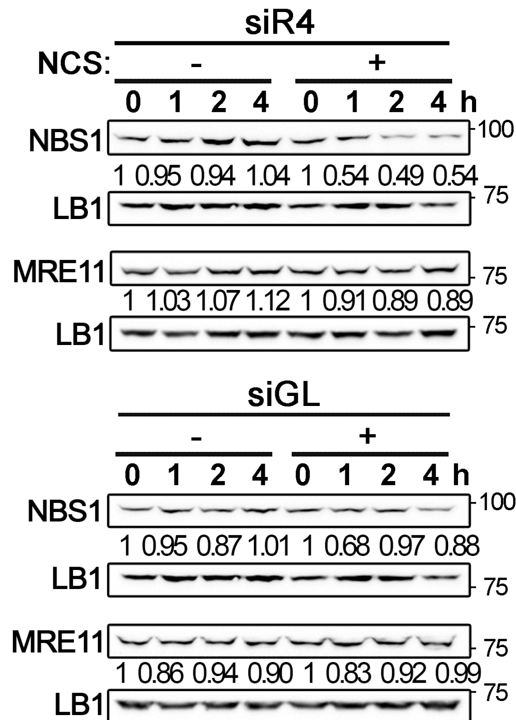



Figure 7. The stability of NBS1 protein is decreased in RECQL4-depleted cells treated with NCS.

Stability of NBS1 and MRE11 proteins in RECQL4- or mock-depleted U2OS cells treated with NCS for indicated times. All cells were treated with 50 μg/mL cycloheximide. The levels of MRE11 and NBS1 proteins normalized to that of Lamin B1(LB) from three independent experiments are shown below each lane.

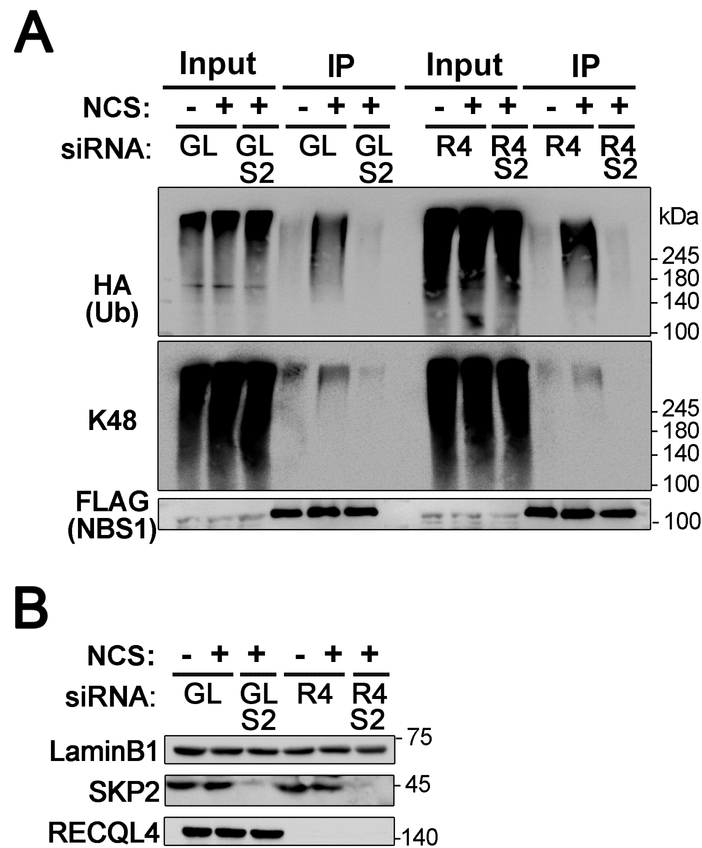



Figure 8. SCF$^{SKP2}$-dependent ubiquitination of NBS1 occurs in cells treated with NCS.

(A) Ubiquitination of NBS1 in HEK293T cells depleted of RECQL4 and/or SKP2, 1 h after NCS or mock treatment. NBS1 proteins were immunoprecipitated with anti-FLAG antibody, and anti-HA (for ubiquitin), anti-K48 ubiquitin (K48), and anti-FLAG (for NBS1) antibodies were used for western blotting.

(B) Western blots showing depletion of SKP2 and RECQL4 in cells used in (A).

Because lysine at amino acid residue 735 (K735) of NBS1 was mapped as a major K63−ubiquitination site by $SCF^{SKP2}$ E3 ligase in a previous study (Wu et al. 2012), I examined whether lysine−to−arginine substitution at this site (K735R) can also prevent K48−linked ubiquitination. As shown in Figure 9, K48−linked ubiquitination was significantly reduced in K735R NBS1 compared to wild type NBS1, suggesting that K735 is a major K48−ubiquitination site in NBS1. Therefore, K48−linked ubiquitination of NBS1 by $SCF^{SKP2}$ E3 ligase indeed occurs in cells treated with NCS and appears to be responsible for the premature disassembly of the MRN complex from DSB sites in RECQL4−depleted cells.

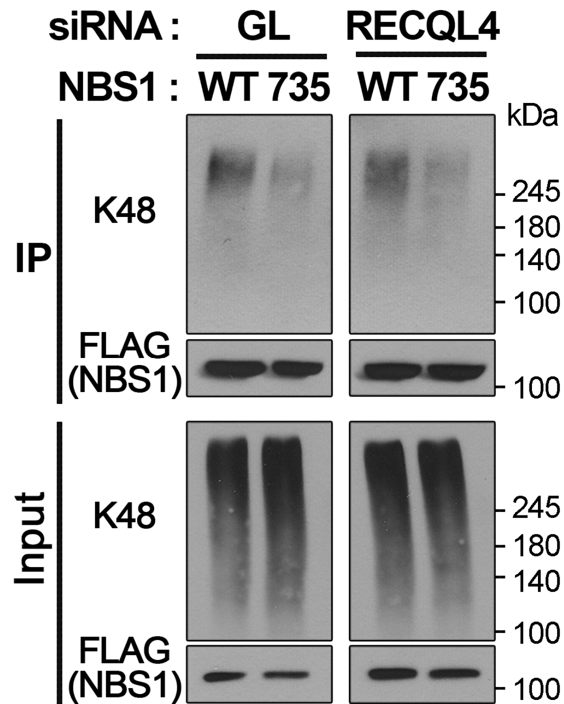



Figure 9. SCF$^{SKP2}$−dependent ubiquitination of NBS1 is prevented by lysine−to−arginine substitution at amino acid residue 735.

Ubiquitination of wild type (WT) or K735R NBS1 (735) in mock− or RECQL4−depleted HEK293T cells treated with NCS. NBS1 proteins were immunoprecipitated with anti−FLAG antibody, and anti−K48 ubiquitin (K48) and anti−FLAG (for NBS1) antibodies were used for western blotting.

### 3.1.5. Stable maintenance of the MRN complex is sufficient to restore the DSB response in RECQL4-defective cells.

If ubiquitination of NBS1 and premature disassembly of the MRN complex from DSB sites are responsible for the known defects in RECQL4-defective cells, preventing their ubiquitination or degradation would be sufficient to restore the DSB response. A possible way to prevent ubiquitin-dependent degradation would be the overexpression of a deubiquitinase that can act on the target protein. Therefore, I explored deubiquitinases that could prevent premature disassembly of the MRN complex after overexpression in RECQL4-defective cells. After testing several deubiquitinases that were shown to be associated with DNA metabolism, I found that USP28 overexpression resulted in the stable maintenance of the MRN complex on DSB sites in RTS and RECQL4-depleted U2OS cells, whereas overexpression of a catalytically inactive USP28 (C171A) did not (Figure 10 and 11). Furthermore, formation of phospho-ATM and RAD51 foci, which are markers of ATM activation and HR repair, respectively, was significantly increased by the overexpression of USP28 (Figure 10). I also found that USP28 overexpression in RTS cells increased resistance to DSB inducing reagents (Figure 12). Therefore, USP28 overexpression almost completely restored ATM activation and HR repair abilities in RECQL4-defective cells.

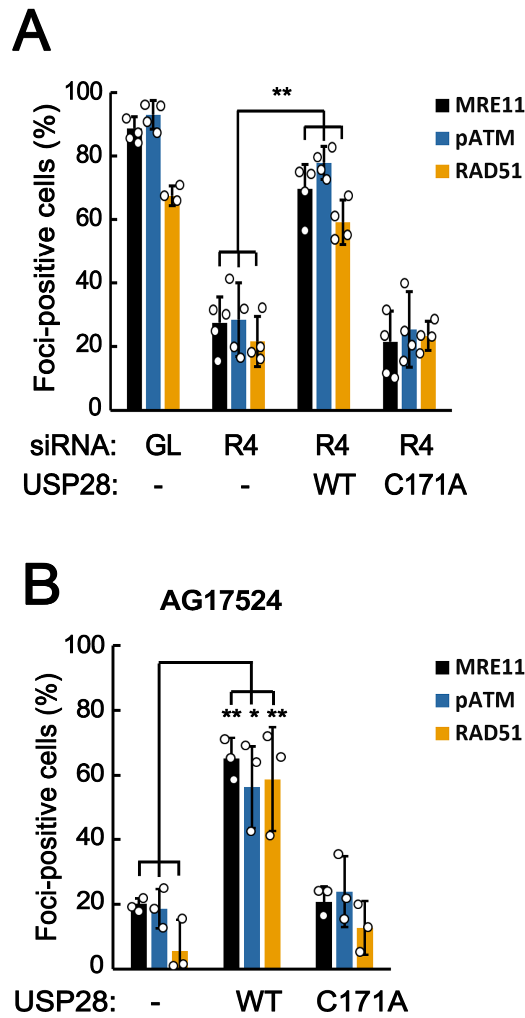



Figure 10. Defects in DSB response are restored by the expression of USP28 in RECQL4-defective cells.

(A and B) Percentages of MRE11-, phospho-ATM-, or RAD51 foci-positive cells after NCS treatment in RECQL4-depleted U2OS cells (A) and RTS (AG17524) cells (B) transfected with empty (-), wild type USP28 (WT), or C171A mutant USP28 plasmids. Data in graphs are means ± SD; n = 4 (for A) or 3 (for B and C). ** $P < 0.01$, * $P < 0.05$.

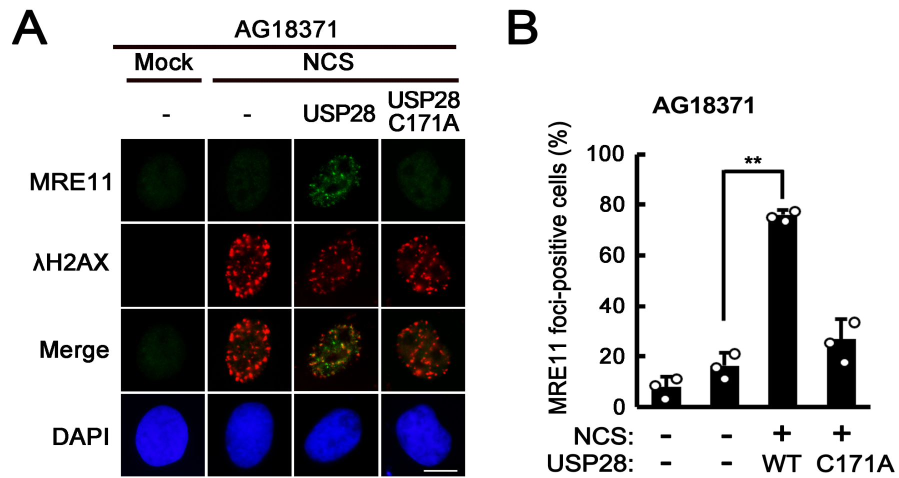



Figure 11. The stability of the MRN complex on double-strand break sites is restored in RTS AG18371 cells by overexpression of USP28.

(A and B) AG18371 cells transfected with plasmids expressing USP28 WT, C171A mutant, or empty vector were treated with NCS, and immunofluorescence staining was performed. Representative images (A) and graph for percentage of foci-positive cells (B) are shown. Scale bar: 10 μm. Data are means ± SD; n = 3. ** $P < 0.01$

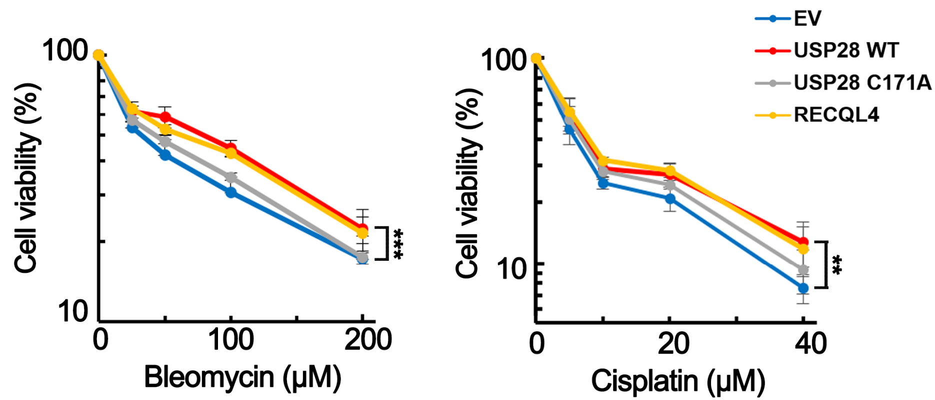



Figure 12. Expression of USP28 as well as RECQL4 decreases sensitivity of RTS cells to DSB inducing reagents.

