Master's Thesis of Education

The role of the putative PAR binding motif in RECQL4 during the DNA double-strand break response

DNA 이중 가닥 절단에 대한 세포 반응에서 RECQL4의 putative PAR binding motif의 역할

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Abstract

RECQL4 protein belongs to the RecQ helicase family and plays crucial roles in genome maintenance. RECQL4 is particularly important for ATM activation and HR repair at DNA double-strand break (DSB) sites. Previous research has shown that RECQL4 is recruited to DSB sites in a manner dependent on poly ADP-ribosylation (PARylation). Notably, the amino acid residues 360-437 region of RECQL4 have been identified as responsible for recruiting RECQL4 to DSB sites in a PARylation-dependent manner, and this region directly interacts with PAR. This suggests the presence of a PAR binding motif (PBM) in the amino acid residues 360-437 region of RECQL4. However, the significance of the putative PBM region in RECQL4 for its recruitment to DSB sites and whether PARylation-dependent recruitment of RECQL4 plays critical roles in the DSB response remain unknown.

In this study, I replaced the putative PBM of RECQL4 protein with a well-known PBM and its mutant variant. Using these modified RECQL4 proteins, I examined the recruitment of RECQL4 at DSB sites and its role in the cellular response to DSB. The results indicated that the putative PBM region of RECQL4 is crucial for its PARylation-dependent recruitment to DSB sites and for facilitating ATM activation, MRN stability, and HR repair. These findings suggest the importance of the interaction between RECQL4 and PAR through PBM in the DSB response.

Keyword : RECQL4, PAR, PARylation, PAR binding motif, DNA double-strand break, ATM, MRN, HR repair

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Chapter 1. Introduction

1.1. Cellular response to DNA double-strand breaks in mammalian cells

DNA double-strand breaks (DSBs) are severe damages that can lead to genome rearrangements and cell death. Defective repair of DSBs is closely linked to developmental, immunological, and neurological disorders, as well as being a significant factor in cancer (Jackson & Bartek, 2009; McKinnon, 2009). Consequently, organisms have developed diverse mechanisms to repair DSBs and mitigate their detrimental effects.

Upon encountering any form of DNA damage, the initial response of a cell is to swiftly detect the presence of the damage. Within cells, DSBs are rapidly recognized. The MRN (Mre11/Rad50/Nbs1) complex serves as the primary sensor for detecting DSBs by binding to the broken DNA ends. Subsequently, the MRN complex facilitates the recruitment and activation of ATM, an essential step in the cellular response to DSBs (Lee & Paull, 2005). Following the induction of DSBs, Ataxia telangiectasia mutated (ATM) kinase promptly undergoes activation in response to DNA damage (Lavin et al., 2005). Within minutes after the induction of DNA damage, ATM is recruited and activated in the proximity of DSBs. In this localized environment, ATM initiates the phosphorylation of various proteins crucial for DNA damage response and repair. These phosphorylated proteins include those within the MRN (Mre11/Rad50/NBS1) complex, p53, SMC1, and the histone variant H2AX (Bakkenist & Kastan, 2003).

Upon detecting a DSB, the histone variant H2AX undergoes targeted phosphorylation in chromatin near the site of the break. The phosphorylated form of H2AX, referred to as γH2AX, acts as a molecular beacon, indicating the presence of damage by marking nucleosomes in a region spanning one or more megabases of DNA surrounding the DSB (Rogakou et al., 1998). γH2AX plays a central

role in bridging damaged chromatin with the DNA repair machinery, facilitating the recruitment of multiple DNA repair and signaling proteins to repair centers, which are visible as nuclear aggregates known as "foci" (Lukas et al., 2011).

In the repair of two-ended DSBs, two pathways are predominant: non-homologous end joining (NHEJ) and homologous recombination (HR). NHEJ is a rapid and high-capacity pathway in mammalian cells, where it directly joins two DNA ends with minimal regard to DNA sequence. However, NHEJ can accommodate limited base-pairing between the processed DNA ends, allowing for repair joints with up to 4 bp of "microhomology." On the other hand, HR requires extensive sequence homology between the broken DNA and a donor DNA molecule and involves templated DNA synthesis as a crucial step in the repair process (Wyman & Kanaar, 2006).

1.2. Homologous recombination repair

Homologous recombination repair (HR repair) is a DNA repair mechanism that accurately repairs DSBs and other types of DNA damage (Li & Heyer, 2008). It is a complex process that involves the use of an undamaged DNA template as a guide to repair the broken DNA strands. The DSBs are recognized and processed by various sensor proteins, including the MRN complex (MRE11-RAD50-NBS1) and CtIP (C-terminal binding protein-interacting protein) (Cejka, 2015; Mao et al., 2008). Phosphorylated ATM is recruited to the sites of DNA damage and phosphorylates various downstream effectors, including key HR proteins such as BRCA1, CtIP, and NBS1 (Anand et al., 2019). This phosphorylation cascade leads to the activation of HR pathway components and facilitates their proper function during DNA repair. Phosphorylated ATM phosphorylates CtIP, which promotes DNA end resection. DNA end resection is a crucial step in HR repair, as it generates the necessary 3' single-stranded DNA (ssDNA) overhangs that initiate the strand invasion and homology search process. The 3' ssDNA tails invade the homologous region of an intact sister chromatid or

homologous chromosome. This invasion is mediated by the recombinase enzyme RAD51, which forms a nucleoprotein filament on the ssDNA (Sung, 1994). The invading 3' end primes DNA synthesis using the intact DNA template. This results in the formation of a displacement loop (D-loop) structure, with the invading strand acting as the primer for DNA synthesis. The D-loop structure undergoes branch migration, in which the Holliday junction is formed and moves along the DNA helix. Eventually, the Holliday junction is resolved by specialized enzymes, such as resolvases, to generate two intact DNA molecules. The remaining nicks are sealed by DNA ligase, resulting in the complete repair of the DSB (Heyer, 2007; Pâques & Haber, 1999; Sung & Klein, 2006; Syed & Tainer, 2018; West, 2003; Wyman & Kanaar, 2006).

