Master's Thesis of Education

Characterization of USP2 recruitment to DNA damage site

USP2의 DNA 손상 부위 결합 특성 연구

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Abstract

DNA double strand break (DSB), the most detrimental DNA lesion in human genome, triggers DNA damage response such as DNA repair, cell cycle regulation, replication stress response. In response to DNA damage, the MRE11-RAD50-NBS1 (MRN) complex contributes to the sensing and repair of DNA damage, which is critical for genomic integrity and cell survival. Thus, MRN complex could be a target of various post-translational modification (PTM) to control its stability or function. Recently, USP2 was identified as a new deubiquitinase that prevents NBS1 ubiquitination to stabilize MRN complex at DSB site. In this study, I showed the mechanism of USP2 recruitment to DSB site through performing live-cell imaging of eGFP fused USP2 after laser-microirradiation treatment. Then, I found out USP2 recruitment to DSB site is dependent on ATM, PARP, and RECQL4 during the DNA damage response. Furthermore, I showed ATM-dependent phosphorylation of two critical residues, Serine 2 and Threonine 137, in N-terminus of USP2 is essential for its recruitment to the DSB site. Collectively, this study discovered a new mechanism of USP2 recruitment to the DSB site which is a prerequisite process for MRN complex stability.

Keyword : Post-translational modification (PTM), Ubiquitination, Deubiquitination, Double strand break (DSB), USP2, ATM, PARP, RECQL4

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1. Introduction

1.1. Study Background

The human genome is continuously threatened by various exogenous and endogenous factors that promote different types of DNA lesions such as DNA single strand-breaks (SSBs) and DNA double- strand breaks (DSBs) (Han & Huang, 2020). DSBs are the most deleterious lesion that cause genome instability and lead to cell death (Da Silva, 2021). To combat this, cells have evolved to activate DNA damage response in which sense and respond to DNA damage and mediate cell cycle regulation, apoptosis, and DNA repair (Pilié et al., 2019). There are two major pathways – non-homologous end joining (NHEJ) and homologous recombination (HR) – that resolve the DSBs. NHEJ activates throughout the cell cycle, whereas HR, considered as an error-free repair pathway, only occurs in S/G2 phase due to the usage of sister chromatid as a repair template (Chatterjee & Walker, 2017). In these cellular responses to DSBs, phosphatidylinositol 3-kinases, such as ataxia telangiectasia-mutated (ATM), Ataxia telangiectasia and Rad3-related (ATR), and DNA-dependent protein kinase (DNA-PK) are activated to recruit various proteins factor related to DNA repair (Blackford & Jackson, 2017; Kim et al., 2022).

MRE11-RAD50-NBS1 (MRN), which contributes to the detecting and repair of DNA damage, initiates HR process at the DSBs. After the MRN complex senses and binds to the DNA damage site, it recruits and assists ATM kinase, which phosphorylates a number of downstream effector proteins such as H2AX and MDC1, to amplify the MRN-ATM signaling (Chatterjee & Walker, 2017). ATM also directly phosphorylates CtIP, an important factor required for end resection in HR, to bind with MRN

complex. Then, MRE11, with the help of CtIP, initiates short range resection of DSB ends to generate 3' overhang (Kim et al., 2022). This single strand is coated by single-strand DNA binding protein (RPA), and this RPA is replaced by RAD51 to start DNA strand invasion to accomplish HR (Liu et al., 2010).

As MRN complex acts as a hub of the DNA damage response, this complex is a major target of post-translational modification, such as phosphorylation, methylation, and ubiquitination to participate in DNA repair process (Lu et al., 2021). phosphorylation of all the members of MRN complex is very critical in initiating the downstream signaling of DNA repair pathway (Lavin et al., 2015). Moreover, MRN complex is precisely controlled by ubiquitination to maintain its stability and function during DNA damage response (Ha et al., 2019; Wu et al., 2012). In particular, the ring finger protein RNF8 promotes optimal binding between NBS1 and DSB site through ubiquitination of Lysine (K) 6 residue in NBS1, and K63-linked ubiquitination of NBS1 by SKP2-SCF E3 ligase enhances the ATM kinase activation and HR repair (Lu et al., 2012; Wu et al., 2012).

Recent study of MRN complex stability at DNA damage site revealed that MRN complex is prematurely disassembled from DSB sites by SKP2-SCF-dependent ubiquitination of NBS1 in RECQL4 deficient cell, and this event is restored, including ATM kinase activity and HR repair, by overexpressing deubiquitinases USP2 or USP28 (Kim et al., 2021; Kim et al., 2022). As K48-linked ubiquitination of NBS1 by SKP2-SCF is reduced in normal cell by overexpressing USP2 or USP28, ubiquitination and deubiquitination of NBS1 may have a critical role in stabilizing MRN complex during the DNA damage response. To clarify how deubiquitinase USP2 and USP28 stabilize MRN complex by counteracting SKP2-SCF-

dependent ubiquitination of NBS1, the mechanism of deubiquitinase recruitment, such as USP2 and USP28, to DNA damage site must be studied.

1.2. Purpose of Research

MRE11-RAD50-NBS1 (MRN) complex has a key role in DNA damage responses not only in DNA detection and signaling but also in succeeding HR repair pathway (Qiu & Huang, 2021). Mutations in any of these three genes possibly cause genomic instability such as loss of genetic information, increasing sensitivity to genotoxic agents that produce DNA lesions. Also, in MRN deficient cells, Activation of ATM, a major checkpoint kinase against DSBs that phosphorylates several DDR factors, is defective during the DNA damage response (Bian et al., 2019). For that reason, sustaining stable structure and function of MRN complex is one of the critical for maintaining human genome integrity.

As I mentioned in study background, MRN complex is controlled by various post-translational modification. In particular, SKP2-SCF-dependent ubiquitination of NBS1, one of the members of MRN complex, cause the premature disassembly of the MRN complex in RECQL4 defective cell. However, overexpressing deubiquitinase USP28 restored the stability of the MRN complex at DSB sites, ATM activation, and HR repair in these cells (Kim et al., 2021). On account of that biological phenomenon, understanding the control mechanism of MRN complex stability by deubiquitinating or ubiquitinating NBS1 is required to study. However, the mechanism of deubiquitinase recruitment at DNA damage site is still obscure.

In this study, I performed live-cell imaging of eGFP-USP2 after laser-microirradiation treatment to identify the mechanisms of USP2, a new deubiquitinase acting on stabilizing MRN complex, recruitment to DNA damage site by exploring the essential domain and indispensable two

phosphorylating residue of USP2. I also identified the interactors of UPS2, in which help USP2 to recruit at DSB site, by conducting immunoprecipitation assay between USP2 and the factors acting on the DNA damage response.

2. Materials and Methods

2.1. Cell culture, transfection of DNA, and antibodies

Human U2OS cells were cultured at 37 ℃ in a humidified incubator with 5% CO_2 in Dulbecco's modified Eagle's medium (DMEM; welgene, Korea) supplemented with 10% fetal bovine serum (FBS; Welgene, Kore) and 1% antibiotics (Welgene, Korea).

DNA plasmids was transfected into U2OS cells by polyfect (QIAGEN) or Lipofectamine 3000 (Invitrogen). For protein depletion, siRNAs were transfected using a Neon electroporator (Invitrogen) and incubated for 48 h. siGL2 and siRECQL4 were synthesized by Bioneer (Daejeon, Korea). The sequences of the sense strand of siRNAs used in this study were following: GL2 (targeting firefly luciferase), 5′-AACGUACGCGGAAUA CUUCGA-3′; RECQL4, 5′- GACUGAGGACCUGGGCAAA-3′;

The ATM inhibitor, KU-55933 (SML1109), was purchase from Sigma-Aldrich. The DNA-PK inhibitor, NU7441 (Axon 1463), was purchased from Axon Medchem (Axon2678). The PARP inhibitor, olaparib, was purchased from Sellekchem and the PARG inhibitor, PDD00017273, was purchased from Sigma-Aldrich.