AG18371 cells transfected with empty, wild type USP28, C171A mutant USP28 or RECQL4 plasmids were treated with various concentration of bleomycin or cisplatin for 48 h. WST-1 assay was carried out to measure the percentage of viable cells relative to undamaged cells. Data in graphs are means ± SEM.; n = 4. P values for USP28 WT and empty vector control were presented. *** $P < 0.001$, ** $P < 0.01$.

USP28 protein was localized in DSB sites after NCS treatment (Knobel et al. 2014) and interacted directly with NBS1 proteins in co-immunoprecipitation experiments (Kim et al. 2021). So, I investigated whether USP28 could counteract ubiquitination of NBS1. As shown in Figure 13, ubiquitination of NBS1 is significantly reduced by overexpression of wild type USP28, but not by catalytically inactive USP28. Therefore, overexpression of USP28 appeared to restore MRN stability on DSB sites in RECQL4-defective cells by counteracting the ubiquitination of NBS1.

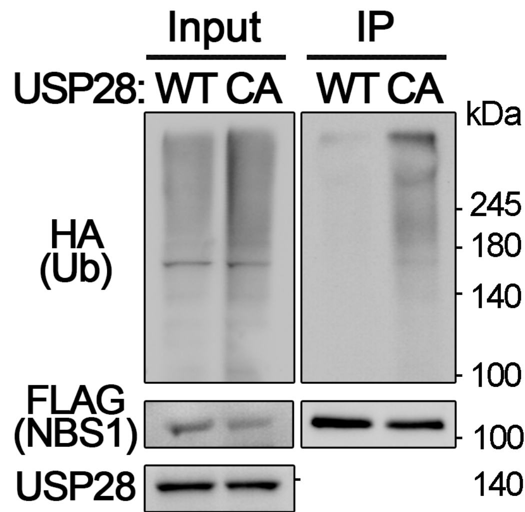



Figure 13. USP28 counteracts the ubiquitination of NBS1 to stabilize the MRN complex.

Ubiquitination of NBS1 in HEK293T cells expressing WT or C171A mutant USP28, 1 h after NCS treatment. NBS1 proteins were immunoprecipitated with anti-FLAG antibody, and anti-HA (for ubiquitin), anti-K48 ubiquitin (K48), and anti-FLAG (for NBS1) antibodies were used for western blotting.

## 3.2. USP2 stabilizes the MRN complex at DNA DSB sites by counteracting the ubiquitination of NBS.

### 3.2.1 Overexpression of USP2 restores the DSB response in RECQL4-defective cells.

Restoration of MRN stability and DSB response in RECQL4 defective cells by USP28 overexpression raised the possibility that USP28 may play an important role in controlling the stability of the MRN complex during DSB response in cells with intact RECQL4 proteins. However, depletion of USP28 has previously been shown not to affect the stability of the MRN complex and DSB response in U2OS cells (Zhang et al. 2006; Knobel et al. 2014). So, I explored other deubiquitinases that influence the stability of the MRN complex during the DSB response in RECQL4-depleted cells. Several deubiquitinases that have been shown to be associated with DNA damage responses, cell cycle, or carcinogenesis were individually overexpressed in RECQL4-depleted cells, and MRE11 foci were examined after treating cells with NCS. I found that overexpression of USP2 increased MRE11 foci-positive cells, as with the overexpression of USP28, suggesting that premature disassembly of the MRN complex at DSB sites is prevented by USP2 (Figure 14A). HR repair and ATM activation abilities, which were determined by the appearance of RAD51 and pATM foci, respectively, were also restored by USP2 overexpression in RECQL4-depleted cells (Figure 14B).

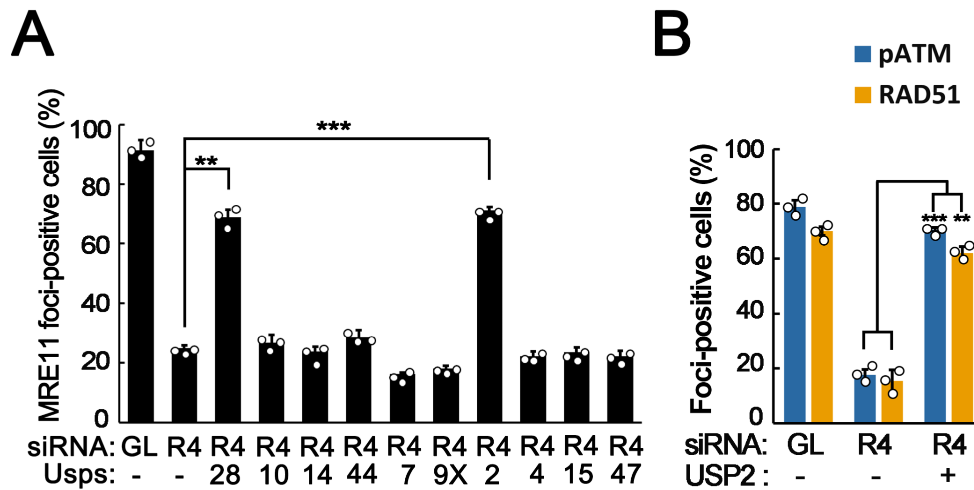



Figure 14. Overexpression of USP2 restores the DSB response in RECQL4-depleted U2OS cells.

(A) Screening of deubiquitinases increasing stability of the MRN complex in RECQL4-depleted cells. U2OS cells transfected with the indicated siRNAs and plasmids were treated with NCS for 15 min and incubated in fresh medium for 1 h. MRE11 immunostaining was carried out and more than 50 cells were counted for foci number in each experiment. Data in graphs are means ± SD; n =3. ** $P < 0.01$; *** $P < 0.001$. R4, RECQL4.

(B) Immunostaining of pATM and RAD51 in mock- (siGL) or RECQL4-depleted (siR4) U2OS cells transfected with empty or USP2 plasmids. Cells were treated with NCS and incubated in fresh medium for 1 h. Data in graphs are means ± SD; n =3. ** $P < 0.01$; *** $P < 0.001$.

The effect of USP2 overexpression on HR repair ability was further confirmed by an HR assay using DR-GFP HR reporter cells (TRI-DR-U2OS) (Figure 15). Restoration of MRE11, pATM, and RAD51 foci by USP2 overexpression was also observed in RTS cells (Figure 16). Taken together, these results suggest that overexpression of USP2 prevented premature disassembly of the MRN complex from DSB sites and restored the DSB response in RECQL4-defective cells.

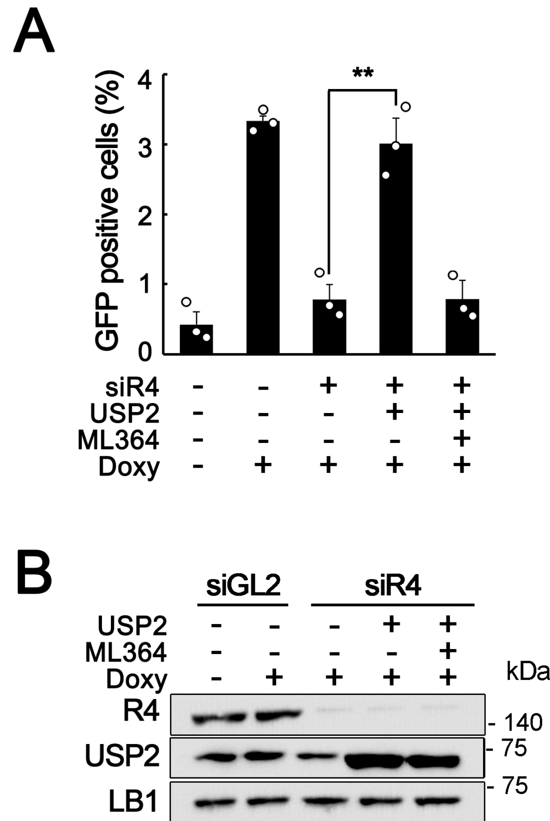



Figure 15. Overexpression of USP2 restores the HR ability in RECQL4-depleted cells.

(A) HR repair assay using TRI-DR-U2OS cells. Mock- or RECQL4-depleted cells were transfected with empty or USP2 plasmids. After 24 h incubation, cells were treated with doxycycline to induce I-*Sce*1 and incubated for additional 48 h. For inhibiting USP2, 20 μM of ML364 was treated 1 h before doxycycline treatment. Data in graphs are means ± SD.; n =3, ** $P < 0.01$.

(B) The western blots showing depletion of RECQL4 and USP2 expression. LB1, lamin B1.

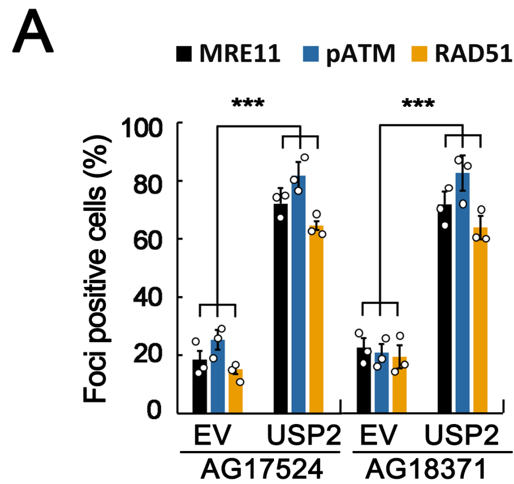


Figure 16. Overexpression of USP2 restores the DSB response in RTS cells.

(A) Immunostaining of MRE11, pATM or RAD51 in RTS cells (AG17524 and AG18371) transfected with empty (EV) or USP2 plasmids. Cells were treated with NCS and incubated in fresh medium for 1 h. Data in graphs are means ± SD; n =3. *** $P < 0.001$.

(B) Lower panel is the western blots showing expression of HA-tagged USP2.

### 3.2.2. USP2 is recruited to DSB sites and counteracts the ubiquitination of NBS1.

Because premature disassembly of the MRN complex from DSB sites was caused by $SCF^{SKP2}$ dependent ubiquitination of the NBS1 protein in RECQL4-defective cells (Figure 5-8), I tested the possibility that USP2 increases the stability of the MRN complex at DSB sites by counteracting the ubiquitination of NBS1. First, I examined the recruitment of USP2 to DSB sites. While EGFP-fused USP2 proteins expressed in U2OS cells were mostly located in the cytoplasm, small amounts of these proteins appeared in the nucleus and were rapidly bound to laser micro-irradiation sites (Figure 17). The level of protein binding peaked at approximately 150 s, and then gradually decreased until 600 s. This result indicates that USP2 plays a role in the DSB response by directly acting on the targets at DSB sites. Co-immunoprecipitation analysis of U2OS cells overexpressing USP2 and MRE11 or USP2 and NBS1 indicated that USP2 barely interacted with MRE11 in the absence or presence of DNA damage (Figure 18A). On the other hand, USP2 stably interacted with NBS1 upon NCS treatment, and their interaction persisted regardless of NCS treatment (Figure 18B).

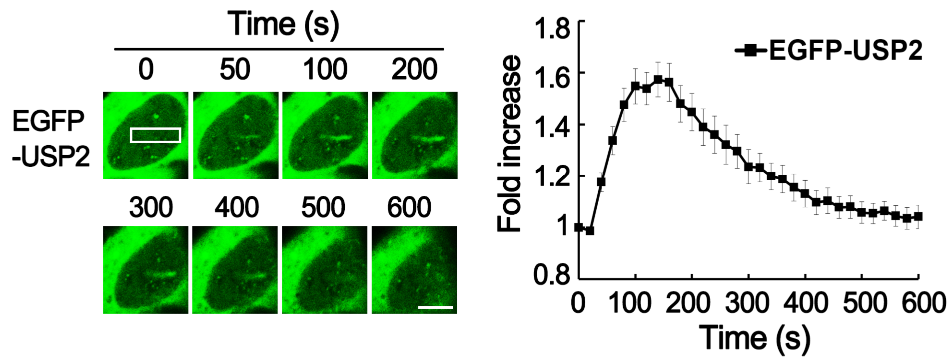



Figure 17. USP2 is recruited to laser-induced DSB sites.

EGFP-USP2 binding to micro-irradiation sites in U2OS cells. Representative images (left panel) and graph for relative green fluorescence intensity in damaged area (right panel) are shown. Data in graphs are means ± SEM; n =20. The scale bar represents 10 μm.