HR repair is particularly important during the S and G2 phases of the cell cycle when a sister chromatid or homologous chromosome is available as a template for repair. It ensures accurate repair without loss of genetic information. HR repair is critical for maintaining genome stability and preventing the accumulation of deleterious mutations (Wyman & Kanaar, 2006).

1.3. Poly(ADP-ribosyl)ation in DSB repair

Poly ADP-ribosylation (PARylation) is a crucial post-translational protein modification that rapidly occures at DNA damage sites (Gibson & Kraus, 2012; Perina et al., 2014; Seet et al., 2006; Walsh, 2006). In human, ADP-ribosylation is catalyzed by poly (ADP-ribose) polymerases (PARPs) (Diefenbach & Bürkle, 2005; Hakmé et al., 2008; Luo & Kraus, 2012). PARPs predominantly covalently attach the ADP-ribose (ADPR) unit to the carboxyl group of acidic residues, such as glutamate or aspartate, on the target proteins through an ester bond (d'AMOURS et al., 1999; Tallis et al., 2014). However, cysteine and lysine residues can also act as acceptors (Altmeyer & Hottiger, 2009; Vyas &

Chang, 2014). Nevertheless, most PARPs can only transfer a single mono (ADP-ribose) group onto their target proteins. So far, PARP1, 2, and 3 have been identified as catalyzer of PARylation in response to DNA damage (De Vos et al., 2012). Additionally, tankyrases, including tankyrase-1 (PARP5a) and tankyrase-2 (PARP5b), have been shown to contribute to genomic stability (Dregalla et al., 2010). Among these PARPs, PARP1 serves as the founding member of the PARP family, responsible for synthesizing PAR chains. The mechanism of PARP1 activation by single-strand and double-strand DNA breaks is well- established (Juarez-Salinas et al., 1982). Using NAD^+ as substrate, PARPs repeatedly catalyze the transfer of successive units of ADPR moieties via a unique 2′ ,1″ -*O*-glycosidic ribose-ribose bond to target proteins, ultimately producing PAR chain (Gibson & Kraus, 2012). Several reports have shown that PAR chains can consist of up to 200 ADPR units in length (Miwa et al., 1979; Tanuma & Kanai, 1982).

In cells, PAR polymers are primarily degraded by PAR glycohydrolase (PARG), which possesses both exoglycosidic and endoglycosidic activities (Kim et al., 2012; Miwa et al., 1979; Slade et al., 2011). PARG efficiently cleaves the unique 2′,1″-glycosidic ribose-ribose bonds of the PAR chains and releases the free ADPR moieties (Niere et al., 2012; Slade et al., 2011). Similar to various other post-translational modifications, the generation and breakdown of PAR chains are tightly and dynamically regulated in living organisms, with a short half-life of only a few minutes. If PAR chains are not efficiently hydrolyzed, the accumulation of protein-free PAR chains can trigger a form of cell death resembling apoptosis, known as parthanatos (Barkauskaite et al., 2013).

PARylaiton mediates DNA damage repair. PARPs engage in both physical and functional interactions with diverse DNA damage factors, facilitating their recruitment to sites of DNA damage. Specifically, PARP1 can physically and functionally interact with the single-strand break repair (SSBR) factor X-ray repair cross-complementing protein 1 (XRCC1). XRCC1 plays a crucial role in

the SSBR signal pathway, facilitating the assembly and recruitment of the SSBR machinery (Caldecott, 2008).

A recent study indicates that the BRCA1 C Terminus (BRCT) domain of XRCC1 directly binds to PAR chain, mediating the early recruitment of XRCC1 to DNA lesions (Li & Yu, 2013). PARP1 has been associated with HR-mediated repair and reactivation of stalled replication forks, thereby promoting faithful DNA replication (Haince et al., 2008). Moreover, PARP1 facilitates the recruitment of MRE11 and RAD51, which are essential for the BRACA1/2-dependent early DNA damage response, particularly in the restart of stalled replication (Zhang et al., 2015).

The BRCTs of BARD1, the oligonucleotide/oligosaccharide binding-fold of BRCA2, and the N-terminus of exonuclease 1 (EXO1) containing the PIN domain are the PAR-binding modules that target these HR repair machineries to DSBs for efficient damaged DNA repair. These findings support the notion that PAR chains can serve as a landing platform for the recruitment of DNA repair complexes (Masson et al., 1998).

1.4. PAR binding modules

In order to recruit DNA repair complexes, PAR chains must be recognized by diverse proteins, including DNA damage response factors. To date, several distinct classes of PAR-binding modules have been identified. These modules include the PAR binding-motif (PBM), the PAR-binding zinc finger (PBZ), the Macro domain, the WWE domain, the BRCT domain, the forkhead-associated (FHA) domain, the OB-fold domain, the PIN domain, and the RNA recognition motif (RRM) domain(Andegeko et al., 2001; Žaja et al., 2012).

The PBM is remarkably present in a wide of proteins, including DNA damage response proteins and enzymes involved in chromatin remodeling and RNA processing (Gibson & Kraus, 2012; Hassa & Hottiger, 2008; Herceg & Wang, 2001; Krishnakumar & Kraus, 2010). Proteins such as p53, histones, and XRCC1 contain the PBM.

The PBM consists of a 20-amino acid sequence with a cluster enriched with basic residues and a pattern of hydrophobic amino acids alternating with basic residues (Ahel et al., 2008; Gagné et al., 2003; Pleschke et al., 2000).