The primary antibodies used in this study are followings: anti-NBS1 (A7703, ABclonal), anti-USP2 (A10399, ABclonal), anti-HA (AE008, ABclonal), and Anti-RECQL4, which was prepared by Abfrontier by immunizing rabbits with recombinant N-terminal (amino acid residues 1–241) RECQL4.

2.2. Plasmid preparation

Wild-type USP2 cDNA and its domain for N-terminal region cDNA (1-160 aa for CD1, 1-258 aa for CD2, 1-360), and C terminal region (150-605 aa for ND1, 250-605 aa for ND2, 350-605 aa for ND3) were amplified by polymerase chain reaction and subcloned into the pcDNA3.1 (-) plasmid 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. Immunoprecipitation

For immunoprecipitation assay, U2OS cells were lysed by a buffer containing 40 mM Tris-HCl (pH 7.5), 100 mM NaCl, 2.5 mM MgCl2, 1 mM dithiothreitol, 5% glycerol, 0.2% NP-40, 20 mM NaF, 0.1 mM sodium orthovanadate, and protease and phosphatase inhibitors. The lysate was sheared by sonication. After shearing by sonication, cell lysate was treated with benzonase (90 units/mL) at 4 °C for 4 h. The lysate was cleared by centrifugation at 18,000 × g Journal Pre-proof 20 for 10 min and the supernatant was used for immunoprecipitation.

2.4. Laser microirradiation and live cell imaging of fluorescent proteins

For laser micro-irradiation, U2OS cells were weeded and grown on a dish with a thin glass bottom (SPL, Korea, 101350) and were treated with 5 μg/mL Hoechst 33342 for 10 min before micro-irradiation treatment. Cells were locally irradiated with a fixed laser wavelength (405nm, 100% laser output at a scan speed of 32.77 Journal Pre-proof 21 μs/pixel with 1

iteration) using a LSM880 laser confocal microscope system with a temperature-controlled CO2 chamber (Zeiss, Germany). The Plan-Apochromat 63X oil objective lens was used to observe the time-lapse images, and the fluorescence intensities of irradiated areas relative to non-irradiated areas within the nucleus were measured and analyzed by using ZEISS ZEN 2.3 SP1 software (Zeiss, Germany).

2.5. Statistics analysis

Statistical significance between groups was determined by two-tailed Student's t-test using Journal Pre-proof 22 GraphPad Prism5 software. Data are presented as mean ± SD (standard deviation) or SEM (standard error of the means). All statistical tests are indicated in the figure legends.

2.6. Table of plasmid DNA used in this study

Plasmid DNA

Plasmid DNA	Transfection amount	Vector
eGFP-USP2	1 μg	PCDNA3.1(-)
eGFP-USP2-CD1	1 μg	PCDNA3.1(-)
eGFP-USP2-CD2	1 μg	PCDNA3.1(-)
eGFP-USP2-CD3	1 μg	PCDNA3.1(-)
eGFP-USP2-ND1	1 μg	PCDNA3.1(-)
eGFP-USP2-ND2	1 μg	PCDNA3.1(-)
eGFP-USP2-ND3	1 μg	PCDNA3.1(-)
eGFP-USP2 (S2A)	1 μg	PCDNA3.1(-)
eGFP-USP2 (S94A)	1 μg	PCDNA3.1(-)
eGFP-USP2 (T137A)	1 μg	PCDNA3.1(-)
eGFP-USP2 (S142A)	1 μg	PCDNA3.1(-)
eGFP-USP2 (AA)	1 μg	PCDNA3.1(-)
eGFP-USP2 (4A)	1 μg	PCDNA3.1(-)
eGFP-USP2 (EE)	1 μg	PCDNA3.1(-)
eGFP-USP2 (4E)	1 μg	PCDNA3.1(-)
2HA-USP2	1 μg	PCDNA3.1(-)

2.7. Abbreviation

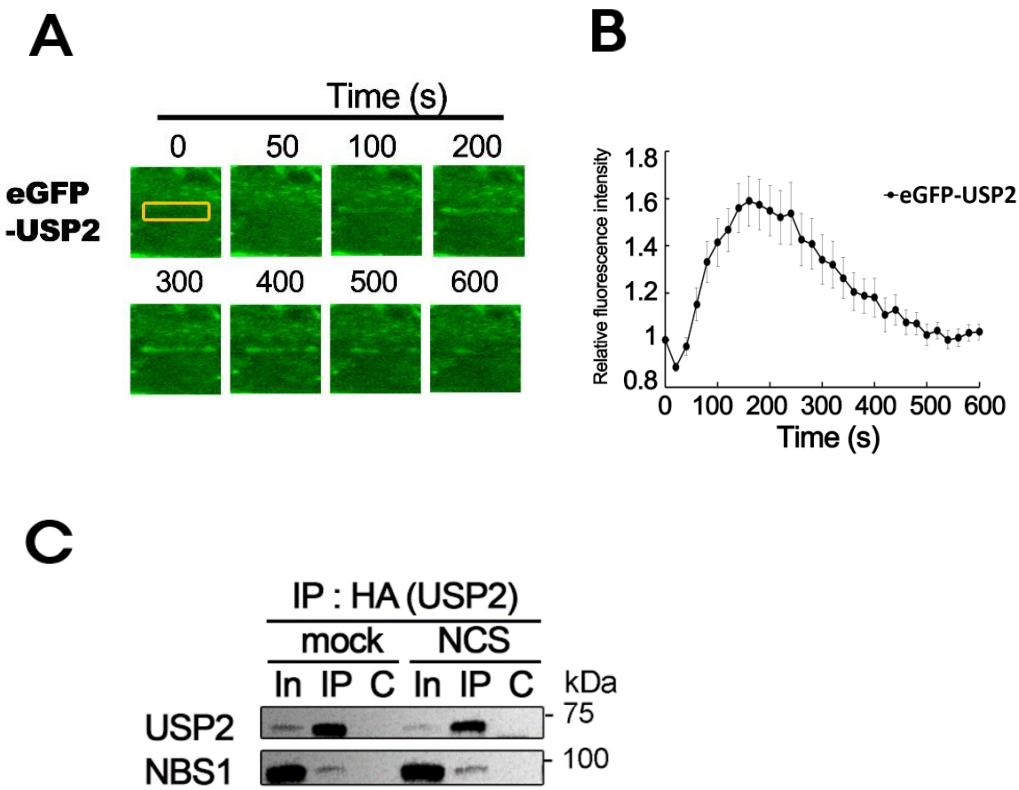
The abbreviations used are:

MRN, MRE11-RAD50-NBS1; DDR, DNA damage response; DSB, double-strand break; HR, homologous recombination; ATM, ataxia telangiectasia-mutated; DNA-PK, DNA-dependent protein kinase; NCS, neocarzinostatin; PARylation, poly(ADP-ribosyl)ation; EGFP, enhanced green fluorescent protein.

3. Results

3.1 USP2 is recruited to laser-induced DNA damage site and interacts with NBS1.

USP2 counteracts the ubiquitination of NBS1 to prevent the premature disassembly of MRN complex, and this event stabilizes MRN complex in which contribute to the DNA damage detection and repair (Kim et al., 2022). However, the control mechanism of USP2 recruitment and stabilizing the MRN complex during the DNA damage response is unknown. To address this, I thus attempt to examine how USP2 is recruited to DSB site by performing live-cell imaging of eGFP-USP2. For live-cell imaging, I fused the eGFP to USP2 protein to chase its localization at DSB site, then U2OS cells were subjected to laser-microirradiation. eGFP-USP2 was rapidly bound to laser-induced DNA damage site. To be more specific, the retention of USP2 binding at DSB site peaked at 150 s, and the signal was gradually decreased until 600 s (Fig. 1A and Fig. 1B). This result suggests that USP2 may play a role in the DNA damage response by directly acting on the target at DSB site. Therefore, I conducted the immunoprecipitation of HA tagged USP2 with NBS, one of the members of MRN complex, to confirm if USP2 is directly involved with MRN complex stability. HA tagged USP2 was immnunoprecipitated with endogenous NBS1 upon NCS treatment, and their interaction was continued regardless of NCS treatment (Fig. 1C). These data suggest that USP2 directly bind to laser-induced DNA damage site, and USP2 possibly acts on NBS1 to stabilize MRN complex.