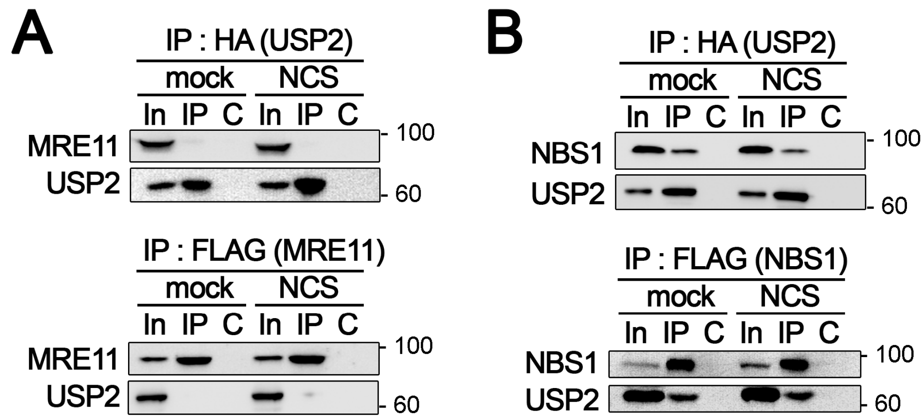



Figure 18. USP2 interacts with NBS1.

(A and B) Interaction of USP2 with MRE11 (A) or NBS1 (B) in mock- or NCS-treated U2OS cells. FLAG-tagged MRE11 or FLAG-tagged NBS1 was expressed with HA-tagged USP2 in U2OS cells, and immunoprecipitation (IP) was carried out with anti-HA antibodies (upper panel) or anti-FLAG-M2-agarose beads (lower panel). Anti-FLAG (for MRE11 or NBS1) and anti-HA (for USP2) antibodies were used for western blotting. Lanes: In, 10% of input for IP; C, control IP with nonspecific IgG.

I then examined whether USP2 influences DSB-dependent ubiquitination of NBS1 proteins. As shown in Figure 19, DSB-dependent ubiquitination of NBS1 was reversed by USP2 overexpression to almost the same level as that in non-damaged cells. Treatment with ML364, a specific inhibitor of USP2 deubiquitinase, masked the effect of USP2 overexpression, indicating that increased activity of USP2 is responsible for the decrease in the ubiquitination of NBS1 (Figure 19). Taken together, these results suggest that USP2 prevents premature disassembly of the MRN complex in RECQL4-defective cells by counteracting the ubiquitination of NBS1.

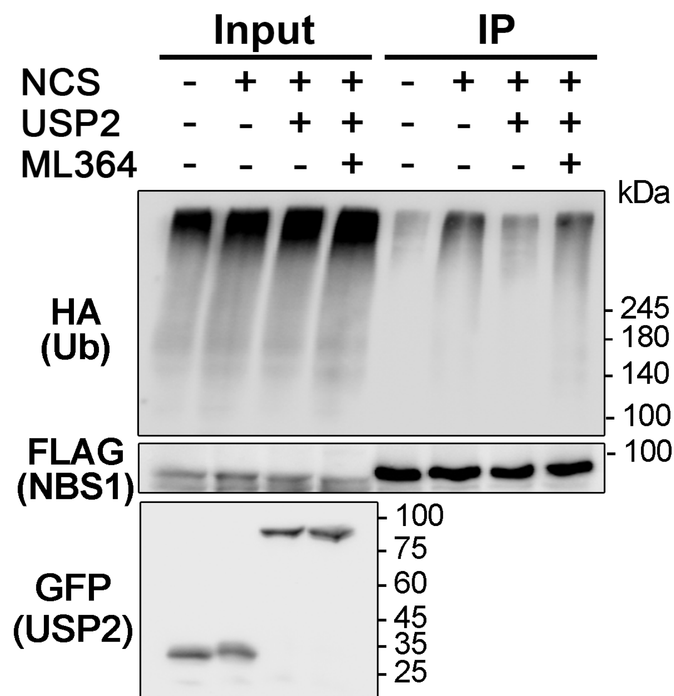



Figure 19. USP2 counteracts the ubiquitination of NBS1.

Ubiquitination of NBS1 in HEK293T cells treated as indicated. Cells transfected with HA-ubiquitin, FLAG-NBS1, and GFP-USP2 plasmids as indicated were pretreated with ML364 and MG132 1 h before NCS treatment. Anti-FLAG M2 agarose beads were used for IP, and anti-HA (for ubiquitin), anti-FLAG (for NBS1), and anti-GFP (for USP2) antibodies were used for western blotting.

### 3.2.3. USP2 is recruited to DSB sites in a manner dependent upon ATM, PARP, and RECQL4.

As USP2 can act at DSB sites, I examined how its recruitment to DSB sites was controlled during the DSB response. In the DSB response, ATM and DNA-PK play important roles to initiate signaling cascade and to recruit many factors to DSB sites by phosphorylating various target proteins (Blackford and Jackson 2017). To determine the role of these kinases in the recruitment of USP2, specific inhibitors of ATM and DNA-PK, KU55933 and NU7441, respectively, were individually treated in cells expressing EGFP-USP2 proteins, and recruitment of USP2 to the laser micro-irradiation site was analyzed by live-cell imaging. USP2 binding to the micro-irradiation site was still observed in the DNA-PK inhibitor-treated cells (Figure 20). In contrast, treatment with the ATM inhibitor almost completely reduced USP2 binding to the micro-irradiation site (Figure 20), suggesting that ATM kinase activity is required for recruitment of USP2 to DSB sites.

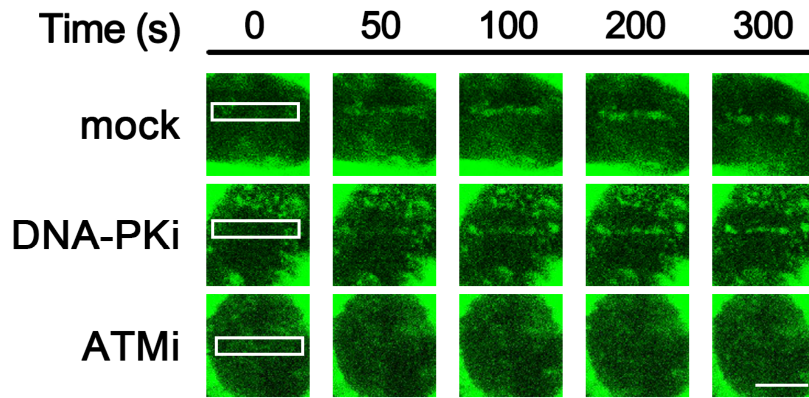



Figure 20. USP2 is recruited to DSB sites in a manner dependent on ATM.

Recruitment of EGFP-USP2 to laser micro-irradiation sites was analyzed in U2OS cells treated with inhibitors (20 μM) of ATM (KU55933) or DNA-PK (NU7441) 1 h before micro-irradiation. Representative images for green fluorescence in damaged area are shown. The scale bar represents 10 μm.

Another important player in the DSB response is poly(ADP-ribosyl)ation (PARylation), which is produced by poly(ADP-ribose) polymerases (PARPs) and removed by poly(ADP-ribose) glycohydrolase (PARG). PARylation also appeared to be critical for the recruitment of USP2 to DSB sites because treatment with olaparib, an inhibitor of PARP1 and PARP2, almost completely abolished the binding of USP2 to laser-induced DSB sites, and treatment with the PARG inhibitor, PDD00017273, prevented dissociation of USP2 from DSB sites (Figure 21). Furthermore, depletion of RECQL4 abolished the recruitment of USP2 to laser-induced DSB sites (Figure 22, A and B). The expression of helicase-defective RECQL4 mutant proteins (D605A and E606A in the Walker B motif) as well as wild-type RECQL4 proteins restored USP2 binding to laser-induced DSB sites in RECQL4-depleted cells (Figure 22, C and D). Therefore, physical presence of RECQL4, instead of its helicase activity, appeared to be required for the recruitment of USP2 to DSB sites. However, dissociation of USP2 from DSB sites was faster in Walker B mutant-expressing cells than in cells with wild-type RECQL4 (Figure 22C), suggesting that the helicase activity of RECQL4 may play a role in the maintenance of USP2 at DSB sites. Taken together, these results indicate that recruitment of USP2 to DSB sites depends on RECQL4 protein, PARylation, and ATM kinase activity. Interestingly, recruitment of RECQL4 proteins to DSB sites also requires

PARylation (Aleksandrov et al. 2018). Therefore, the PARylation dependency of USP2 recruitment to DSB sites might be caused by the PARylation dependency of RECQL4 recruitment to DSB sites. To support this notion, USP2 interacted with RECQL4 in co-immunoprecipitation experiments, and NCS treatment significantly increased this interaction (Figure 23A). Furthermore, inhibition of ATM kinase activity almost completely abolished this interaction in NCS-treated cells (Figure 23B). Taken together, these results suggest that USP2 may be recruited to DSB sites via its interaction with RECQL4, which is dependent on ATM kinase activity.

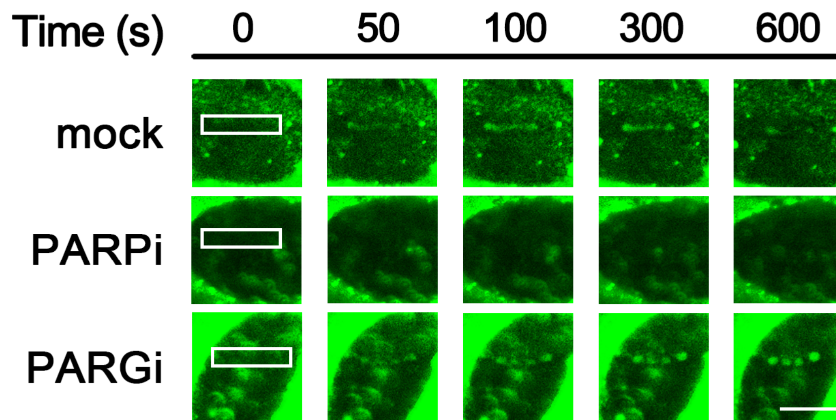



Figure 21. USP2 is recruited to DSB sites in a manner dependent on PARylation.

Recruitment of EGFP-USP2 to laser micro-irradiation sites was analyzed in U2OS cells treated with inhibitors (20 μM) of PARP (olaparib) or PARG (PDD00017273) 1 h before micro-irradiation. Representative images for green fluorescence in damaged area are shown. The scale bar represents 10 μm.

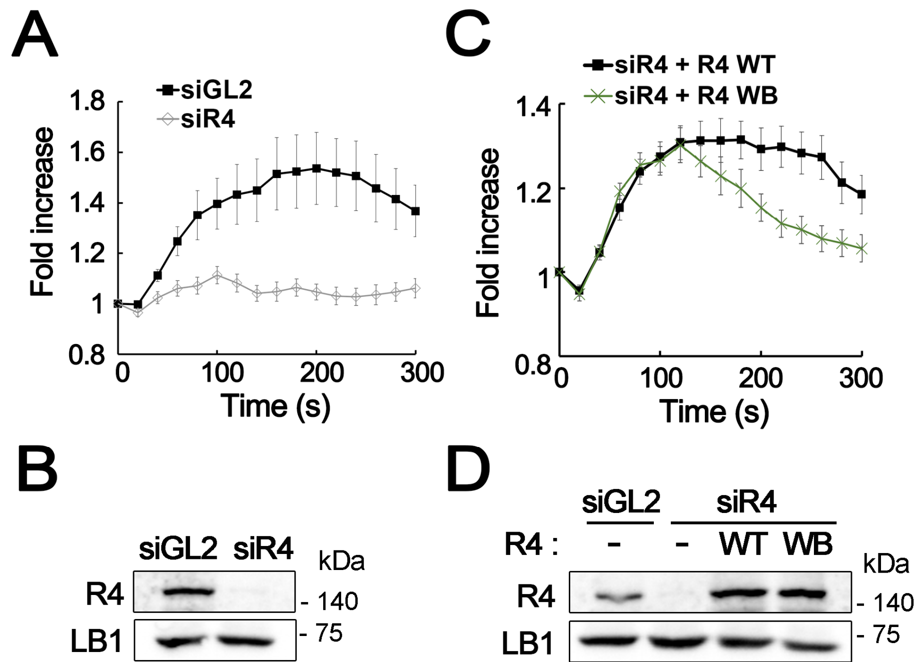



Figure 22. USP2 is recruited to DSB sites in a manner dependent on RECQL4.

(A and B) Recruitment of USP2 to micro-irradiation sites in mock-(siGL) or RECQL4-depleted (siR4) U2OS cells. Graph for relative green fluorescence intensity in damaged area (A) and western blot for RECQL4 protein levels (B) are shown.

(C and D) RECQL4-depleted U2OS cells were transfected with wild type (WT) or Walker B mutant (WB) RECQL4 plasmids and used for analyzing USP2 binding to micro-irradiation sites. Graph for relative green fluorescence intensity in damaged area (C) and western blot for RECQL4 protein levels (D) are shown. Data in graphs are means ± SEM; n =20.