The typical PAR binding motif has a consensus pattern of -hxbxhhbbhhb-. In the pattern, "h" represents residues with hydrophobic side chains, "b" indicates a preference for basic amino acids, and "x" can be any amino acid. The number of basic residues in the consensus pattern can range from 2 to 4. Additionally, a cluster of amino acids with positive residues often precedes the typical PAR binding motif (Pleschke et al., 2000). The overall positive charge of PBMs facilitates electrostatic interactions with negatively charged PAR chains, promoting their interaction (Teloni & Altmeyer, 2015).

The recently identified PBZ domains possess the consensus sequence [K/R]xxCx[F/Y]GxxCxbbxxxxHxxx[F/Y]xH (Ahel et al., 2008). PBZs are less common in mammalian proteins involved in DNA repair and cell cycle checkpoint, although they are much more widespread in some other eukaryotes (Ahel et al., 2008; Mehrotra et al., 2011; Oberoi et al., 2010; Ruscetti et al., 1998). The WWE domain is the most recently discovered PAR-binding domain, named after the three strictly conserved amino acid residues, tryptophan-tryptophan-glutamate (WWE). The WWE domains can recognize iso-ADPR of PAR chains with high affinity, tightly links ubiquitination and PARylation signal pathways (Wang et al., 2012). The Macro domain, which consists of 130–190 amino acid residues, is evolutionarily conserved and widely spread throughout all kingdoms of organisms (Feijs et al., 2013). It is well known that FHA and BRCT domains can bind to phosphorylated proteins and modify protein–protein interactions(Reinhardt & Yaffe, 2013). The OB-fold is an ssDNA or ssRNA binding domain found in proteins from all three kingdoms (Zhang et al., 2015). The PIN domain-containing proteins serve as nucleases that cleave ssDNA/ssRNA in a sequence-specific manner (Arcus et al., 2011). The RRM is one

of the most abundant protein domains in eukaryotes and can serve as a versatile RNA-binding platform to regulate post-transcriptional gene expression (Maris et al., 2005).

Distinct PAR reader domains possess the ability to distinguish between PAR and between soluble and protein-linked PAR. Consequently, the regulation of distinct protein sets by PAR depends on the specificity of the PAR domain on the target protein (Karras et al., 2005; Qi et al., 2019).

1.5. RECQL4 in DSB response

RECQL4 is a DNA helicase protein that plays a crucial role in various DNA metabolic processes, including DNA replication, recombination, and repair. It belongs to the RecQ family of helicases, enzymes involved in unwinding DNA helix structures (Bernstein et al., 2010; Croteau et al., 2014). The RECQL4 gene is located on chromosome 8 (8q 24.3) and consists of 21 exons (Kitao et al., 1999). Mutations in this gene have been associated with Rothmund-Thomson syndrome (RTS), a rare genetic disorder characterized by premature aging, skeletal abnormalities, and an increased risk of developing cancer (Bernstein et al., 2010; Kitao et al., 1999; Larizza et al., 2010; Lu et al., 2017).

RECQL4 functions as a molecular motor that utilizes the energy from ATP hydrolysis to unwind DNA duplexes, thereby resolving DNA structures and facilitating DNA replication and repair processes (Castillo-Tandazo et al., 2019; Suzuki et al., 2009). It interacts with various proteins involved in DNA metabolism, including DNA polymerases, DNA ligases, and components of the HR pathway (Mo et al., 2018; Yokote et al., 2017).

RECQL4 plays a crucial role in the activation of ATM in the DSB response (Park et al., 2019). It facilitates the activation of ATM, which initiates downstream signaling pathways involved in DNA repair and cell cycle checkpoints (Khanna et al., 2001). Through its interaction with ATM, RECQL4 contributes to the

proper coordination of DNA repair processes and the maintenance of genome integrity.

Previous studies have shown that RECQL4 plays a critical role in the HR repair. It promotes DNA end resection during HR-dependent DSB response (Lu et al., 2016). However, recent studies have indicated that in the absence of RECQL4, end resection can still occur as long as the MRN (Mre11-Rad50-Nbs1) complex remains stable. The MRN complex is essential for end resection (Nimonkar et al., 2011), and in the absence of RECQL4, the stability of MRN decreases, leading to a failure in end resection. (Kim et al., 2021). RECQL4 indirectly mediates DNA end resection by regulating MRN stability, rather than directly participating in the process. In fact, RECQL4 plays a crucial role in the deubiquitination control of MRN stability (Kim et al., 2023).

1.6. Purpose of Research

The recruitment of RECQL4 to DSB sites is dependent on the activity of PARP1, and the fact that RECQL4 continues to reside at DSB sites upon treatment with a PARG inhibitor indicates the important role of PARylation by PARP1 in the recruitment of RECQL4 to DSBs (Su et al., 2010; Woo et al., 2006). In previous research, it was found that the amino acid residues 360– 437 portion of RECQL4 could bind to DSB sites in a PARylation-dependent manner (Kim, 2021). Moreover, this region was shown to directly interact with PAR (Kim, 2022). Based on these results, it is anticipated that the putative PAR binding motif located in the amino acid residues 360– 437 portion of RECQL4 may be crucial for its role in DSB response. However, this has not been experimentally confirmed.

In my study, I aimed to explore the significance of the interaction between RECQL4's N-terminus and PAR in relation to its recruitment to DSBs and its role in DNA repair mechanisms. To achieve this goal, I generated variant RECQL4 proteins by replacing the putative PBM region with the well-known PBM of hnRNP A2 and its inactive mutant PBM. I examined the recruitment of these proteins to DSB sites and their function in response to DSBs.

Chapter 2. Method

2.1. Plasmid constructions and Primer

To generate RECQL4-hnRNP, site-directed mutagenesis by overlap extension using the polymerase chain reaction (PCR) was performed to replace the 361-382 region of RECQL4 with the PAR binding motif of hnRNP. Two DNA fragments with overlapping ends were generated by using complementary oligodeoxyribonucleotide (oligo) primers (#1947, #1948) and PCR (Table 1). The resulting fusion product was further amplified by PCR. Subsequently, 2flag-RECQL4-pcDNA3.1(-) and RECQL4-hnPAR PCR products were digested with BsrGI (BioLabs, R0575S) and AfeI (BioLabs, R0652S) restriction enzymes, followed by ligation. To generate RECQL4-hnPAR-A^m, complementary oligodeoxyribonucleotide primers (#1952, #1953) were used.