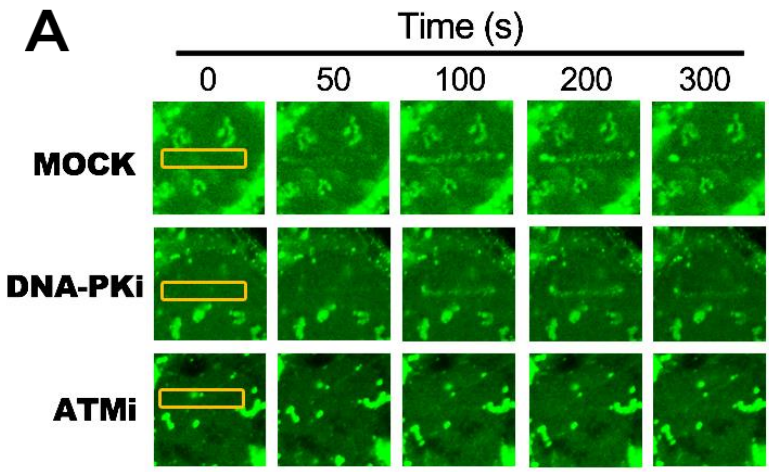
[Figure 1] USP2 is recruited to laser induced DSB site and directly interacted with NBS1.

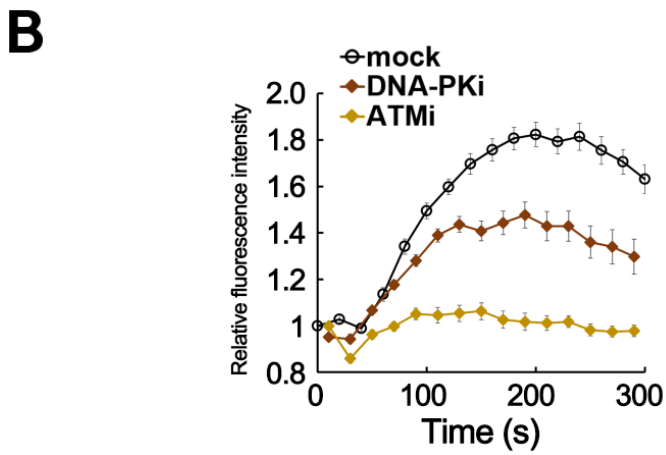
(A-B) eGFP-USP2 recruitment to laser-induced DSB site. U2OS cells, overexpressed with eGFP-USP2 construct, were laser-microirradiated at indicated region. Representative time-lapse images of eGFP-USP2 (A) and quantification of fluorescent intensity of eGFP-USP2 binding to laser-induced DSB sites in U2OS cells (B). Data in graphs are means ± SEM; n =20. (C) USP2 interaction with NBS1, U2OS cells were overexpressed by 2HA-USP2 and were immunoprecipitated with anti-HA antibody. Anti-USP2 (for USP2) and anti-NBS1 (for NBS1) were used for Western blotting. Lines: In, 10% of input for IP; C control IP with nonspecific IgG.

3.2 USP2 recruitment to DSB site is dependent on ATM, PARP, and RECQL4, respectively.

I figured out that USP2 directly act on the DSB site, but I still need to know how its recruitment to DSB site is controlled by DNA damage response factors. In DNA damage response, it is generally accepted that ATM and DNA-PK regulate DNA damage response as well as homologous recombination by phosphorylating and recruiting various proteins involved in DNA repair pathway at DSB site (Blackford & Jackson, 2017; Menolfi & Zha, 2020). To test if these kinases have roles in USP2 recruitment in DSB site, I performed a live-cell imaging with laser microirradiation after treating ATM and DNA-PK specific inhibitors, KU55933 and NU7441, in eGFP-USP2 overexpressed U2OS cells. The retention of eGFP-USP2 binding to the laser induced DSB site in DNA-PKi treated cell showed the same patterns with eGFP-USP2, but the binding intensity was decreased compared to non-treated cell (Fig. 2A and Fig. 2B). In contrast, ATMi treated cell showed almost abolished USP2 binding to the laser induced DSB site (Fig. 2A and Fig. 2B). These results indicate that ATM kinase activity is required for USP2 recruitment at DSB site. Despite these findings, it is indispensable to find other factors controlling USP2 recruitment at DSB site. Poly ADP-ribosylation (PARylation), which is catalyzed by poly(ADP-ribose) polymerases (PARPs) and executed by poly(ADP-ribose) glycohydrolase (PARG), is another important process that facilitates the recruitment of DNA damage factors such as MRE11 and RECQL4 (Peng et al., 2021). Then, I proved that PARylation is one of the important modification for USP2 recruitment to the DSB site by conducting microirradiation induced live-cell imaging with the treatment of PARP1 and

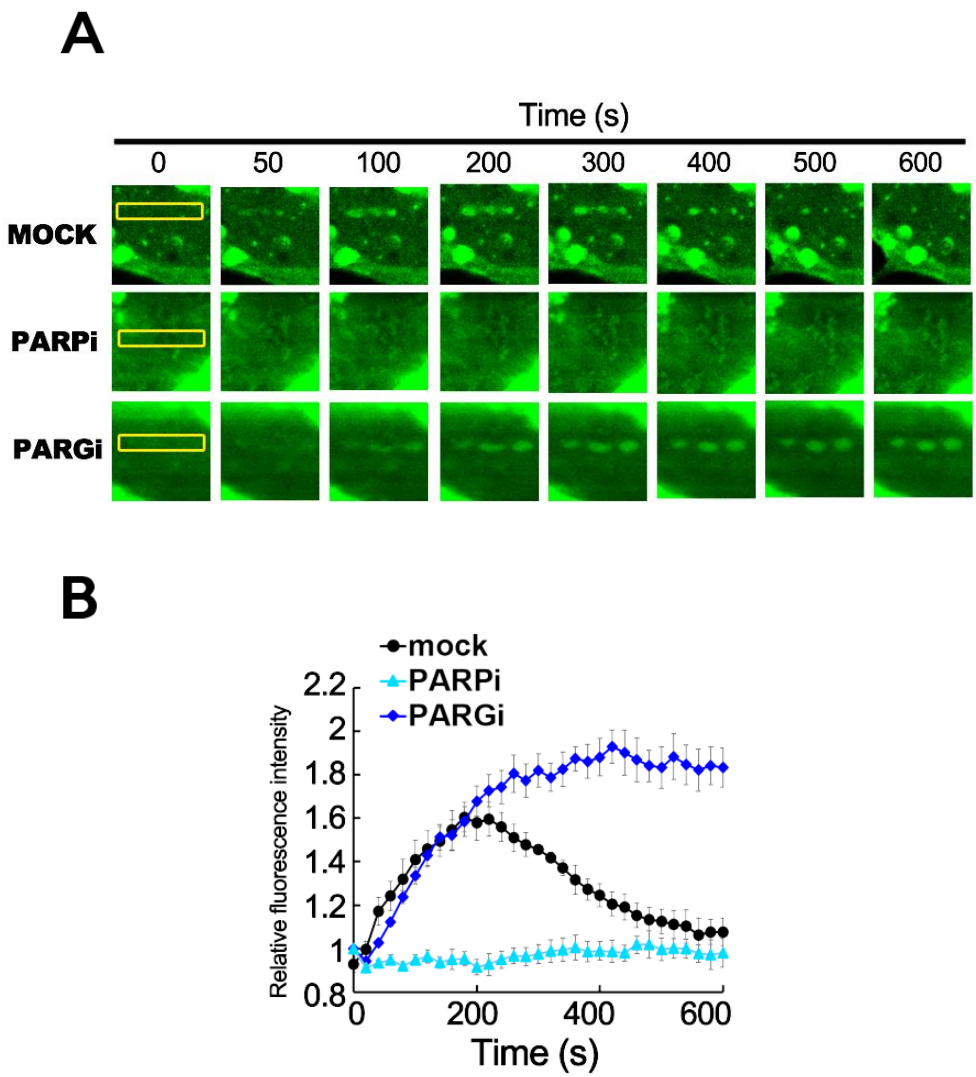
PARP2 specific inhibitor, olaparib, and PARG specific inhibitor, PDD0017273. In the live-cell imaging, PARP inhibitor completely abolished USP2 recruitment, and PARG inhibitor prevented the dissociation of USP2 from DSB site (Fig. 3A and Fig. 3B). This result raises the possibility that USP2 recruitment at DSB site has dependency of interaction with RECQL4. It is because RECQL4, a member of RecQ family helicase that has a crucial role in DNA damage response, is regulated by PARylation in response to DSB (Kim et al., 2021). Indeed, USP2 was interacted with RECQL4 and the interaction between two proteins was significantly intensified upon NCS treatment (Fig. 4A). Furthermore, inhibited ATM kinase activity completely disturbed USP2 interaction with RECQL4 (Fig. 4B). Also, the recruitment of USP2 at laser-induced DSB site was blocked when RECQL4 was depleted by siRNA (Fig 4C). These results revealed that the PARylation dependency of USP2 recruitment might be due to the RECQL4 recruitment at DSBs which is PARylated by PARP1. Collectively, USP2 is recruited to DSB site through the interaction with RECQL4, which is dependent on ATM kinase activity.