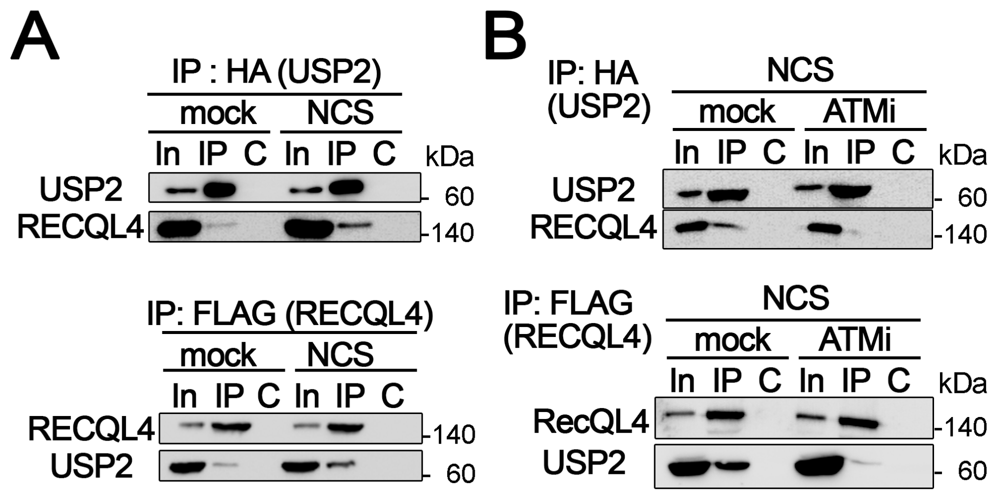



Figure 23. USP2 interacts with RECQL4 in a manner dependent on DSB and ATM.

(A and B) Interaction between USP2 and RECQL4 in U2OS cells. U2OS cells transfected with FLAG-RECQL4 and HA-USP2 plasmids were used for IP with anti-HA antibodies (upper panels) or anti-FLAG-M2-agarose beads (lower panels). Cells treated with NCS (A) or NCS and ATM inhibitor (KU55933, 20 μM) (B) were used for IP. Anti-FLAG (for RECQL4) and anti-HA (for USP2) antibodies were used for western blotting. Lanes: In, 10% of input for IP; C, control IP with nonspecific IgG.

### 3.2.4. The N-terminus of USP2 is sufficient for ATM-dependent recruitment to DSB sites.

To clarify the mechanism of USP2 recruitment to DSB sites, I determined the minimal USP2 region required for binding to DSB sites. For this purpose, several EGFP-fused truncated USP2 proteins were expressed (Figure 24A) and their binding to the laser micro-irradiation site was examined by live-cell imaging. As shown in Figure 24B, deletion of the USP domain at the C-terminus did not significantly affect the binding to laser-induced DSB sites. In contrast, truncated proteins without N-terminus were unable to bind to laser-induced DSB sites, and the N-terminus of USP2 containing amino acid residues 1 to 160 (CD1) was sufficient to bind to DSB sites (Figure 24). Similar to the full-length USP2 protein, this minimal region of USP2 bound to laser-induced DSB sites in a manner dependent on PARylation and ATM kinase activity (Figure 25). Furthermore, RECQL4 depletion also prevented recruitment of this minimal region of USP2 (Figure 26), suggesting that this region of USP2 contains all the requirements for recruitment to DSB sites. As this minimal region for DSB binding also stably interacted with RECQL4 in a DSB-dependent manner (Figure 27), USP2 might be recruited to DSB sites via interaction with RECQL4.

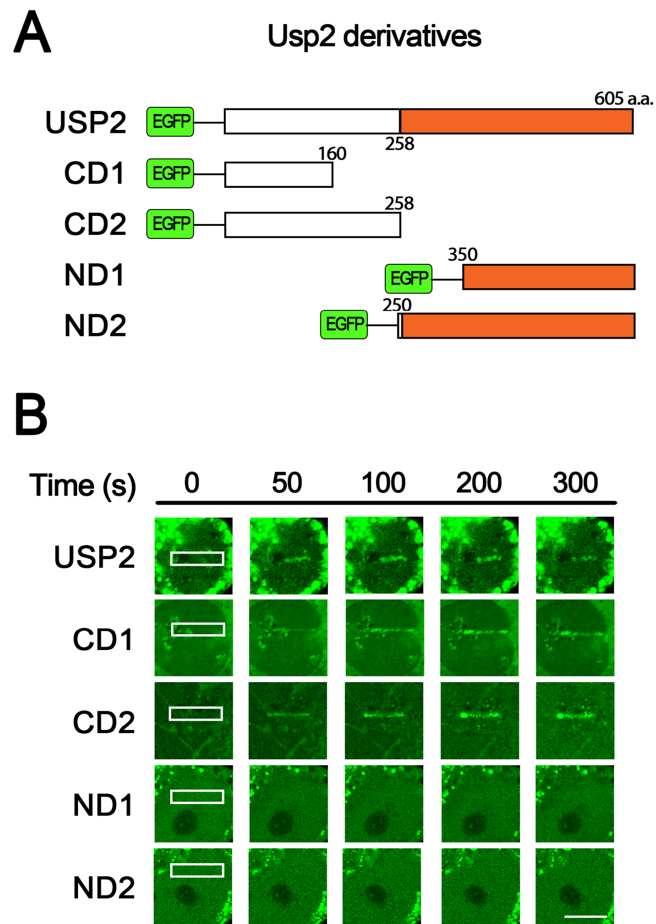



Figure 24. USP2 binds to DSB sites through its N-terminus.

(A) Schematic diagram of truncated USP2 proteins and their binding to laser-induced DSB sites. The USP2 C-terminus (orange) indicates conserved USP domain.

(B) EGFP-fused USP2 derivatives were expressed in U2OS cells and their binding to laser micro-irradiation sites were analyzed. Representative images for green fluorescence in damaged area are shown. The scale bar represents 10 μm.

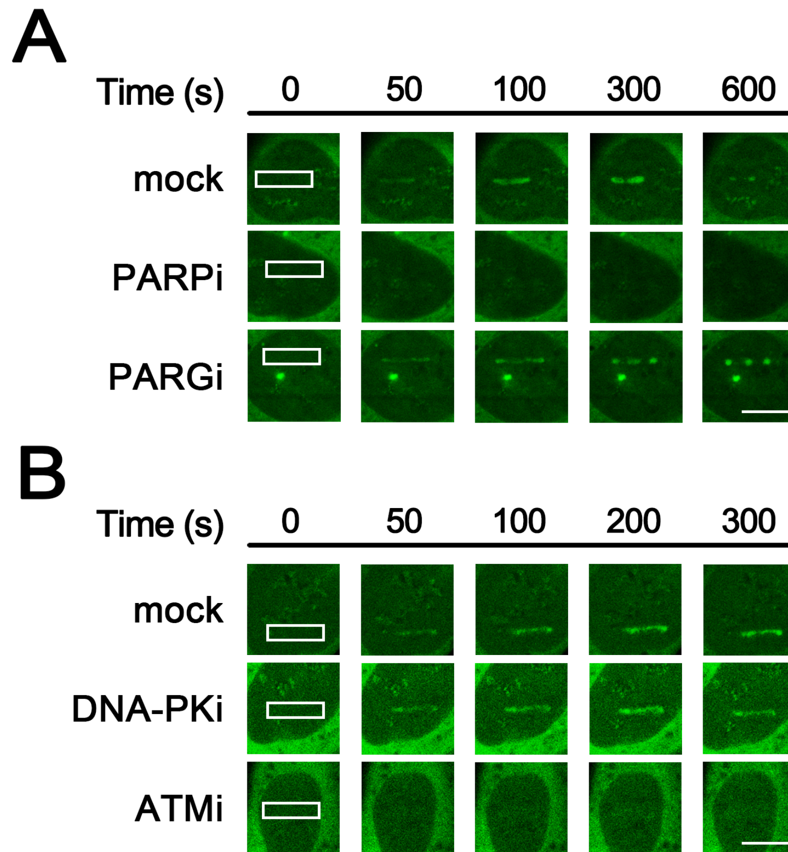



Figure 25. N-terminus of USP2 is recruited to DSB sites in a manner dependent on PARylation and ATM.

(A and B) Influences of PARylation (A) and inhibition of DSB inducing kinases (B) on the recruitment of EGFP-fused CD1 to DSB sites. Recruitment of EGFP-CD1 to laser micro-irradiation sites was analyzed in U2OS cells treated with inhibitors (20 μM) of ATM (KU55933), DNA-PK (NU7441), PARP (olaparib) or PARG (PDD00017273) 1 h before micro-irradiation. Representative images for green fluorescence in damaged area are shown. The scale bar represents 10 μm.

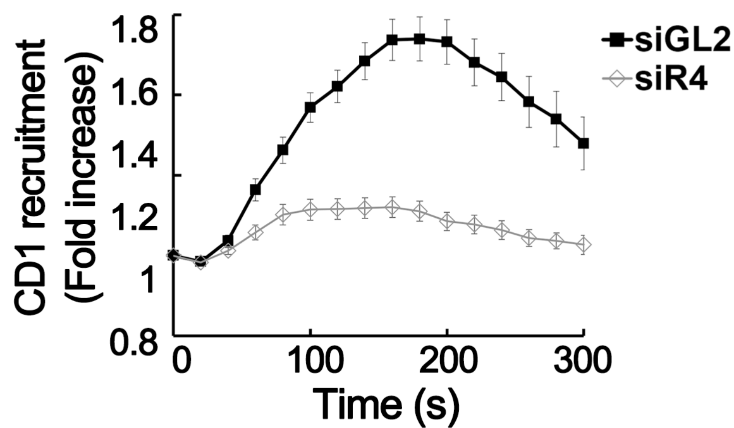



Figure 26. N−terminus of USP2 is recruited to DSB sites in a manner dependent on RECQL4.

Influence of RECQL4 depletion on the recruitment of EGFP−fused CD1 to DSB sites. Inhibitors were treated 1 h before micro−irradiation. Graph for relative green fluorescence intensity in damaged area is shown. Data in graphs are means ± SEM; n =20.

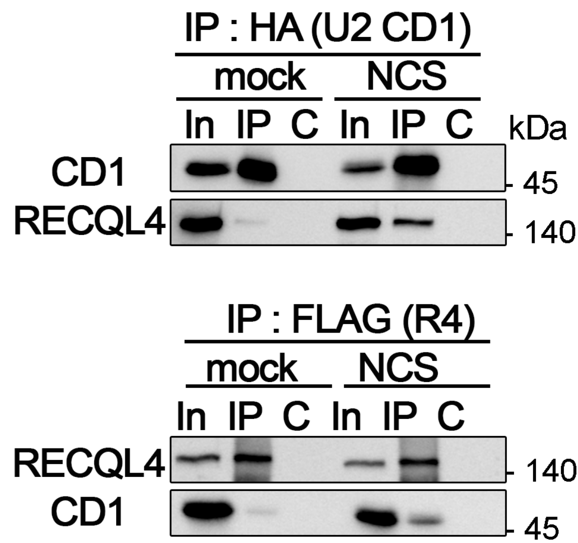



Figure 27. N−terminus of USP2 interacts with RECQL4 in a manner dependent on DSB.

Interaction between HA−CD1 and FLAG−RECQL4 in mock− or NCS−treated U2OS cells. IP was carried out with anti−HA antibodies (upper panel) or anti−FLAG−M2−agarose beads (lower panel). Anti−FLAG (for RECQL4) and anti−HA (for CD1) antibodies were used for western blotting. R4, RECQL4. Lanes: In, 10% of input for IP; C, control IP with nonspecific IgG.

### 3.2.5. Phosphorylation of two critical residues in USP2 N-terminus is required for its recruitment to DSB sites and interaction with RECQL4.

As the N-terminal domain of USP2 showed ATM-dependent recruitment to DSB sites, I further investigated whether phosphorylation of USP2 N-terminus is important for its recruitment to DSB sites. The minimal region of the USP2 N-terminus for DSB binding contains four SQ/TQ sites (S2, S94, T137, S142) that can be recognized and phosphorylated by ATM kinase. Therefore, I generated USP2 proteins with various combinations of alanine substitutions in these four S/T residues and determined their binding to laser-induced DSB sites. While individual alanine substitutions of serine or threonine in these putative phosphorylation sites did not significantly affect the recruitment of USP2 to DSB sites, alanine substitution of all four serine and threonine residues (4A) resulted in complete loss of DSB binding activity (Figure 28). Furthermore, alanine substitution of two amino acid residues, S2A and T137A (AA), also led to the loss of DSB-binding activity (Figure 28), suggesting that phosphorylation of these two residues is critical for the recruitment of USP2 to DSB sites. The phospho-mimetic mutant USP2 with glutamic acid substitution at these two phosphorylation sites, S2E and T137E (EE), was able to bind to DSB sites, but its binding was disturbed by ATM inhibition (Figure 29), suggesting that the phosphorylation

of these residues is required but not sufficient for recruitment to DSB sites.