To generate eGFP-RECQL4-hnPAR and eGFP-RECQL4-hnPAR-A^m, eGFP DNA fragments was amplified by PCR from eGFP-2flag-RECQL4-pcDNA3.1(-). The eGFP DNA fragments were then inserted into the constructed RECQL4-hnPAR and RECQL4-hnPAR-A^m plasmids using restriction enzyme sites. The eGFP DNA fragments were treated with NheI (Enzynomics, R016S) and XbaI (Enzynomics, R013S) restriction enzymes, while the vector was treated with NheI restriction enzyme.

Plasmid constructions were verified by sequence analysis (BIONICS, Korea). Primer design was carried out considering restriction enzyme sites, T_m values, GC%, and other factors, and the primers were chemically synthesized by Bioneer (Daejeon, Korea).

Table 1. Primer used in this study

The term "Primer Number" refers to the identification numbers assigned to the primers in our laboratory. Primer #1351 was used for sequencing the RECQL4-hnPAR and RECQL4-hnPAR-A^m. The sequencing of EGFP-RECQL4-hnPAR and EGFP-RECQL4-hnPAR-A^m was performed using the CMV promotor. Primer #1790 and #1793 were used to amplify the EGFP DNA fragment. Primers #1946, #1947, #1948, #1949, #1952 and #1953 were employed for site-direct mutagenesis.

Primer Number	Sequence
#1351	5′ -CTGATCTAGGCTCAGAGGAA-3′
#1790	5′ -GTCAGCTAGCACCATGGTGAGCAAGGG- 3′
#1793	5′ -GTCATCTAGACTTGTACAGCTCGTCCATG- 3′
#1946	5′ -GGAACCTGTACAGGCACAGCCACC- 3′
#1947 RECQL4-PAR	5′ - CTTCTTCACAGTCACATGAGCACCAGGCTTACCACTCTCTTC TCTCTGCTTCATGTTGAGCCGTACGTAATTGC - 3′
#1948 RECQL4-PAR	5′ - GCCTGGTGCTCATGTGACTGTGAAGAAGCTTTTCGTTGGTGG TATCAAGTGGCGGAAGAAAGGGGAGTGTTTTG - 3
#1949	5′ - GAGCAGCGCTGGGAGCTGGTAG - 3′
#1952 RECQL4-PAR(M)	5′ - CCACCAACCGAAAGCAGCAGCAGCATAGCAGCAGCACCAGGC TTACCACTCTCTTCTCTC - 3′
#1953 RECQL4-PAR(M)	5′ - CTGCTGCTGCTGCTTTCGTTGGTGGTATCGCTTGGCGGAAGA AAGGGGAGTGTTTTGGG - 3′

2.2. Cell culture

Human osteosarcoma (U2OS) cells were cultured in Dulbecco's modified Eagle's medium (DMEM) (Welgene, LM001-05, Korea) supplemented with 10%(v/v) fetal bovine serum (FBS) (Welgene, S001-01, Korea) and 1%(v/v) antibiotic Antimycotic Solution, 100X(penicillin/streptomycin/amphotericin B) (Welgene, LS 203-01, Korea). The cells were grown in humidified atmosphere of 5% CO_2 at 37℃.

2.3. Small-interfering RNA transfection

To deplete proteins, we used siRNAs transfected with an electroporator (Invitrogen) and incubated for 48 hours. At 24 hours after siRNA transfection, the medium was replaced with fresh medium. All siRNA oligonucleotides utilized in this study were custom-synthesized by Bioneer (Daejeon, Korea). The sense strand sequences of siRNA duplexes are as follows:

siGL2, 5′ -AACGUACGCGGAAUACUUCGA- 3′

siRECQL4 UTR2, 5′ -GACUGAGGACCUGGGCAAA- 3′

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

The cells were seeded on a 35㎜ glass-bottomed confocal dish(SPL #101350). Transfections of 500ng EGFP-tagged RECQL4 or RECQL4-hnPAR, RECQL4-hnPAR-A^m plasmids were performed with an lipofectamin™ 3000(Invitrogen), following the manufacturer' s instruction. 24 hours following transfection, Cells were treated with 5㎍/㎖ Hoechst 33342 (ThermoFisher Scientific, Korea) for 10 min, then analyzed.

An LSM880 laser confocal microscope system with a

temperature controlled CO_2 chamber (Zeiss) was used. Fixed wavelength of laser (405㎚) at a scan speed of 8.19㎲/pixel with four iterations and Plan-Apochromat 63X oil objective lens were used. Defined regions of interest were irradiated with 100% laser output. Time-lapse images were captured and fluorescence intensities of irradiated areas relative to those of nonirradiated areas within the nucleus were obtained using the ZEISS ZEN 2.3 SP1 software (Zeiss).

2.5. Immunoblotting

To prepare whole-cell extracts for immunoblotting, cells were lysed in a buffer containing 40mM Tris-HCl, pH 7.5, 150mM NaCl, 1% NP-40, 1mM ethylenediaminetetraacetic acid (EDTA), 0.25% sodium deoxycholate, 20mM NaF, 0.1mM sodium orthovanadate, and protease inhibitors (GenDEPOT, P3100-01). The cells were disrupted with sonication, and the concentrations of proteins were measured by Bradford assay. Approximately 20㎍ of protein was subjected to sodium dodecyl sulfate-polyacrylamide gel electrophoresis.