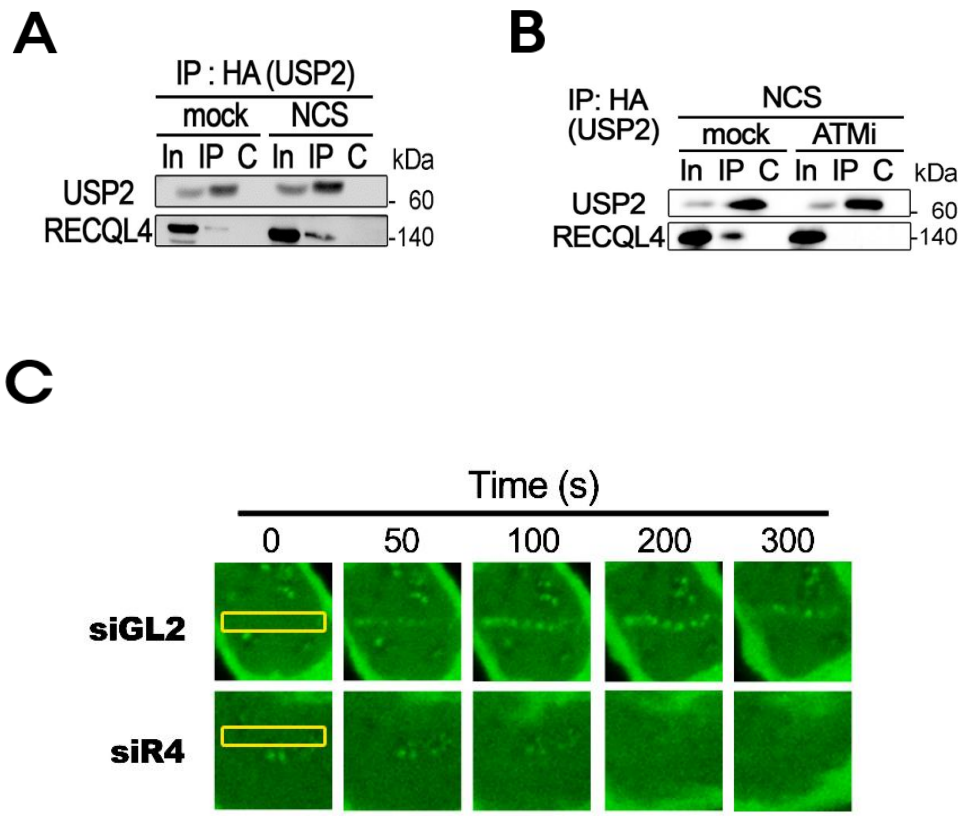
[Figure 2] USP2 is recruited to DSB site is dependent on ATM activity.

(A-B) eGFP-USP2 recruitment to DSB site upon DNA-PKi and ATMi. U2OS cells were overexpressed with eGFP-USP2 construct and treated with inhibitors (20 μM) of ATM (KU55933) or DNA-PK (NU7441) 1 h before the test. Then, the cells were laser-microirradiated at indicated region. Representative time-lapse images of eGFP-USP2 (A) and quantification of fluorescent intensity of eGFP-USP2 binding to laser-induced DSB sites in U2OS cells (B). Data in graphs are means ± SEM; n =20.



[Figure 3] USP2 is recruited to DSB site is dependent on PARP.

(A-B) eGFP-USP2 recruitment to DSB site upon DNA-PKi and ATMi. U2OS cells were overexpressed with eGFP-USP2 construct and treated with inhibitors (20 μM) of PARP (olaparib) or PARG (PDD00017273) 1 h before the test. Then, the cells were laser-microirradiated at indicated region. Representative time-lapse images of eGFP-USP2 (A) and quantification of fluorescent intensity of eGFP-USP2 binding to laser-induced DSB sites in U2OS cells (B). Data in graphs are means ± SEM; n =20.

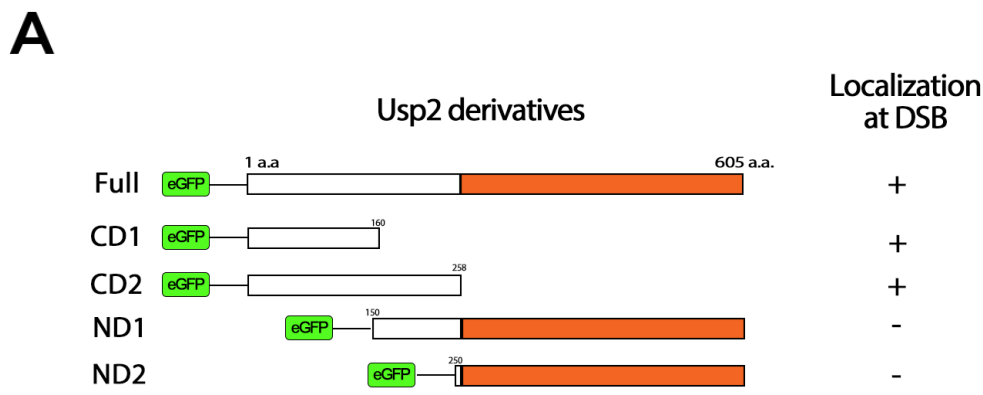


[figure 4] USP2 is recruited to DSB site is dependent on RECQL4.

(A-B) Interaction between USP2 and RECQL4. U2OS cells were overexpressed by 2HA-USP2 plasmid and were immunoprecipitated with anti-HA antibody. Cells were treated with NCS (A) or NCS and ATMi (KU55933, 20 μM) (B). Anti-USP2 (for USP2) and anti-RECQL4 (for RECQL4) were used for Western blotting. Lines: In, 10% of input for IP; C control IP with nonspecific IgG. (C) eGFP-USP2 recruitment to laser-induced DSB site in RECQL4 depleted cells. U2OS cells were transfected with siRECQL4 by electroporation and then overexpressed with eGFP-USP2 plasmid. The cells were laser-microirradiated at indicated region.