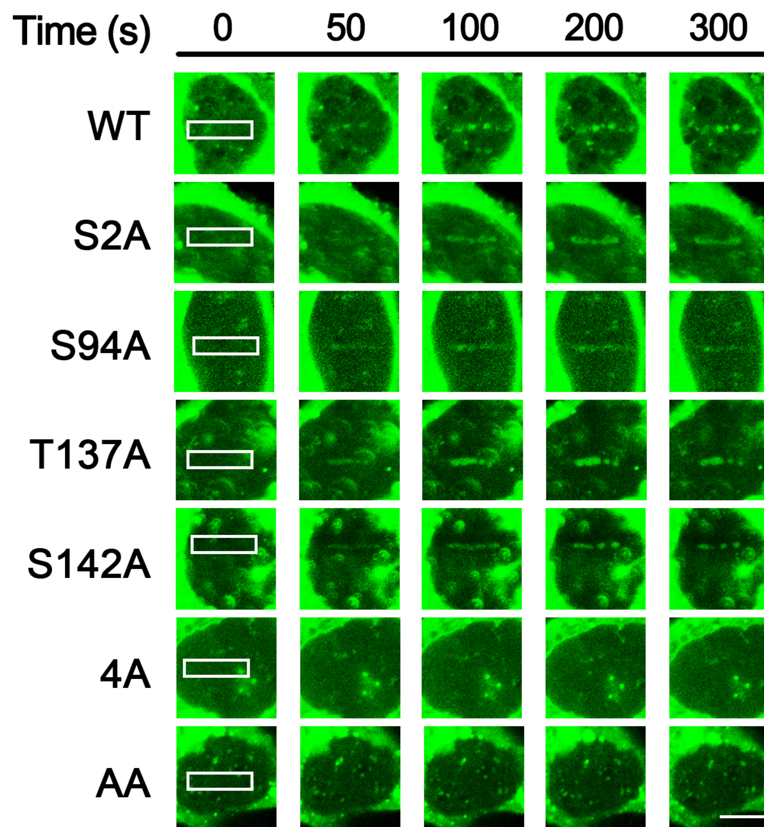



**Figure 28. Phosphorylation of two critical residues in USP2 N-terminus by ATM is required for recruitment to DSB sites.**

Recruitments of various alanine-substituted full length USP2 proteins to DSB sites were examined by laser micro-irradiation followed by live cell imaging. WT, wild type USP2; 4A, USP2 with substitution of 4 ATM phosphorylation sites (S2, S94, T137, S142) to alanine; AA, substitution of S2 and T137 to alanine. The scale bar represents 10 μm.

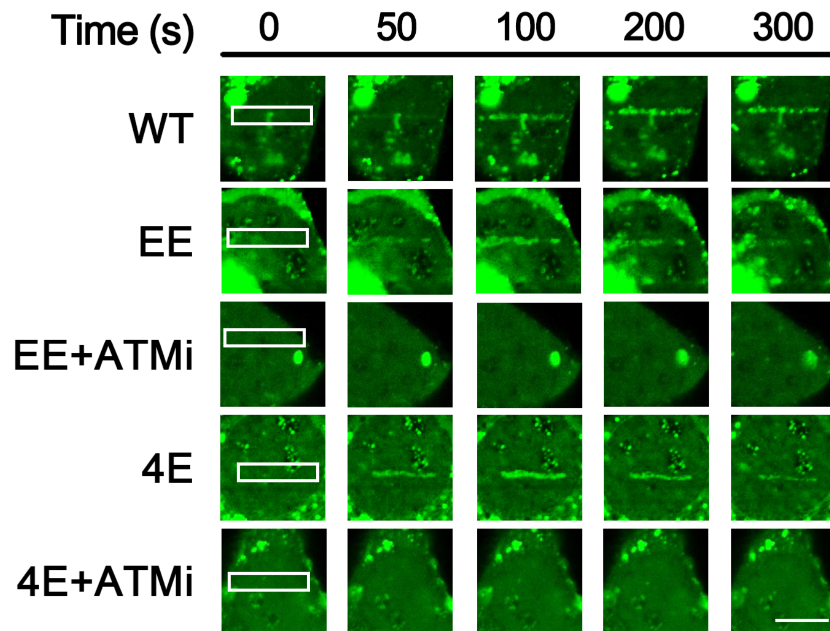



Figure 29. Phospho−mimetic mutant USP2 is recruited to DSB sites, but this recruitment is prevented by inhibition of ATM.

Recruitments of glutamic acid−substituted full length USP2 proteins (EE and 4E) to DSB sites were examined by laser micro−irradiation followed by live cell imaging. ATM inhibitor (ATMi), KU55933, was treated 1 h before micro−irradiation. WT, wild type USP2; EE, substitution of S2 and T137 to glutamic acid; 4E, USP2 with substitution of 4 ATM phosphorylation sites (S2, S94, T137, S142) to glutamic acid. The scale bar represents 10 μm.

I then examined whether the phosphorylation of USP2 influences its interaction with RECQL4. As shown in Figure 30A, interaction between phospho-deficient USP2 (AA) and RECQL4 was not observed in the co-immunoprecipitation analysis. In contrast, phospho-mimetic USP2 (EE) proteins stably interacted with RECQL4 both in the absence and presence of DNA damage (Figure 30A), and inhibition of ATM kinase activity did not disrupt their interaction, although the interaction appeared to be slightly weak (Figure 30B). Taken together, these results suggest that interaction of USP2 with RECQL4 through ATM-dependent phosphorylation of these two sites is not sufficient for the recruitment of USP2 to DSB sites. As the mutant USP2 with phospho-mimetic substitution of all four ATM phosphorylation sites in the N-terminus (4E) still showed ATM dependency in the recruitment to DSB sites (Figure 29), phosphorylation of other proteins by ATM might also play an important role in the recruitment of USP2 to DSB sites. Collectively, these data suggest that ATM is a key regulator for USP2 recruitment to DSB sites by phosphorylating USP2 at S2 and T137, which is essential for the interaction between USP2 and RECQL4.

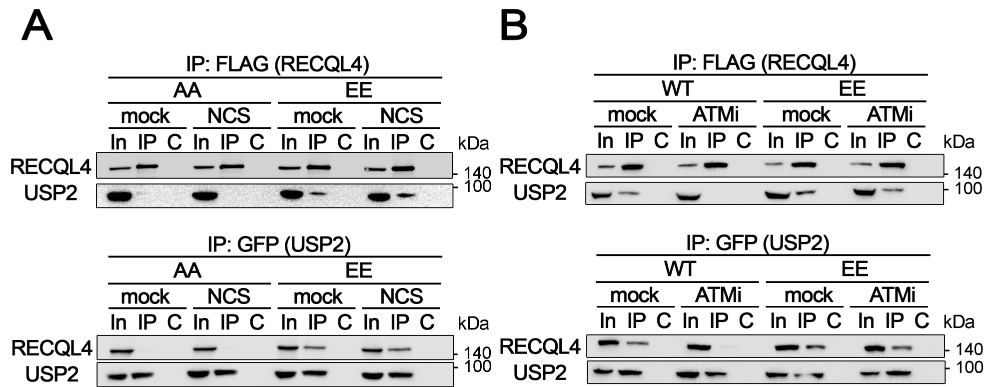



Figure 30. Phosphorylation of two critical residues in USP2 N-terminus by ATM is required for interaction with RECQL4.

(A and B) Interaction of phospho-deficient or mimetic mutant of USP2 with RECQL4. FLAG-RECQL4 and EGFP-USP2 mutant proteins were expressed in U2OS cells and treated as indicated. IP was carried out with anti-FLAG-M2-agarose beads (upper panel) or anti-GFP antibodies (lower panel). Anti-FLAG (for RECQL4) and anti-GFP (for CD1) antibodies were used for western blotting. Lanes: In, 10% of input for IP; C, control IP with nonspecific IgG.

### 3.2.6. USP2 and USP28 play a redundant role in the stable maintenance of the MRN complex at DSB sites during the DSB response.

As USP2 counteracted the ubiquitination of NBS1 to increase the stability of the MRN complex in RECQL4-defective cells, I examined whether USP2 plays a role in the DSB response in cells with intact RECQL4 helicase. For this purpose, U2OS cells were treated with ML364 to inhibit the deubiquitinase activity of USP2, and the stability of the MRN complex was examined after NCS treatment. As shown in Figure 31 and 32, neither EGFP-fused MRE11 (EGFP-MRE11) recruitment to micro-irradiation site nor foci formation of MRE11 were significantly influenced by inhibition of USP2 deubiquitinase activity, suggesting that USP2 activity may not be essential for the stable maintenance of the MRN complex at DSB sites in cells with intact RECQL4.

I also observed that USP28 was also able to counteract NBS1 ubiquitination and prevent premature disassembly of the MRN complex from DSB sites in RECQL4-defective cells (Figure 10-12). However, depletion of USP28 was shown to hardly influence the DSB response in many human cells, including U2OS (Zhang et al. 2006). As both USP2 and USP28 can oppose NBS1 ubiquitination, I hypothesized that these two deubiquitinases play a redundant role in preventing premature disassembly of the MRN complex during the DSB response. To test this possibility, I examined the stability

of the MRN complex at DSB sites in cells lacking the activity of both USP2 and USP28. EGFP-MRE11 proteins rapidly bound to the micro-irradiation site, and the level of binding peaked at approximately 100 s (Figure 31). The level of EGFP-MRE11 proteins at the micro-irradiation site gradually decreased in cells treated with both USP2 inhibitor and USP28 siRNA, whereas the level of binding was stably maintained in mock- or single-treated cells (Figure 31). Early removal of the MRN complex from DSB sites was also confirmed by immunostaining of MRE11 foci after NCS treatment in cells treated with both USP2 inhibitor and USP28 siRNA (Figure 32).

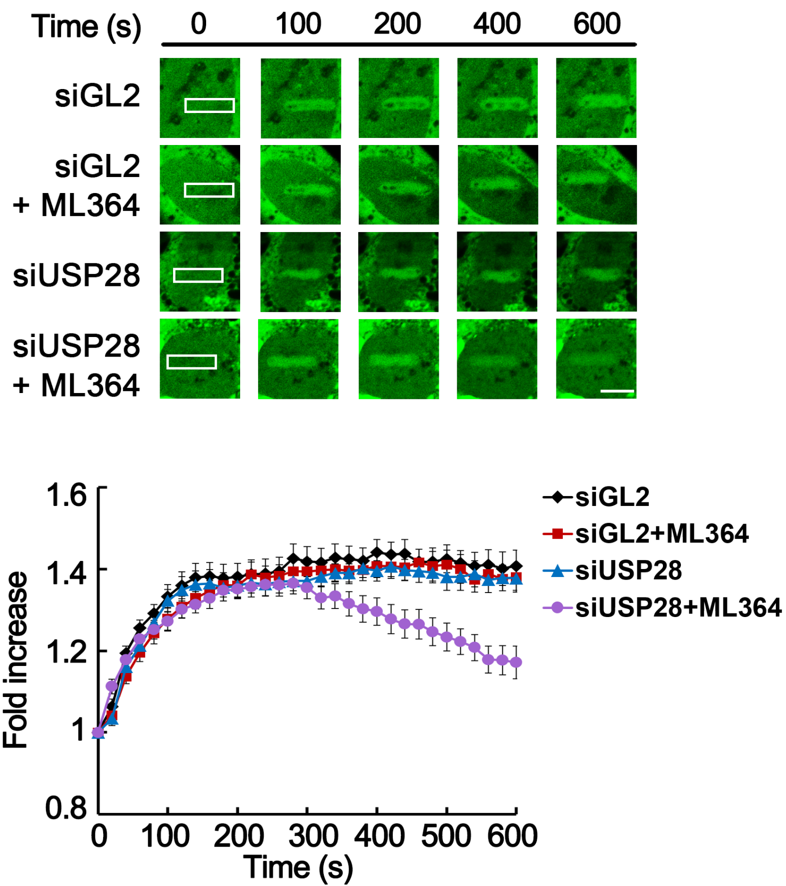



Figure 31. Inactivation of both USP2 and USP28 activities results in decrease of EGFP−MRE11 recruitment to micro−irradiation site.

Binding of EGFP−MRE11 to micro−irradiation induced DSB sites in USP2 and/or USP28 inactivated cells. Cells transfected with mock (GL) or USP28 siRNAs were treated with USP2 inhibitor (ML364, 20 μM) 1 h before micro−irradiation. Representative images (upper panel) and graph for relative green fluorescence intensity in damaged area (lower panel) are shown. The scale bar represents 10 μm. Data in graphs are means ± SEM.; n =20.