The following antibodies were used in this study: anti-RECQL4 (AB frontier, AR05-PA0007), anti-LaminB1 (abcam, ab16048), anti-GFP (Santa Cruz Biotechnology, SC-9996), anti-FLAG (AB frontier, AC063), anti-Mouse IgG antibody (GeneTex, GTX213111-01), anti-Rabbit IgG antibody (GeneTex, GTX213110-01)

2.6. Immunofluorescence

The siRNA-transfected U2OS cells were seeded at 50,000 cells per well in a 35ml dish, with each well containing a coverslip. The next day, the cells were transfected with an empty plasmid, plasmid expressing RECQL4 or RECQL4-hnPAR, RECQL4-

hnPAR-A^m under CMV promoter. At 24 hours following transfection, the cells were treated with or without 200ng/mL NCS for 15 minutes. Subsequently, the cells were washed with PBS, and fresh medium was added. The treated cells were allowed to recover for 1hour.

The coverslip was then pretreated with a cold buffer containing non-ionic detergent (10mM PIPES pH 7.0, 100mM NaCl, 300mM sucrose, 3mM MgCl2, 0.5% Triton X-100) on ice for 5 minutes and fixed with 4% paraformaldehyde in PBS for 10 minutes at 25℃. After fixation, the sample was permeabilized with PBS containing 0.25% Triton X-100 for 10 minutes at 25℃ and then incubated in a blocking buffer (5% BSA and 0.25% Tricon X-100 in PBS) for 30 minutes at 25℃. The indicated proteins were labeled with the respective primary and secondary antibodies in the blocking buffer for 1 hour at 25℃. The nuclei of cells were stained with 0.1ug/mL 4'-6'-diamidino-2-phenylindole (DAPI) in mounting solution (VECTASHIELD, H-1200, Vector, USA) in the dark. Fluorescent images were obtained by fluorescence microscopy (Zeiss LSM880), and cells containing 10 or 20 were counted as foci-positive cells for quantitation.

In immunofluorescence experiments, anti-pATM antibody (Cell Signaling, S1981) was used in a 1:1,500 dilution, anti-Mre11 antibody (GeneTex, GTX30294) in a 1:1,000 dilution, anti-Rad51 antibody (GeneTex, N1C2) in a 1:500 dilution, mouse anti-γH2AX antibody (Santa Cruz Biotechnology, Sc-517348) in a 1:200 dilution, and rabbit anti-γH2AX antibody (BETHYL, A300-081A-M) in a 1:1,500 dilution.

2.7. WST-1 assay for cell viability

The assay was carried out as described in the manufacturer's instructions (EZ-Cytox, DoGenBio). In brief, U2OS cells were transfected with siRNAs (siGL2 was also used as a negative control). The siRNA transfected U2OS cells were seeded at

100,000 cells per well in a 60 ml dish in each well. The next day, the cells were transfected with an empty plasmid, plasmid expressing RECQL4, RECQL4-hnPAR, or RECQL4-hnPAR-A^m under CMV promoter. After 24 hours of transfection, each sample were cultured in 96-well plates for one day. Cells were exposed to different concentrations of bleomycin (AbMole BioScience, M2100) and incubated for 24 hours. After incubation, cells were treated with 10% WST-1 reagent for 1 hour, and absorbance was measured using Epoch2 Microplate Spectrophotometer (BioTek).

Chapter 3. Result

3.1. Generation of protein by replacing the putative PAR binding motif of RECQL4 with the well-characterized hnRNP A2 PBM and its mutant PBM

To determine the role of RECQL4's putative PAR binding motif (PBM) in its recruitment to DSB sites and DNA damage response, I generated modified RECQL4 proteins by replacing the putative PBM with a well-studied PBM (Figure 1). If the putative PBM of RECQL4 indeed functions as a PBM, replacing it with a well-characterized PBM should not affect the function of RECQL4. However, if the PBM is replaced with a mutant form that does not interact with PAR, it is expected to impair the function of RECQL4.

The selection of the replacement region within the amino acids 360-437 region of RECQL4 was based on a comparison with the typical sequence of a PAR binding motif (Figure 1. A). The typical PAR binding motif sequence, -hxbxhhbbhhb-, contains several Lys-Arg-clusters interspersed with hydrophobic amino acids (Pleschke et al., 2000). It was observed that the 361-372 region exhibits a similar pattern of basic amino acids interspersed between hydrophobic amino acids. Therefore, I replaced the amino acid sequence 361-372 with the well-characterized PBM sequence from hnRNP A2 (Gagné et al., 2003). The mutant PAR binding motif used in this study was created by replacing some of the hydrophobic and basic amino acids in hnRNP A2's PBM with alanine residues (Figure 1. B). Upon analyzing the secondary structure using a secondary structure analysis program, there were no significant changes in the surrounding secondary structure when comparing RECQL4's putative PBM before and after substitution of amino acid residues. The modified RECQL4 was then transfected into U2OS cells, and successful expression was confirmed (Figure 2). For the micro-irradiation experiments, EGFP was fused to the N-terminus of each RECQL4 variant proteins (Figure 2. B).

Moving forward, the RECQL4 protein with the PAR binding

motif of hnRNP A2 will be referred to as RECQL4-hnPAR. On the other hand, the RECQL4 protein with the inactive mutant hnRNP A2 PAR binding motif will be referred to as RECQL4-hnPAR-A^m.