3.3 N-terminus of USP2 domain is crucial for ATM dependent recruitment to DSB sites.

To understand which region of USP2 is crucial for its recruitment at DSB site, I constructed six eGFP fused USP2 domains, which were truncated to CD1 (a.a. 1-160), CD2 (a.a. 1-258), CD3 (a.a. 1-360), ND1 (a.a. 150-605), ND2(a.a. 250-605), and ND3 (a.a. 305-605) (Fig. 5A). To support this notion, U2OS cells were transfected with these eGFP fused domain and performed the microirradiation induced live cell imaging. Deletion of N-terminus of USP2 domains abolished its recruitment to the DNA damage site whereas deletion of C-terminus domains showed almost identical recruitment pattern to eGFP-USP2 (Fig. 6A and Fig. 6B). Particularly, N-terminus of USP2 containing amino acid residues 1 to 160 (CD1) has the most intensive retention at laser-induced DSB sites. These data demonstrate that USP2-CD1 domain is essential and sufficient for its recruitment at DSB sites. To test if USP2-CD1 domain also shows ATM, PARP, and RECQL4 dependency as USP2 full-length, U2OS cells, overexpressed with eGFP-USP2-CD1, were subjected to laser microirradiation with the treatment of ATMi, DNA-PKi, PARPi, PARGi. The live cell-imaging results exhibited an almost similar recruitment pattern to eGFP-USP2 (Fig. 7 - Fig. 8). Furthermore, immunoprecipitation assay confirmed that USP2-CD1 still interacted with RECQL4. (Fig. 9A and Fig. 9B). Therefore, these data suggest that N-terminus of USP2 has the function to recruit itself to DSB sites in ATM and RECQL4 dependent manner.



[Figure 5] Schematic diagram of truncated USP2 proteins

(A) Schematic diagram of eGFP fused USP2 domain. The USP2 C-terminus (orange) indicates conserved USP domain

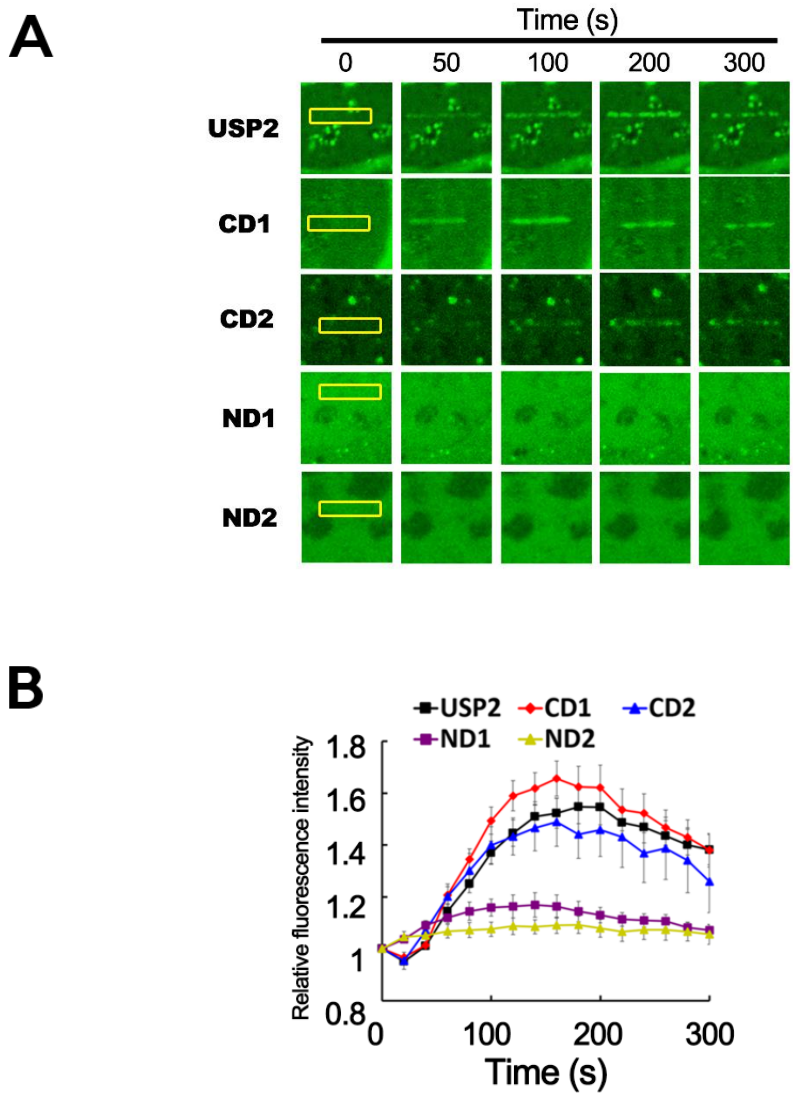
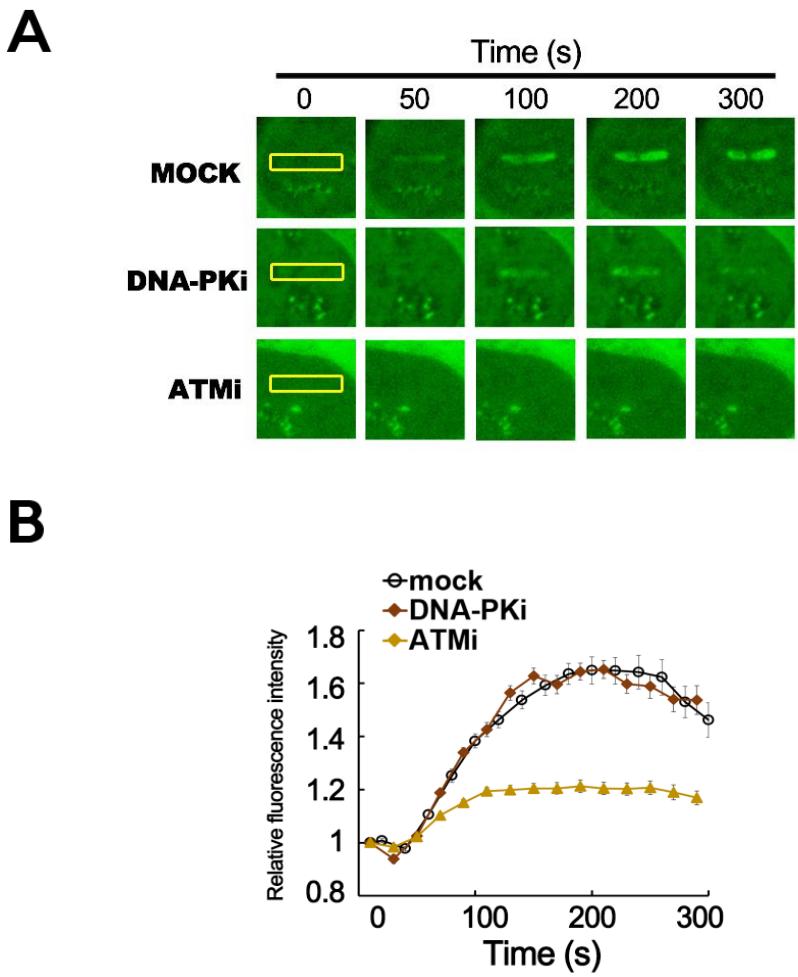