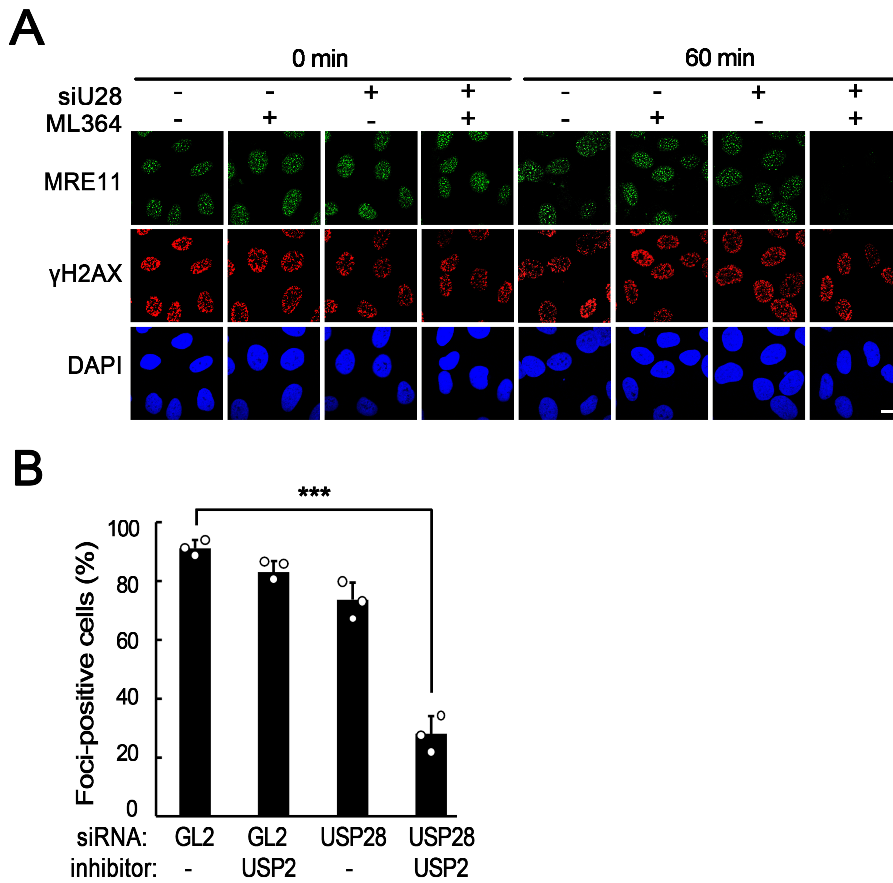



Figure 32. Inactivation of both USP2 and USP28 activities results in premature disassembly of the MRN complex.

(A and B) Immunostaining of MRE11 in USP2 and/or USP28 inactivated U2OS cells. Cells were transfected with the indicated siRNAs and/or treated with USP2 inhibitor (ML364, 20 μM) followed by NCS treatment and incubation in fresh medium for the indicated time. Representative images (A) and graph for the percentage of MRE11 foci positive cells with 1 h incubation after NCS treatment (B) are shown. The scale bar represents 10 $\mu$m. Data in graphs are means ± SD; n = 3. *** $P < 0.001$.

An increase in the NBS1 ubiquitination and a decrease in the NBS1 stability were also observed after NCS treatment in cells inactivated with both USP2 and USP28 (Figure 33 and 34), although a small decrease in the stability of NBS1 was also observed in the absence of DNA damage (Figure 34 mock). Consistent with these observations, defects in ATM activation and HR repair abilities were observed only in cells inactivated with both USP2 and USP28 activities, whereas single inactivation of USP2 or USP28 only slightly decreased these abilities (Figure 35, A and B). Sensitivity to the DSB-inducing reagent, bleomycin, also increased mostly by the inactivation of both USP2 and USP28 deubiquitinases (Figure 35C). Taken together, these results strongly suggest that USP2 and USP28 play a redundant role in the DSB response to stably maintain the MRN complex on DSB sites by counteracting the ubiquitination of NBS1, and their role is essential for the proper cellular response to DSBs.

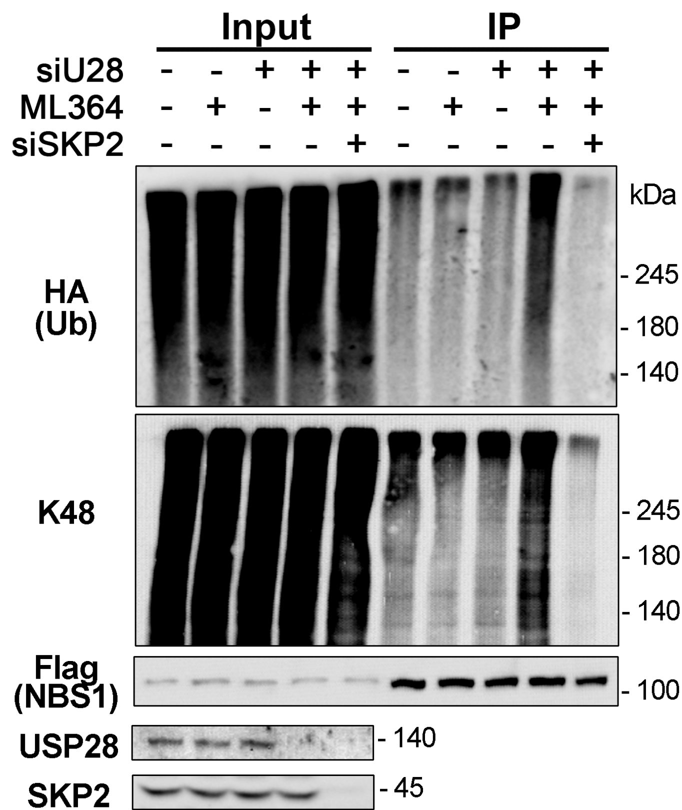



Figure 33. NBS1 ubiquitination is prevented by inactivation of both USP2 and USP28 activities.

Ubiquitination of NBS1 in HEK293T cells transfected with indicated siRNAs and treated with USP2 inhibitor (ML364, 20 μM). Anti-FLAG M2 agarose beads were used for IP, and anti-K48 ubiquitin, anti-HA (for ubiquitin), anti-FLAG (for NBS1), and anti-USP28, anti-SKP2 antibodies were used for western blotting.

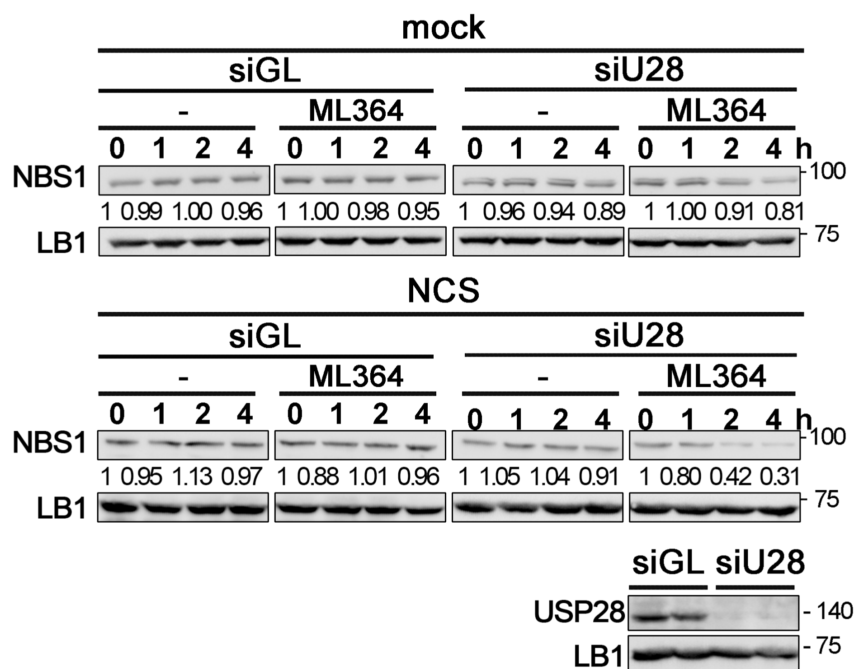
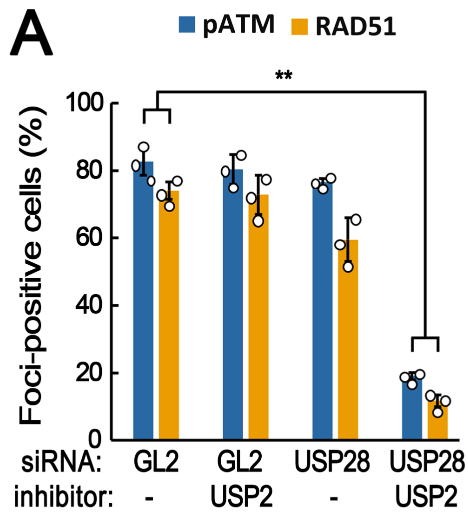
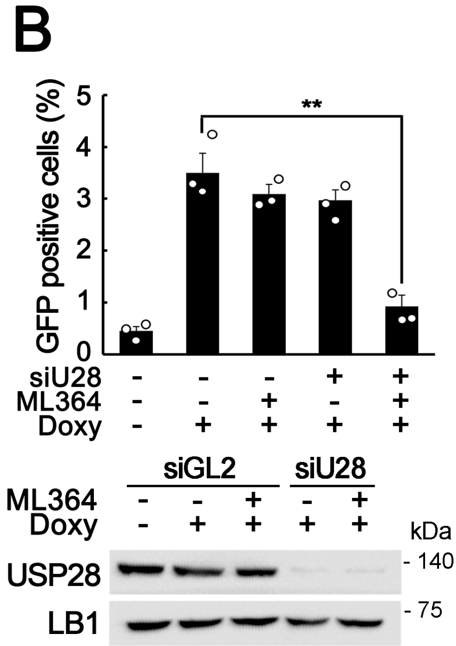



Figure 34. NBS1 protein stability is decreased by inactivation of both USP2 and USP28 activities.

Stability of NBS1 proteins in USP28-depleted and/or USP2 inhibited U2OS cells. Cells treated with NCS were incubated for the indicated time in the presence of cycloheximide (50 μg/mL) and western blotting was carried out to determine the level of NBS1 protein. The levels of NBS1 proteins normalized to that of lamin B1(LB) are shown below each lane.


A
pATM
RAD51
**
Foci-positive cells (%)
100
80
60
40
20
0
siRNA:
GL2
GL2
USP28
USP28
inhibitor:
-
USP2
-
USP2


B
**
GFP positive cells (%)
5
4
3
2
1
0
siU28
ML364
Doxy
siGL2
siU28
ML364
Doxy
kDa
USP28
- 140
LB1
- 75

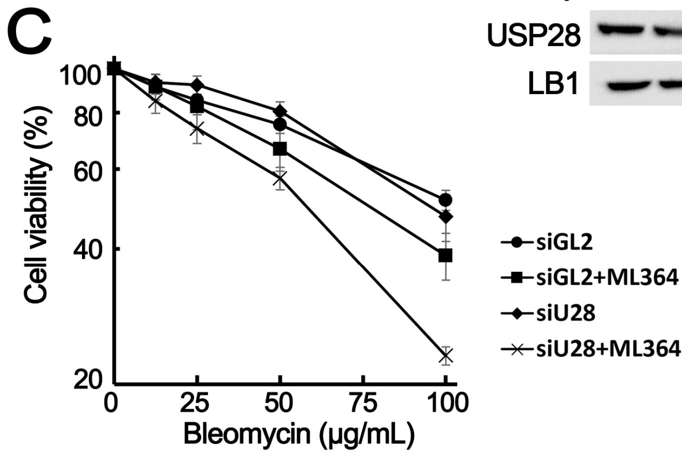

C
Cell viability (%)
100
80
60
40
20
0
25
50
75
100
Bleomycin (μg/mL)
siGL2
siGL2+ML364
siU28
siU28+ML364

**Figure 35. Inactivation of both USP2 and USP28 activities results in defects in ATM activation and HR repair abilities.**

(A) Percentages of pATM or RAD51 foci-positive cells after NCS treatment in USP28 depleted and/or USP2 inhibited U2OS cells. Data in graphs are means ± SD; n = 3. ** $P < 0.01$.

(B) HR repair assay using TRI-DR-U2OS cells. Cells transfected with the indicated siRNAs and incubated for 48 h in the presence or absence of ML164 (20 μM) after induction of I-*Sce*1 by doxycycline treatment. Data in graphs are means ± SD; n =3. ** $P < 0.01$. Lower panel is the western blot showing the depletion of USP28.

(C) Cell viability against bleomycin in USP28 depleted and/or USP2 inhibited cells. Cells were treated with various concentrations of bleomycin for 48 h, and WST-1 assay was carried out to measure the percentage of viable cells relative to undamaged cells. Data in graphs are means ± SEM; n = 3.