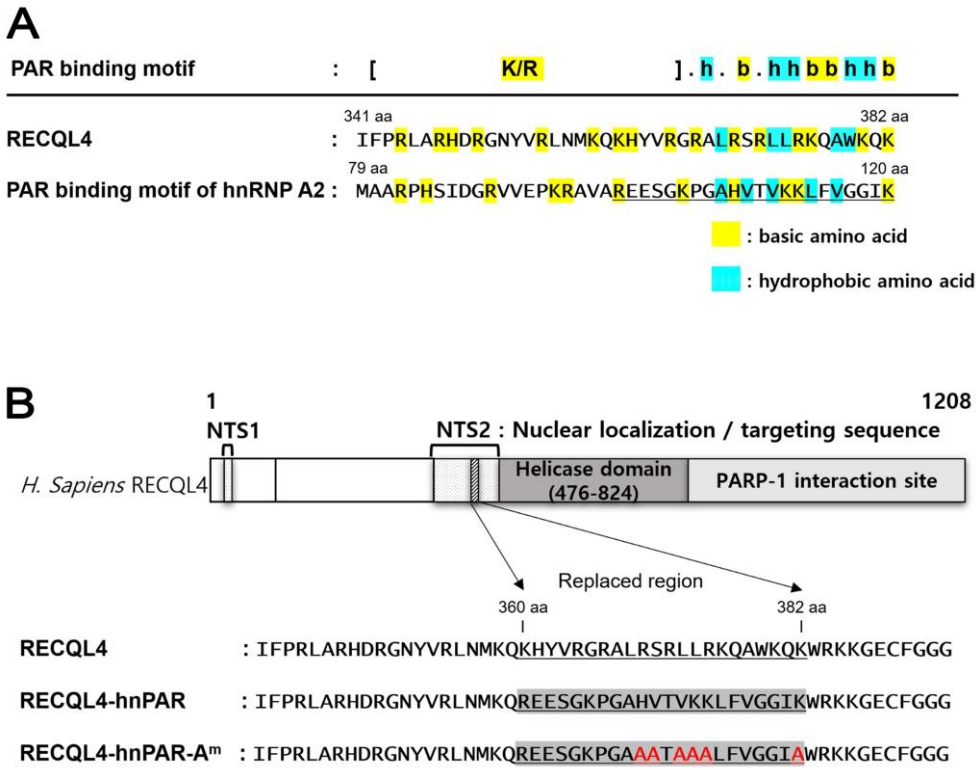

Figure 1. Schematic diagram of the construct of RECQL4 PAR binding motif (PBM) modification

(A) The putative PBM amino acid sequence of RECQL4 and the PBM amino acid sequence of hnRNP A2. A common feature of the amino acid sequences is the presence of hydrophobic amino acids spaced by basic amino acids, along with an accumulation of basic residues at the N-terminal side of the motif. Conserved hydrophobic residues are indicated against a blue background, and neighboring basic amino acids, as well as the residues corresponding to the basic block at the N-terminal part, are shown against a yellow background. Numbers refer to the amino acid positions from the initiation codon. The positions of the amino acids to be replaced are marked with an underline.
(B) Schematic diagram of human RECQL4. Arrows indicate the replaced region. The positions of NTS1, NTS2, Helicase domain, and PARP-1 interaction site are as specified by (Croteau et al., 2012) and (Balajee, 2021). Substituted amino acid positions are indicated against a gray background, and the positions replaced with alanine are indicated in red. Numbers refer to the amino acid positions from the initiation codon.

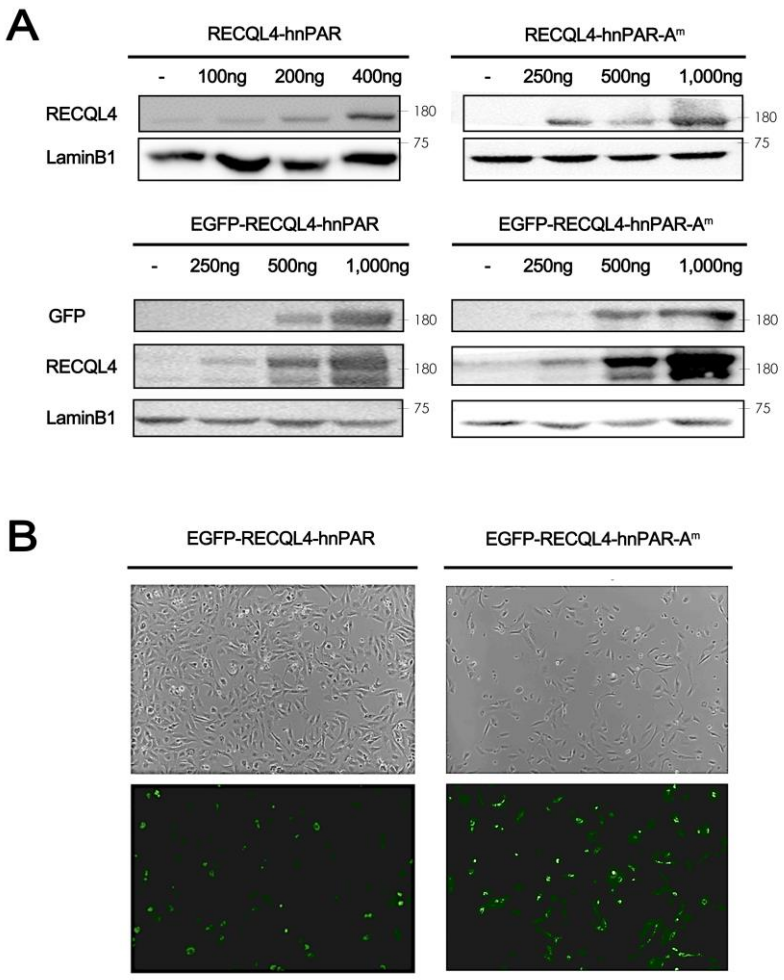

Figure 2. The expression test results of the modified RECQL4 protein

(A) The western blots showing expression level of modified RECQL4 proteins. Modified RECQL4 proteins were transfected into U2OS cells at indicated concentrations and incubated for 24 h. LaminB1 was utilized as a loading control for normalization.
(B) The pictures of fluorescent expression levels after transfection with an EGFP-RECQL4-hnPAR and EGFP-RECQL4-hnPAR-A^m expression vectors at a concentration of 1,000 ng in U2OS cells.