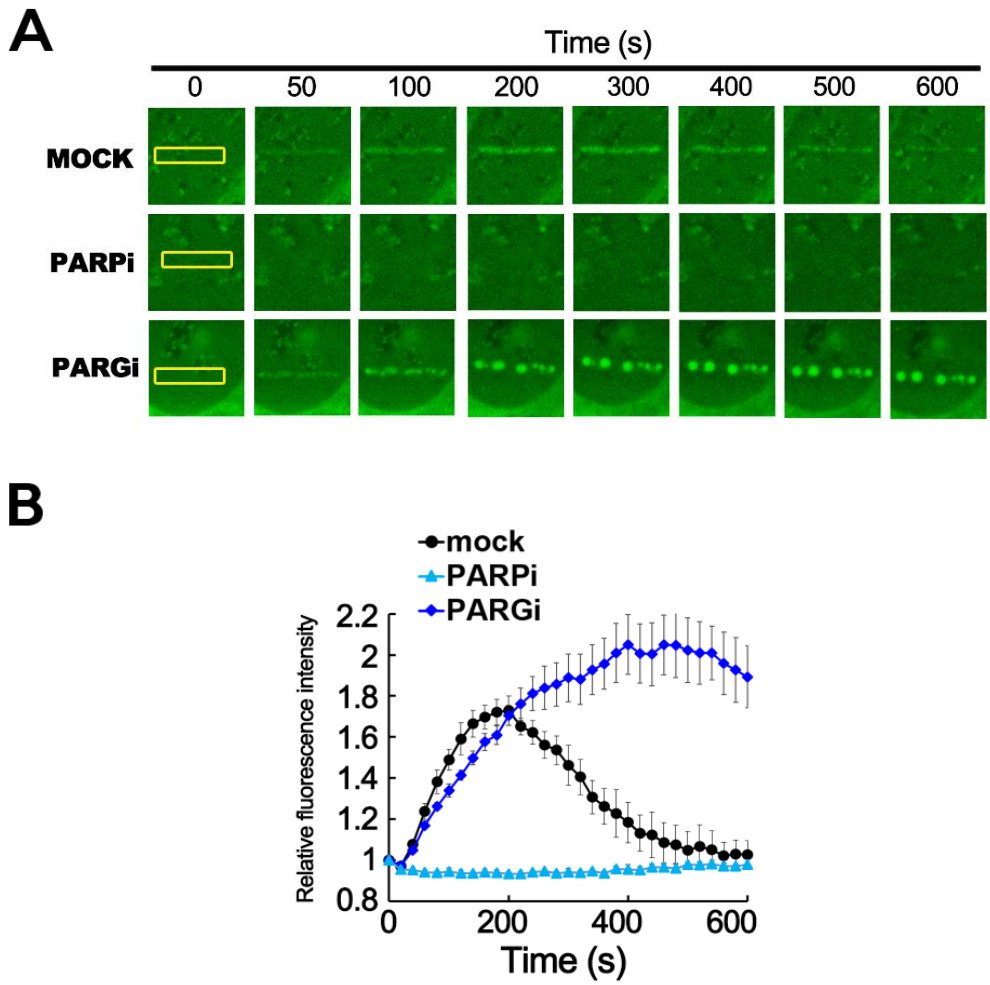
Figure 6] N-terminus domain of usp2 is sufficient for its recruitment to laser-induced DSB site.

(A-B) eGFP-USP2 domains recruitment to laser induced DSB site. U2OS cells were overexpressed with eGFP-USP2 and eGFP-USP2 derivatives construct and the cells were laser-microirradiated at indicated region. Representative time-lapse images of eGFP-USP2 and its derivatives(A) and quantification of fluorescent intensity of eGFP-USP2 and its domain binding to laser-induced DSB sites in U2OS cells (B). Data in graphs are means ± SEM; n =20.



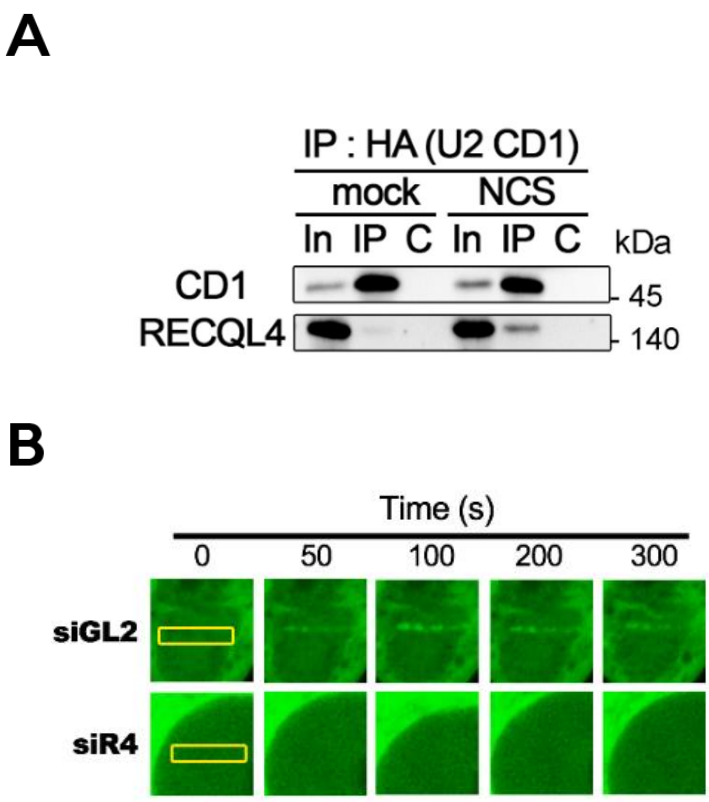
[Figure 7] USP2 CD1 domain recruitment to DSB site is dependent on ATM acitivity.

(A-B) eGFP-USP2-CD1 domain recruitment to DSB site upon DNA-PKi and ATMi. U2OS cells were overexpressed with eGFP-USP2-CD1 construct and treated with inhibitors (20 μM) of ATM (KU55933) or DNA-PK (NU7441) 1 h before the test. Then, the cells were laser-microirradiated at indicated region. Representative time-lapse images of eGFP-USP2 (A) and quantification of fluorescent intensity of eGFP-USP2-CD1 binding to laser-induced DSB sites in U2OS cells (B). Data in graphs are means ± SEM; n =20.



[Figure 8] USP2 CD1 domain recruitment to DSB site is dependent on PARP.

(A-B) eGFP-USP2-CD1 recruitment to DSB site upon DNA-PKi and ATMi. U2OS cells were overexpressed with eGFP-USP2 construct and treated with inhibitors (20 μM) of PARP (olaparib) or PARG (PDD00017273) 1 h before the test. Then, the cells were laser-microirradiated at indicated region. Representative time-lapse images of eGFP-USP2 (A) and quantification of fluorescent intensity of eGFP-USP2-CD1 binding to laser-induced DSB sites in U2OS cells (B). Data in graphs are means ± SEM; n =20.