### 3.2.7. p97/VCP activity is required for dissociation of the MRN complex from DSB sites.

The p97/VCP which unfolds or segregates ubiquitinated substrates from their partners is an important component of the ubiquitin-proteasome system (van den Boom and Meyer 2018), and association of the p97/VCP in the DSB response has been demonstrated in a few studies, such as removal of RNF8 (Singh et al. 2019) or KU 70/80 (van den Boom et al. 2016) from DSB sites. Recently, inactivation of p97 was shown to reduce the disassembly of the MRN complex from DSB sites, resulting in defective DNA repair by excessive end-resection (Kilgas et al. 2021). While MRE11 was postulated as a target of p97/VCP in this study, ubiquitinated NBS1 at the DSB sites can also be a target of p97/VCP. As shown in Figure 36, while dissociation of the MRN complex from DSB sites premature disassembly of the MRN complex caused by inactivation of USP2 and USP28 was not completely prevented by treatment with the p97/VCP inhibitor, DBeQ, was significantly reduced by inhibition of p97/VCP. Therefore, dissociation of the MRN complex from DSB sites by NBS1 ubiquitination also, at least, partly requires p97/VCP activity.

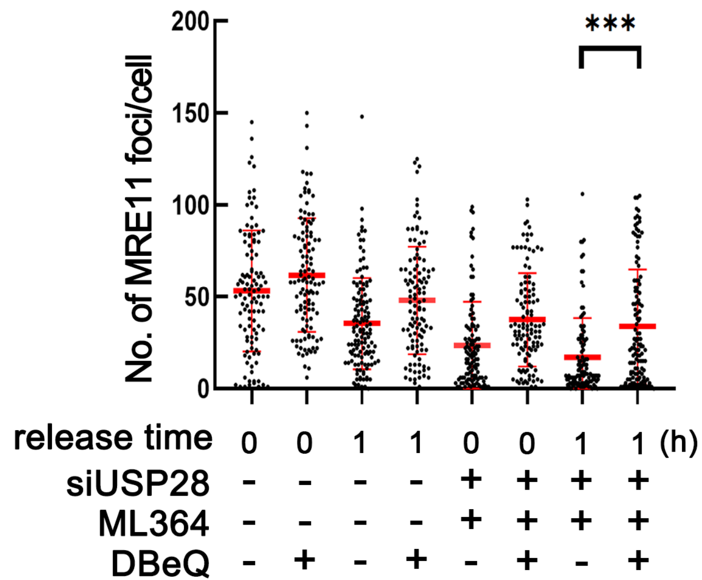



Figure 36. Inhibition of p97/VCP prevents premature disassembly of the MRN complex from DSB sites.

U2OS cells transfected with indicated siRNAs and treated with inhibitors as indicated. Cells were treated with NCS for 15 min and incubated in fresh medium for 0 h or 1 h. MRE11 immunofluorescence staining was performed. For quantitation, 100 cells were counted for number of foci per cell. Bars in graphs are means ± SD; *** $P < 0.001$.

# 4. Discussion

The MRN complex is one of the key factors in the DSB response, and its recruitment to DSB sites and its roles in the DSB response have been well documented in many previous studies. However, the mechanism of its removal from DSB sites is not clearly understood. In this study, I showed that K48-linked ubiquitination of the NBS1 by $SCF^{SKP2}$ E3 ligase was shown to increase after DSBs and to be responsible for premature disassembly of the MRN complex from DSB sites in cells lacking RECQL4 helicase activity. Next, I found that counteracting this ubiquitination by overexpression of deubiquitinases, USP28 or USP2 recovered repair defects in RECQL4-defective cells (Figure 10-12 and 14-16). Furthermore, I figured out that defects in deubiquitinases counteracting the NBS1 ubiquitination resulted in the premature disassembly of the MRN complex from DSB sites in cells with intact RECQL4 (Figure 31-35). Based on this observation, I suggest that K48-linked ubiquitination of the NBS1 protein by $SCF^{SKP2}$ E3 ligase is responsible for the removal of the MRN complex from DSB sites and opposing activity by deubiquitinases plays a critical role in determining the timing of its removal. The suggested mechanism controlling the stability of the MRN complex at DSB sites is summarized in Figure 37.

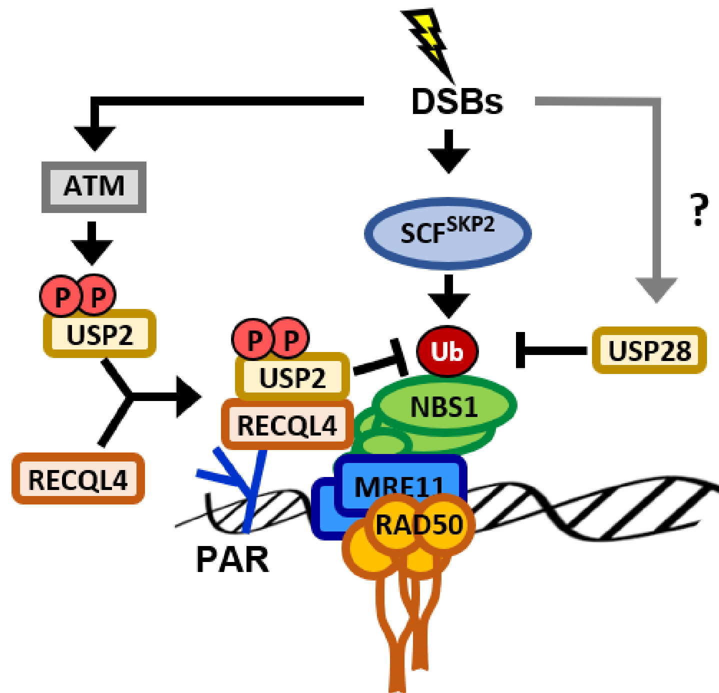



Figure 37. Diagram showing the control of NBS1 ubiquitination during the DSB response.

After DSBs, NBS1 is ubiquitinated by $SCF^{SKP2}$ in K48-dependent manner, which is responsible for disassembly of the MRN complex. Simultaneously, USP2 and USP28 are recruited to DSB sites and counteract this ubiquitination, thereby stabilize the MRN complex. ATM phosphorylation of USP2 promotes interaction with RECQL4 and recruitment to DSB sites. Ub, ubiquitin. P, phosphorylation.

4.1. K48-linked ubiquitination of NBS1 is critical for disassembly of the MRN complex.

Ubiquitination of proteins at DSB sites has been extensively studied and shown to play important roles in DSB response. These roles include promoting the recruitment of checkpoint and repair proteins at damage sites, altering protein-protein interactions, and clearing repair signaling (Schwertman, Bekker-Jensen, and Mailand 2016; Yu, Qin, and Lou 2020). Ubiquitination of NBS1 was previously observed in several studies. RNF8-dependent ubiquitination of NBS1 is reportedly important for optimal binding of NBS1 to DSB sites (Lu et al. 2012), and NBS1 ubiquitination by $SCF^{SKP2}$ E3 ligase or Pellino1 plays an important role in ATM activation (Ha et al. 2019; Wu et al. 2012). However, those reports were on K6- or K63-linked ubiquitination, which are known to affect protein-protein interaction. Therefore, the current study is the first to reveal the K48-linked ubiquitination of NBS1. Although the reported ubiquitination of NBS1 by $SCF^{SKP2}$ E3 ligase was a K63-linked form (Wu et al. 2012), $SCF^{SKP2}$ E3 ligase targets many proteins, including p27, for degradation by K48-linked ubiquitination (Carrano et al. 1999; Wang et al. 2012). Consistent with this notion, K48-linked and $SCF^{SKP2}$ dependent ubiquitination of NBS1 was observed in NCS-treated cells (Figure 8 and 34), and depletion of UBC13, which was shown to be an E2 for K63-ubiquitination of NBS1 (Wu et al. 2012), did not prevent premature

disassembly of the MRN complex in RECQL4-depleted cells, whereas depletion of CDC34, which supports K48-linked ubiquitination (Petroski and Deshaies 2005), did (Figure 6). These results strongly suggest that K-48 linked ubiquitination of NBS1 indeed occurs and is responsible for premature disassembly of the MRN complex from DSB sites in RECQL4-defective cells.

### 4.2. USP28 overexpression recovered repair defects in RECQL4-defective cells, but depletion of USP28 hardly affected DSB response.

Instability of NBS1 proteins and premature disassembly of the MRN complex from DSB sites were observed only in RECQL4-depleted cells (Figure 1 and 7), although K48-linked ubiquitination of NBS1 occurred in both mock- and RECQL4-depleted cells (Figure 8). Additionally, interaction of SKP2 with the MRN complex was observed regardless of the presence of RECQL4 proteins (Kim et al. 2021). Therefore, the absence of RECQL4 may not influence the stability of the MRN complex via affecting the ubiquitination step. As overexpression of USP28 stabilizes the MRN complex, deubiquitination may be a plausible mechanism for stabilization of the MRN complex, and RECQL4 appears to be essential for this control.

USP28 is known to be involved in cancer-related pathways by antagonizing FBW7-dependent ubiquitination of several proteins,

such as c-MYC, C-JUN, ΔNp63, and Hif-1α (Diefenbacher et al. 2015; Flugel, Gorlach, and Kietzmann 2012; Prieto-Garcia et al. 2020). Initially, USP28 was reported to play an important role in the DSB response because depletion of USP28 results in the reduction of protein levels of 53BP1, CHK2, MDC1, and NBS1 after ionizing radiation in H460 lung carcinoma cells (Zhang et al. 2006). The recruitment of USP28 proteins to DSB sites through interaction with the tandem BRCT domains of 53BP1 also supports this notion (Knobel et al. 2014). Furthermore, co-expression of any one of two NBS1 interacting domains in USP28 as competitors, prevented the recovery of MRN stability, ATM activation, and HR repair by the overexpression of USP28 in RECQL4-depleted cells (Kim et al. 2021), which suggested that the overexpression of USP28 restored the DSB response in RECQL4-defective cells by directly acting on the MRN complex. However, the association of USP28 with the DSB response has been controversial because depletion of USP28 did not influence the stability of these proteins or the repair ability in other cell lines, including U2OS cells (Zhang et al. 2006; Knobel et al. 2014). Therefore, results of these studies imply the existence of other players that are defective in H460 cells and influence the stability of proteins involved in DSB response. Since many deubiquitinases are recruited to DSB sites, where they play essential roles, other deubiquitinases may also play a redundant role to stabilize the MRN complex during DSB response.

### 4.3. USP2 plays a redundant role with USP28 in counteracting NBS1 ubiquitination in the DSB response.

USP2 is known to be involved in many cellular processes, including circadian rhythm, cell cycle, and cell proliferation (Yang et al. 2014; Kitamura and Hashimoto 2021). USP2 is strongly associated with cell proliferation control and carcinogenesis by targeting proteins such as MDM2 (Stevenson et al. 2007), cyclin D1 (Davis et al. 2016), cyclin A1 (Kim et al. 2012), Aurora-A (Shi et al. 2011), and β-catenin (Kim et al. 2018). However, its involvement in the DSB response has not been shown before, at least partly due to the presence of another deubiquitinase, USP28, which plays a redundant role with USP2.

In this study, I identified a deubiquitinase, USP2, as a new player acting at the DSB site by opposing the ubiquitination of NBS1, thereby preventing premature disassembly of the MRN complex from DSB sites. Furthermore, by inactivating both USP2 and USP28 simultaneously, I clearly showed that these two deubiquitinases play a redundant role in counteracting NBS1 ubiquitination in the DSB response, and that their activity is critical for proper cellular response to DSBs (Figure 31-35). Therefore, the decrease in NBS1 protein levels in USP28-depleted H460 cells might be caused by defects in or reduced activity of other players acting in the control of NBS1. Consistent with this notion, I found in the Cancer Cell Line Encyclopedia gene expression database that the

expression level of USP2 was much lower in H460 cells than in U2OS or HeLa cells (Table 2).

| cell line | Depmap ID | USP2 (9099) | USP28 (57646) |
|---|---|---|---|
| U2OS | ACH-000364 | 1.86393845 | 4.993674362 |
| HELA | ACH-001086 | 2.266036894 | 3.40599236 |
| NCI-H460 | ACH-000463 | 0.650764559 | 4.175524601 |

**Table 2. Relative expression levels of USP2 and USP28 in U2OS, HELA, or H460 cell lines found in Cancer Cell Line Encyclopedia.**
Relative expression levels of USP2 and USP28 in U2OS, HELA, or H460 cell lines were found in Cancer Cell Line Encyclopedia, which is available online at https://depmap.org/portal/ccle/.