3.2. The putative PAR binding motif of RECQL4 is important for the recruitment of RECQL4 to DSB sites.

I examined the recruitment of wild type EGFP-RECQL4(control), EGFP-RECQL4-hnPAR, and EGFP-RECQL4-hnPAR-A^m proteins in U2OS cells. The respective RECQL4 expression vectors were transfected into U2OS cells. DSBs were induced in live cells using laser micro-irradiation (Figure 3. A).

As shown in previous studies (Kim, 2021), the wild-type EGFP-RECQL4 protein rapidly accumulated at DSB sites and subsequently disappeared within 10 minutes. EGFP-RECQL4-hnPAR showed weaker fluorescence intensity at the laser-induced DSB sites compared to EGFP-RECQL4, but it still displayed a similar pattern of rapid recruitment and disappearance. However, EGFP-RECQL4-hnPAR-A^m did not show a significantly increase in fluorescence intensity at the micro-irradiation sites, indicating a lack of recruitment to the DSBs. When treated with a PARP-1 inhibitor, Olaparib, there was no detectable recruitment of EGFP-RECQL4-hnPAR and EGFP-RECQL4-hnPAR-A^m to the DSB sites induced by laser irradiation (Figure 3). These results suggest that their recruitment is dependent on PARylation.

In the case of RECQL4-hnPAR-A^m, although it does not exhibit a significant increase in recruitment to DSBs, its fluorescence intensity decreases further upon PARP inhibitor treatment (Figure 3. B). This suggests that there may be additional weak PBMs in RECQL4 or PARylation-dependent interaction

ns that contribute to its function.

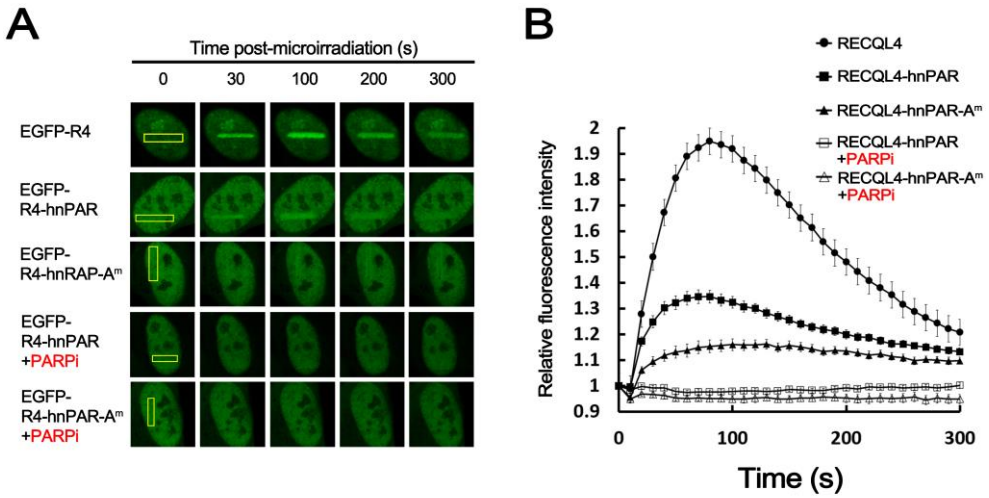

Figure 3. The significance of the putative PBM in the recruitment of RECQL4 to DSB sites

Representative time-lapse images (A) and quantification of relative fluorescent intensity (B) of RECQL4 binding to laser-induced DSB sites in U2OS cells are shown. U2OS cells were transfected with EGFP-RECQL4, EGFP-RECQL4-hnPAR, and EGFP-RECQL4-hnPAR-A^m and incubated for 24 hours. Then, the cells were subjected to laser micro-irradiated at the indicated region, which is marked with a yellow box, for examination. The PARP inhibitor was administered one hour prior to the laser irradiation. Time-laps images were acquired at 10-second intervals, and the fluorescence intensity measurement in the micro-irradiation area was normalized by average fluorescence intensity measurement in the non-irradiation area. Data in graphs are presented as means ±SEM; n=20+.

3.3. The putative PAR binding motif of RECQL4 is important for the DBS response.

I examined whether the putative PBM of RECQL4 is also crucial for its function. Wild type RECQL4(control), RECQL4-hnPAR, and RECQL4-hnPAR-A^m proteins were transfected into U2OS cells depleted of endogenous RECQL4 using siRNA transfection. Immunofluorescence staining was performed to examine ATM activation, MRN stability, and HR repair. To assess the activation of ATM, I stained for pATM. To examine MRN stability, I stained for Mre11. Finally, to evaluate HR repair, I stained for Rad51. γH2AX(phosphorylated H2AX) foci indicate the occurrence of DSBs (Valdiglesias et al., 2013). Treatment with NCS leads to an increase in γH2AX foci, indicating DSB induction.

Figure 4 shows the results of pATM staining. Immunostaining of γH2AX was used as positive control to confirm the occurrence of DSBs. As expected, pATM foci decreased in cells depleted of endogenous RECQL4. In cells transfected with each variant of RECQL4, both wild-type RECQL4 and RECQL4-hnPAR showed a recovery of foci similar to the control (siGL). However, in cells transfected with RECQL4-hnPAR-A^m, the recovery of pATM foci was not observed. These results indicate that the putative PAR binding motif of RECQL4 contributes to the activation of ATM.

Similarly, when DSBs were induced by NCS, cells with RECQL4 depletion exhibited a decrease in Mre11 and Rad51 foci (Figure 5, 6). However, upon the expression of each variant of RECQL4, the foci of both wild-type RECQL4 and RECQL4-hnPAR increased, whereas RECQL4-hnPAR-A^m foci decreased once again. These results indicate that RECQL4's putative PAR binding motif is involved in maintaining the stability of MRN and consequently facilitating HR repair.

I examined the sensitivity to bleomycin treatment. Cell viability was determined using the WST-1 assay after 24 hours of culture (Figure 7). The cells transfected with wild-type RECQL4 and RECQL4-hnPAR showed increased cell viability compared to the

control (siR4). However, RECQL4-hnPAR-A^m, which lacked any transfected components and had RECQL4 depleted, exhibited a similar level of decrease in cell viability as cells with RECQL4 depletion alone.