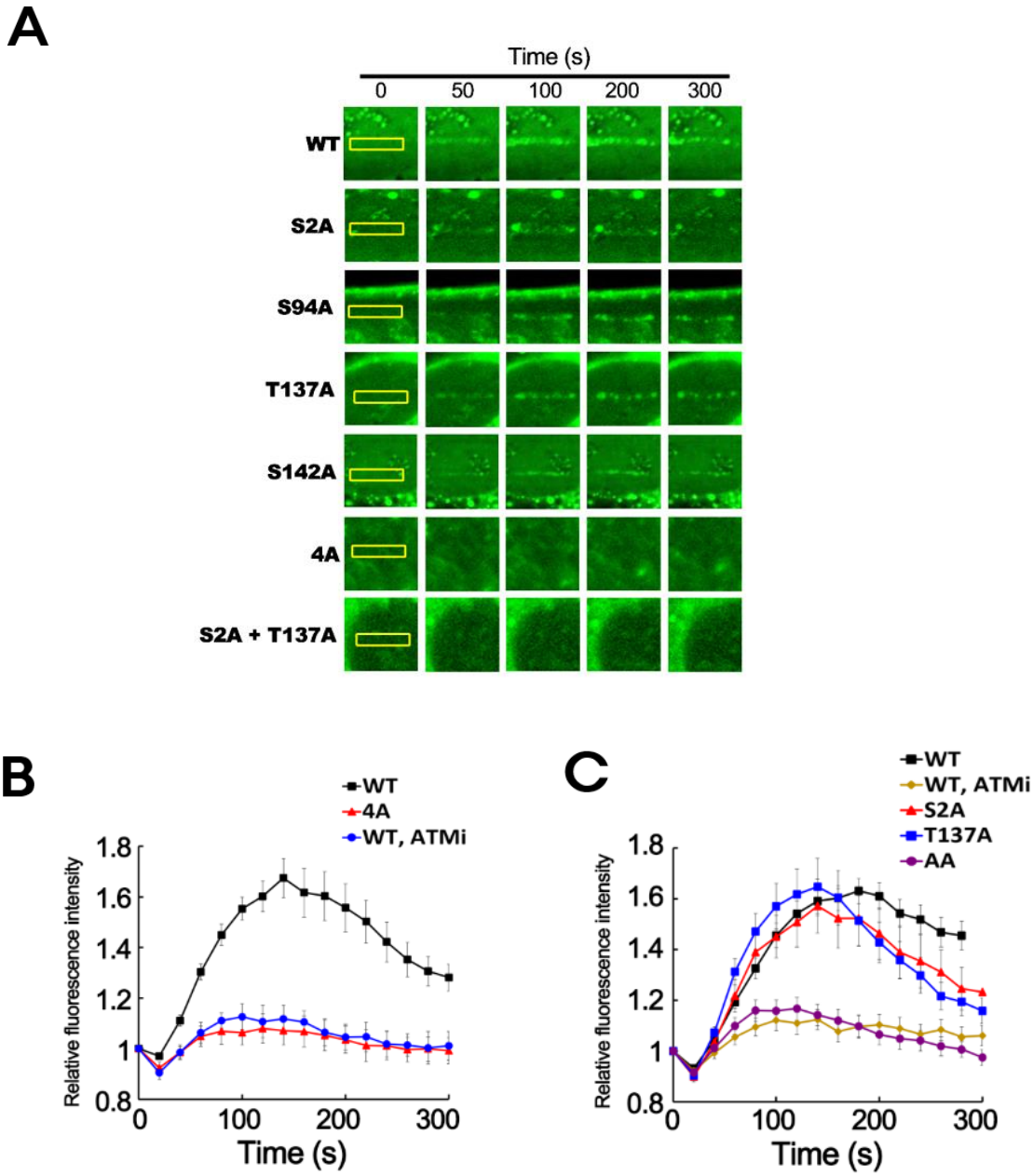


[Figure 9] USP2 CD1 domain recruitment to DSB site is dependent on RECQL4.

(A) Interaction between USP2-CD1 domain and RECQL4. U2OS cells were overexpressed by 2HA-USP2-CD1 plasmid and were immunoprecipitated with anti-HA antibody. Cells were treated with NCS, and Anti-HA (for USP2-CD1) and anti-RECQL4 (for RECQL4) were used for Western blotting. Lines: In, 10% of input for IP; C control IP with nonspecific IgG. (B) eGFP-USP2-CD1 domain recruitment to laser-induced DSB site in RECQL4 depleted cells. U2OS cells were transfected with siRECQL4 by electroporation and then overexpressed with eGFP-USP2-CD1 plasmid. The cells were laser-microirradiated at indicated region.

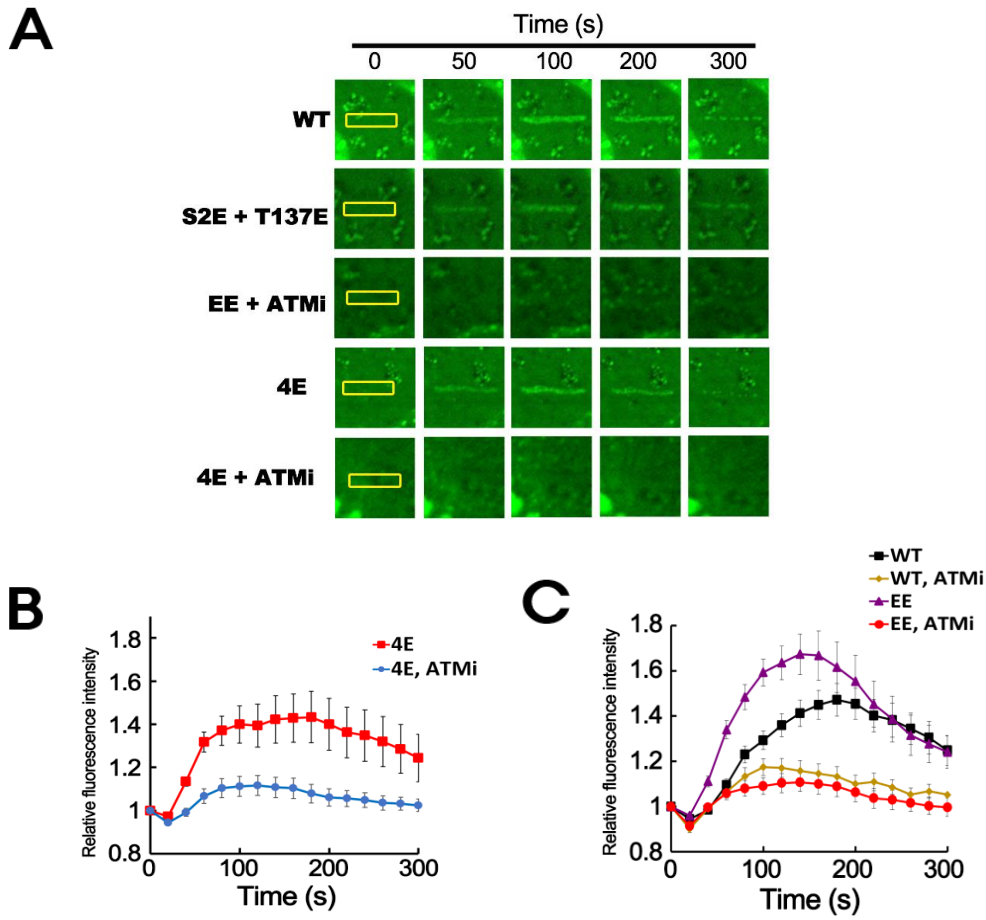
Result 4. ATM phosphorylates two critical residues in USP2 N-termius (S2 and T137) and promotes its recruitment to the DNA damage site.

Given that the N-terminus domain of USP2 is essential for ATM-dependent recruitment to DSB sites, I further investigated whether ATM phosphorylation at N-terminus of USP2 is for its recruitment to DSB sites. ATM kinase is well-documented to phosphorylates SQ/TQ motifs of numerous substrates in response to DNA damage and its activity is disturbed by alanine substitution in Serine or Threonine residue (Dissmeyer & Schnittger, 2011; Traven & Heierhorst, 2005). I thus investigated the phosphorylation sites of USP2 CD1, the most essential domain for its recruitment to DSB site, and I found that it has four SQ/TQ motifs (S2, S94, T137, and S142). Then, I generated phospho-deficient mutants possessing various combination of alanine substitutions in these four S/T residues, and these mutants were conducted to laser micro-irradiation assay. The results demonstrated that individual alanine substitutions of serine or threonine in putative phosphorylation site of USP2 N-terminus did not significantly affect the USP2 recruitment to DSB sites (Fig. 10A). However, alanine substitution of all the serine and threonine residue (4A) in N-terminus of USP2 completely abolished its recruitment to DSB sites (Fig. 10B). I further found out that alanine substitution of two amino acid residues (S2A + T137A, AA) also abolished the DSB-binding activity of USP2 (Fig. 10C). The USP2 phosphor-mimetic with glutamic acid substitution at all the serine and threonine residue (4E) and S2 and T137 residue (S2E + T137E) showed similar recruitment dynamics to DSB sites as eGFP-USP2, but both of their recruitment was abolished by inhibition of ATM kinase activity. (Fig. 11A – Fig. 11C). Collectively, these data suggest that ATM is an important regulator for USP2 recruitment at DSB sties by phosphorylating S2 and T137 residue of USP2 which are indispensable phosphorylation site for the recruitment to the DSB sites.


A
Time (s)
0
50
100
200
300
WT
S2A
S94A
T137A
S142A
4A
S2A + T137A
B
WT
4A
WT, ATMi
Relative fluorescence intensity
1.8
1.6
1.4
1.2
1
0.8
0
100
200
300
Time (s)
C
WT
WT, ATMi
S2A
T137A
AA
Relative fluorescence intensity
1.8
1.6
1.4
1.2
1
0.8
0
100
200
300
Time (s)

[Figure 10] Inhibition of two critical phosphorylation site (S2A and T137A) blocks USP2 recruitment to laser-induced DSB site.

(A-C) various phospho-deficient mutant of USP2 recruitment to laser-induced DSB site. U2OS cells were overexpressed by indicated phospho-deficient mutant of USP2 construct, and the cells were treated with ATM inhibitor (ATMi), KU55933, 1 h before micro-irradiation. Then, laser microirradiation was performed. WT, wild type USP2; 4A, USP2 with substitution of 4 ATM phosphorylation sites (S2, S94, T137, S142) to alanine; S2A + T137A or AA, substitution of S2 and T137 to alanine. Representative time-lapse images of eGFP-USP2 phospho-deficient mutant (A) and quantification of fluorescent intensity of eGFP-USP2-4A (B) and eGFP-USP2-AA (C) construct binding to laser-induced DSB sites in U2OS cells. Data in graphs are means ± SEM; n =20.