**4.4. Counteracting continued NBS1 ubiquitination by deubiquitinases may prevent unwanted accumulation of the MRN complex.**

Although I do not clearly understand the control mechanism of USP28, ATM-dependent control of USP2 recruitment to DSB sites (Figure 20, 23, 25, and 30) suggested that USP2 plays a role in reducing NBS1 ubiquitination and stabilizing the MRN complex only when ATM kinase activity is maintained. Therefore, repair of DSBs and concomitant decrease in ATM activity may result in the dissociation of deubiquitinases and ubiquitination-dependent removal of the MRN complex from DSB sites. Interestingly, in this control of NBS1 ubiquitination, DSBs increase both ubiquitination activity and opposing activity simultaneously at DSB sites. In many ubiquitination-dependent controls of protein stability, timely activation of E3 ubiquitin ligase is a critical step (Nakayama and Nakayama 2006; Ravid and Hochstrasser 2008). However, activation of $SCF^{SKP2}$ E3 ligase for NBS1 ubiquitination appears to occur before its target is removed because the MRN complex should be stably maintained until the repair is completed. As $SCF^{SKP2}$ also plays a role in activating ATM by K63-linked ubiquitination of NBS1 (Wu et al. 2012), early recruitment of $SCF^{SKP2}$ to DSB sites seems inevitable. Therefore, the recruitment of deubiquitinases to prevent premature disassembly of the MRN complex is also required. Furthermore, excessive accumulation of the MRN complex, which may result in genome instability, can be

effectively prevented by activating E3 ligase activity to remove the MRN complex at the beginning of the DSB response. Because MRE11 has nuclease activity (Buis et al. 2008), failure to remove the MRN complex can result in repair defects by excessive MRE11-mediataed end resection (Kilgas et al. 2021). In addition, uncontrolled or enhanced HR activity has been shown to increase genome instability (Traverso et al. 2003; Richardson et al. 2004). Therefore, complete and timely removal of the MRN complex is important for genome integrity, and control of continued ubiquitinating activity by deubiquitinases as long as ATM is active appears to be an efficient strategy to prevent unwanted accumulation of the MRN complex after DNA damage.

### 4.5. USP2 may indirectly reduce NBS1 ubiquitination through interaction with SKP2.

As shown in this study, USP2 stably interacts with NBS1 (Figure 18) and affects ubiquitination of NBS1 (Figure 19). Therefore, USP2 appears to act directly to reduce NBS1 ubiquitination and to stabilize the MRN complex. However, it may also be possible that USP2 acts indirectly to stabilize NBS1. Recently, USP2 was shown to interact with SKP2 via its substrate binding domain of SKP2 and to stabilize substrates of $SCF^{SKP2}$ E3 ligase, such as p21, independently of its deubiquitinase activity (Zhang, Zhao, and Sun 2021). As $SCF^{SKP2}$ E3 ligase is responsible for NBS1 ubiquitination

during the DSB response, USP2 may also be able to reduce NBS1 ubiquitination through interaction with SKP2, although the importance of this mode of action in stabilizing the MRN complex during the DSB response has not yet been determined.

**4.6. USP2 has a potential to influence the MRN stability in the absence of RECQL4.**

As shown in Figure 22 and 23, USP2 stably interacted with RECQL4 in an ATM-dependent manner, and recruitment of USP2 to DSB sites also requires RECQL4. Therefore, the essential role of RECQL4 in the DSB response appears to be mediated by deubiquitinases that counteract NBS1 ubiquitination, such as USP2. Meanwhile, the essential role of RECQL4 in ATM-dependent recruitment of USP2 to DSB sites raises questions about how overexpressed USP2 stabilizes the MRN complex in the absence of RECQL4 as shown in Figure 14-16. In RECQL4-depleted cells, USP2 cannot be recruited to DSB sites through ATM dependent interaction with RECQL4 proteins. However, USP2 can interact with NBS1 protein when both proteins are overexpressed (Figure 18), and direct interaction between USP2 and SKP2 was also observed in a previous study (Zhang, Zhao, and Sun 2021). Therefore, USP2 has a potential to directly influence the MRN stability either by deubiquitinating NBS1 or inhibiting $SCF^{SKP2}$ E3 ligase in the absence of RECQL4. An increase in USP2 proteins due to

overexpression may reinforce these modes of action that is not effective in normal circumstances.

### 4.7. RECQL4 and its helicase activity may indirectly play a role in DSB response.

All RECQ helicases in mammalian cells have been shown to be involved in DNA DSB responses (Bernstein, Gangloff, and Rothstein 2010; Croteau et al. 2014), and direct participation of WRN and BLM have been well documented in many studies; WRN stimulates NHEJ by its helicase and exonuclease activities (Shamanna et al. 2016), and BLM plays both pro- and anti-recombination roles by stimulating the end-resection activity of DNA2 (Nimonkar et al. 2011) and by the displacement of RAD51 from resected DNA intermediates (Patel et al. 2017). As RECQL4 has a conserved helicase domain, and its helicase activity is required for end resection by MRE11-CtIP during HR repair, RECQL4 has also been postulated as participating in the end resection process (Lu et al. 2016). However, the end resection process occurs in RECQL4-defective cells as long as the stability of the MRN complex is maintained by either mutation in ubiquitination sites on NBS1 (Kim et al. 2021) or expression of USP28 (Figure 10). Therefore, it is evident that RECQL4 does not directly participate in the HR repair process as a component of the end-resection complex.

Although I have unequivocally demonstrated the essential role of

RECQL4 in stable maintenance of the MRN complex during DSB response, I still do not understand how RECQL4 and its helicase activity influence the stability of the MRN complex during DSB response. It may be possible that RECQL4 is involved in the stabilization process or in the recruitment of a factor or factors that play a role in the stabilization of the MRN complex, such as deubiquitinases. Previously, RECQL4 proteins were shown to bind rapidly to laser micro-irradiation sites, and MRE11 and its nuclease activity were found to be required for the maintenance of RECQL4 proteins on the micro-irradiation site (Lu et al. 2016). However, RECQL4 proteins are shown to be rapidly recruited to micro-irradiation site but stays there only transiently (peaks around 100 s) (Kim et al. 2021). Moreover, depletion of MRE11 or inhibition of its nuclease activity by mirin treatment does not significantly affect the association and dissociation patterns of RECQL4 (Kim et al. 2021). These observations are further supported by a study on the protein dynamics in DNA lesions (Aleksandrov et al. 2018). In this study, cell lines expressing GFP-fused repair proteins under the control of their own promoters were examined, and RECQL4 was shown to bind to micro-irradiation site faster than RAD50, a component of the MRN complex, and begin to dissociate at the time of RAD50 binding. Therefore, I assume that RECQL4 cannot directly participate in the stabilization or recruitment processes throughout the DSB response. As RECQL4 has intrinsic DNA

helicase activity, it has potential to influence the binding or removal of proteins by affecting the DNA or chromatin structures around DSB sites. Therefore, RECQL4 and its helicase activity may indirectly play a role in MRN stabilization by influencing the binding and/or modifications of proteins around DSB sites during the short stay of RECQL4 at the DSB sites. However, I still cannot rule out the possibility that a small amount of RECQL4 protein remains at DSB site and participates directly in the stabilization process throughout the DSB response.

Interestingly, the helicase-defective mutant (Walker B mutant) of RECQL4 still supports the recruitment of USP2 to DSB sites (Figure 22), which appears to contradict previous observations that support the essential role of RECQL4 helicase activity in the DSB response (Kim et al. 2021; Park et al. 2019; Shamanna et al. 2014; Lu et al. 2017; Lu et al. 2016). While the initial recruitment of USP2 appeared to be normal in cells with RECQL4 lacking helicase activity, dissociation of USP2 from DSB sites was much faster in these cells than in cells with wild-type RECQL4 (Figure 22). Therefore, RECQL4 appears to play an additional role in stabilizing USP2 at the DSB sites, which requires helicase activity. However, it remains unclear how the helicase activity of RECQL4 contributes to the binding and maintenance of repair proteins at DSB sites.

### 4.8. Mouse and human cells showed differences in defects caused by loss of RECQL4.

Importance of RECQL4 helicase activity in DSB response has been demonstrated both here and in previous studies, which all used human culture cells (Lu et al. 2016; Park et al. 2019; Shamanna et al. 2014). However, this notion was challenged by a recent knock-in mouse study. Knock-in mice with an amino acid substitution at the ATP binding motif in the DNA helicase domain of RECQL4 appeared normal in development, hematopoiesis, and B and T cell development. Furthermore, a cell line with this mutant allele was as sensitive to several DNA damage-inducing reagents as the isogenic control cell line (Castillo-Tandazo et al. 2019), suggesting that the helicase activity of RECQL4 may not be required for DNA repair. Although I still do not clearly understand what causes differences between mouse and human cells, our results in this study indicate that defects in DSB response caused by loss of RECQL4 could be complemented by other activities. Different expression levels of deubiquitinases targeting the MRN complex or any differences in the control mechanism governing the stability of the MRN complex between mouse and human systems may influence cellular response to DSBs. Whether premature disassembly of the MRN complex is caused by defects in RECQL4 and is prevented by expressing deubiquitinases in mouse cells remain to be seen.

### 4.9. Differences in the expression level of deubiqutinases in different tissues can be the reason why most of the symptoms of RTS are concentrated in certain tissues.

Although type II RTS is caused by mutations in a single gene, RECQL4, RTS patients showed pleiotropic phenotypes and clinical features are extremely heterogeneous. All patients have a characteristic skin rash called poikiloderma, and have many or a few other features including sparse hair, small stature, dental and nail abnormalities, skeletal abnormalities, and increased risk of cancer (Larizza, Roversi, and Volpi 2010). As RECQL4 has been implicated in many cellular functions (Croteau et al. 2014; Ghosh et al. 2012), multiple and heterogeneous phenotypes may be attributed to multiple functions of RECQL4 and the position of mutations in each RTS patient. However, it is still hard to understand why the majority of the symptoms of RTS are concentrated in certain tissues such as skin and bones, despite RECQL4 playing roles in basic functions required for the maintenance of any cell. I still do not clearly understand what functions of RECQL4 are responsible for each symptom of RTS patients. However, if any of the clinical features of RTS are caused by defects in HR or DSB response, differences in the expression level of deubiqutinases in different tissues or cells may give a clue to answer this question. Additionally, it would be interesting to determine whether the expression of USP28 in these tissues reduces the known symptoms of RTS patients or RTS model animals.

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# Abstract in Korean

## 국문 초록

가장 세포독성이 높은 DNA 병변인 DNA 이중 가닥 절단(DSB)에 대한 적절한 세포 반응은 유전체의 무결성을 유지하는 데 중요하다. MRE11-RAD50-NBS1(MRN) 복합체는 DSB 반응에 필수적인 역할을 하며 다양한 변형 및 제어의 대상이다. 그러나, DSB 반응에서 MRN 복합체의 안정성이 조절되는 기작은 잘 알려져 있지 않다. 본 연구에서는 돌연변이가 로트문드-톰슨 증후군(RTS)과 관련된 RECQL4가 DNA DSB 반응에 필요하고, 그것의 헬리케이스 활성이 DSB 반응 중에 DSB 부위의 MRN 복합체를 안정적으로 유지하기 위해 필요하다는 것을 보여주었다. MRN 복합체는 RECQL4 결함 세포에서 $SCF^{SKP2}$에 의한 NBS1의 라이신-48 매개 유비퀴틴화에 의존적인 방식으로 DSB 부위에서 조기에 해체되었다. 이러한 MRN 복합체의 조기 해체는 탈유비퀴틴화 효소 USP28을 발현함으로써 방지할 수 있으며, 이는 RTS 및 RECQL4 결핍 세포에서 상동 재조합 복구 및 ATM 활성화 능력을 충분히 회복시켰다. 이러한 결과는 DSB 반응에서 RECQL4의 필수적인 역할은 DSB 부위에서 MRN 복합체를 안정적으로 유지하는 것이며, RTS 세포에서 DSB 반응의 결함은 MRN 복합체의 안정성을 조절함으로써 회복될 수 있음을 시사한다.

RECQL4가 손상되지 않은 세포에서 DSB 반응 동안 이 유비퀴틴화가 어떠한 역할을 하며 어떻게 조절되는지 추가로 조사하기 위해 몇 가지 탈유비퀴틴화 효소를 선별하였고, USP2가 DSB 부위에서 작용하여 NBS1 유비퀴틴화에 대항하는 새로운 탈유비퀴틴화 효소임을

확인하였다. USP2는 DSB에 대한 주요 체크포인트 인산화효소인 ATM에 의존적으로 DSB 부위에 결합하며 면역 침강 실험에서 NBS1 및 RECQL4와 안정적으로 상호작용하였다. ATM에 의한 USP2 N 말단의 2개의 중요한 잔기의 인산화는 USP2의 DSB로의 결합 및 RECQL4와의 상호작용에 필요하다. USP28 또는 USP2 각각의 불활성화는 DSB 반응에 큰 영향을 미치지 않지만, 두 탈유비퀴틴화 효소를 동시에 불활성화하면 DSB 부위로부터 MRN 복합체의 조기 해체와 ATM 활성화 및 상동 재조합 복구 능력의 결함을 초래한다. 이러한 결과는 NBS1 유비퀴틴화에 대응하는 탈유비퀴틴화 효소가 MRN 복합체의 안정적인 유지와 DSB에 대한 적절한 세포 반응을 위해 필수적임을 시사한다.

**주요어:** DNA 이중 가닥 절단, RECQL4, MRE11-RAD50-NBS1 복합체, USP28, USP2, 로트문드-톰슨 증후군

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