[Figure 11] The phospho-mimetic mutant USP2 at two critical phosphorylation sites (S2E and T137E) was able to bind to DSB sites.

(A-C) various phospho-mimetic mutant of USP2, with glutamic acid substitution at these two phosphorylation sites, S2E and T137E (EE), recruitment to laser-induced DSB site. U2OS cells were overexpressed by indicated phospho-mimetic mutant of USP2 construct, and the cells were treated with ATM inhibitor (ATMi), KU55933 1 h before micro-irradiation. Then, laser microirradiation was performed. WT, wild type USP2; 4E, USP2 with substitution of 4 ATM phosphorylation sites (S2, S94, T137, S142) to glutamic acid; S2E + T137E or EE, substitution of S2 and T137 to glutamic acid. Representative time-lapse images of eGFP USP2 phospho-mimetic mutant (A) and quantification of fluorescent intensity of eGFP-USP2-4A (B) and eGFP-USP2-AA (C) construct binding to laser-induced DSB sites in U2OS cells. Data in graphs are means ± SEM; n =20.

4. Discussion

In DNA damage response, there are inseparable relations between ubiquitination and deubiquitination to control the recruitment to DNA damage site, molecular interaction, and dissociation of factors involved in DNA damage during DNA damage response. USP2 is a deubiquitinase that is associated in various cellular process, including circadian rhythm, tumorigenesis, cell cycle progression (Kitamura & Hashimoto, 2021). However, its involvement in the DSB response has not been shown before.

In this study, I identified the characteristics of USP2 recruitment at DNA damage site, which is prerequisite phenomenon for the MRN complex stability in the DSB site, by analyzing eGFP-USP2 recruitment at laser-induced DSB site. Premature disassembly of the MRN complex from DSB sites has been shown to be caused by SKP2 dependent ubiquitination of the NBS1 protein in RECQL4-defective cells. Then, deubiquitinase USP2 plays to oppose the NBS1 ubiquitination to prevent premature disassembly of the MRN complex from DSB site. Therefore, USP2 recruitment at the DSB site, as new player acting at the DSB site by opposing the ubiquitination of NBS1, is crucial for genomic stability

As shown in my result, USP2 could be recruited to the DSB site and interacted with NBS1 regardless of NCS treatment (Fig. 1), and this binding could suggest that USP2 directly control NBS1 ubiquitination. However, it is also possible that USP2 indirectly acts to stabilize NBS1. This insight is supported by a recent document which describes that USP2 interacts with SKP2-SCF E3 ligase to stabilize the substrate of SKP2-SCF, p21 (Zhang et al., 2021). Therefore, given NBS1 is a substrate of SKP2-SCF E3 ligase, USP2 may regulate NBS1 stability through interaction with

SKP2 SCF E3 ligase

The MRN complex plays essential roles in the DNA damage response, and its roles have been extensively studied by many researchers. However, the mechanism of the removal of MRN complex from DSB site is not entirely understood. SKP2-SCF-dependent NBS1 ubiquitination resulted in the premature disassembly of the MRN complex from DSB site (Wu et al., 2012). I thus suggest that SKP2-SCF dependent NBS1 ubiquitination accelerate the removal of MRN complex from DSB site. As deubiquitinase USP2 acts on SKP2-SCF-dependent NBS1 ubiquitination, it is possible that USP2 has a critical role in determining the removal timing of MRN complex. In this study shows USP2 recruitment to DSB site critically requires ATM activity, not activity of DNA-PK, during DNA damage response (Fig. 2B and Fig. 2C). This indicates that role of USP2 is only available at the DSB site when ATM kinase activity is present, and this means the repair of DNA damage may decrease the ATM activity, in which results the dissociation of deubiquitinases and ubiquitination-dependent removal of the MRN complex from DSB sites. In DNA damage response, both ubiquitination and deubiquitination are needed to be increased and activated to control protein stability. As SKP2 also has a role to activate ATM kinase by promoting lysine 63 NBS1 ubiquitination (Wu et al., 2012), SKP2-SCF recruitment to DSB site at early time point is fated. therefore, recruiting deubiquitinases to block premature disassembly of the MRN complex is a crucial step for the proper function of MRN complex in DNA damage response. Moreover, increased level of MRN complex at DNA damage site may cause genomic instability due to the excessive MRE11 nuclease activity during end resection, and this excessive activity of MRE11 possibly results genomic instability. (Buis et al., 2008). Collectively,

complete and timely removal of the MRN complex is important for genome integrity and control of continued activity of ubiquitinase by deubiquitinases as long as active ATM, which promotes deubiquitinase recruitment, appears to be an efficient strategy to prevent unwanted accumulation of the MRN complex after DNA damage

eGFP-USP2 also showed a PARP- and RECQL4- dependent recruitment to DSB site (Fig. 2 and Fig. 3). PARP is a protein that has crucial roles in multiple DNA damage repair pathway, and this catalyze the polymerization of poly(ADP-ribose)(PAR), which process called PARylation (Ray Chaudhuri & Nussenzweig, 2017). On the other hand, PARG promotes hydrolysis of PAR chain which prevent PARylation (Feng & Koh, 2013; Gogola et al., 2018). In response to DNA damage, this post-translational modification contributes to recruit many DDR factors to DNA damage site. RECQL4, a member of RecQ family helicase that is involved in DSB repair pathway, is recruited to DSB site through PARylation by Poly(ADP-ribose)polymerase (PARP1), and this protein is suggested to participate in 5'-end resection, promoted by MRE11 and CtIP protein dynamics (Aleksandrov et al., 2018; Lu et al., 2016). Therefore, PARP-dependent recruitment of USP2 is possibly due to the PARylation dependency of RECQL4 recruitment to DSB site. To support this notion, USP2 was not able to bind with RECQL4 when ATM activity was inhibited (Fig 4B), and USP2 recruitment was completely abolished in RECQL4 depleted cells (Fig 4C) Collectively, USP2 is recruited to DSB site along the interaction with RECQL4, which is dependent on ATM activity.

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국문초록

인간 유전체에서 가장 해로운 DNA 손상인 DNA 이중나선손상(DSB)은 DNA 수선과 같은 DNA 손상 반응을 유발한다. 인간의 유전체 항상성과 세포 생존에 필수적인 MRE11-RAD50-NBS1(MRN) 복합체는 DNA 손상을 감지하고 수선에 기여한다. 그러므로 MRN 복합체는 자신의 안정성 및 기능 유지를 위해 다양한 번역 후 변형(Post-translational modification)의 타깃이 될 수 있다. 최근에 밝혀진 문헌에 따르면, USP2는 DSB 부위에서 NBS1의 유비퀴틴화를 억제하므로 MRN 복합체를 안정화시키는 새로운 탈유비퀴틴화 효소로 밝혀졌다. 이에 본 연구에서는 DSB가 일어난 상황에서 MRN 복합체를 안정화시킬 수 있는 USP2의 DNA 손상부위 결합 메커니즘을 보여주고 있다. 연구자는 USP2 단백질을 과발현 시킨 U2OS 세포에 레이저 미세 조사 통해 실시간 세포 영상 촬영하고, 이를 분석하여 이중가닥손상 부위에 대한 USP2 결합이 ATM, PARP, 및 RECQL4에 의존적으로 일어난다는 것을 발견하였다. 또한, USP2가 이중가닥손상 부위에 모집되는 데에 USP2 N-말단에 위치한 2번 Serine 과 137번 Threonine의 인산화가 필수적임을 확인했다. 따라서 본 연구는 MRN 복합체 안정성을 위한 전제 과정인 DNA 이중가닥손상 부위에 대한 USP2의 새로운 모집 메커니즘을 규명하였다.