Taken together, these results indicate that the PAR binding motif is not only crucial for the recruitment of RECQL4 but also plays a significant role in ATM activation, maintenance of MRN stability, and HR repair.

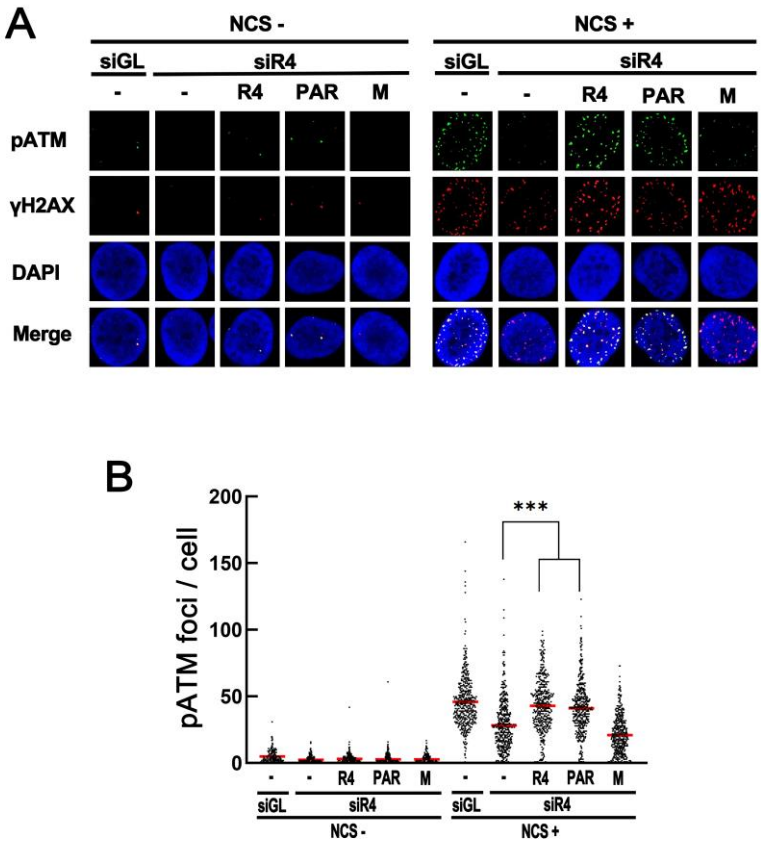

Figure 4. The necessity of putative PBM in the activation of ATM during the cellular response to DSBs

(A) The images show immunostaining of pATM and γH2AX foci within the nuclei of cells following knockdown of RECQL4 using siRNA with expression of RECQL4, RECQL4-hnPAR, and RECQL4-hnPAR-A^m. siGL2 was used as a control.
(B) The graph represents the quantification of the number of pATM within the nuclei(n=440). The red line indicates the mean value, and the error bars represent the standard error. $p < 0.001$
R4 : RECQL4, PAR : RECQL4-hnPAR, M : RECQL4-hnPAR-A^m

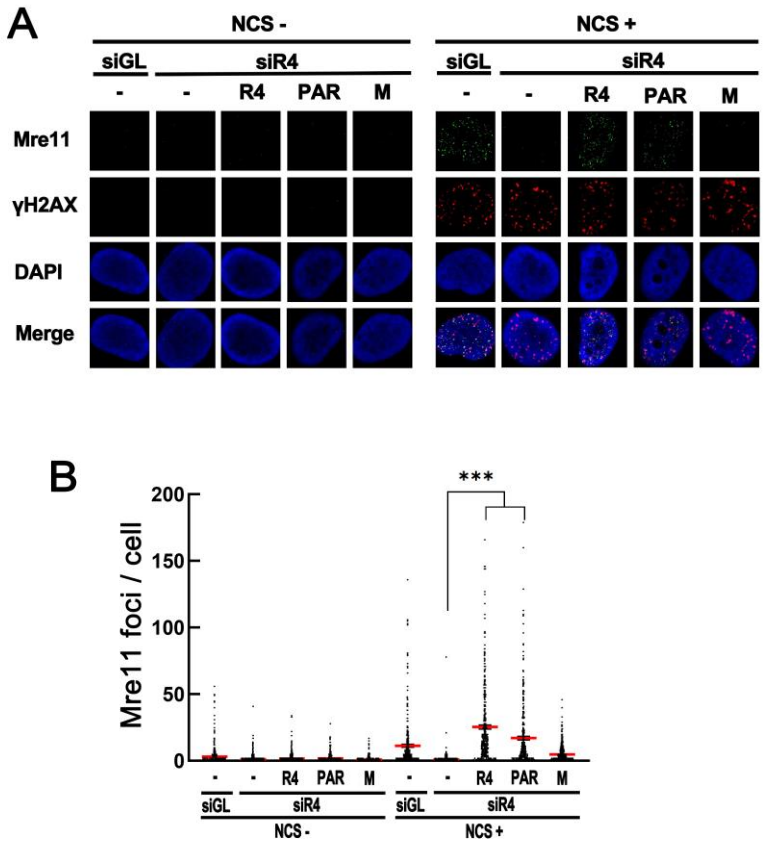

Figure 5. The necessity of putative PBM for the recruitment of Mre11 during the cellular response to DSBs

(A) The images show immunostaining of Mre11 and γH2AX within the nuclei of cells following knockdown of RECQL4 using siRNA with expression of RECQL4, RECQL4-hnPAR, and RECQL4-hnPAR-A^m. siGL2 was used as a control.
(B) The graph represents the quantification of the number of Mre11 foci within the nuclei(n=300). The red line indicates the mean value, and the error bars represent the standard error. $p < 0.001$
R4 : RECQL4, PAR : RECQL4-hnPAR, M : RECQL4-hnPAR-A^m

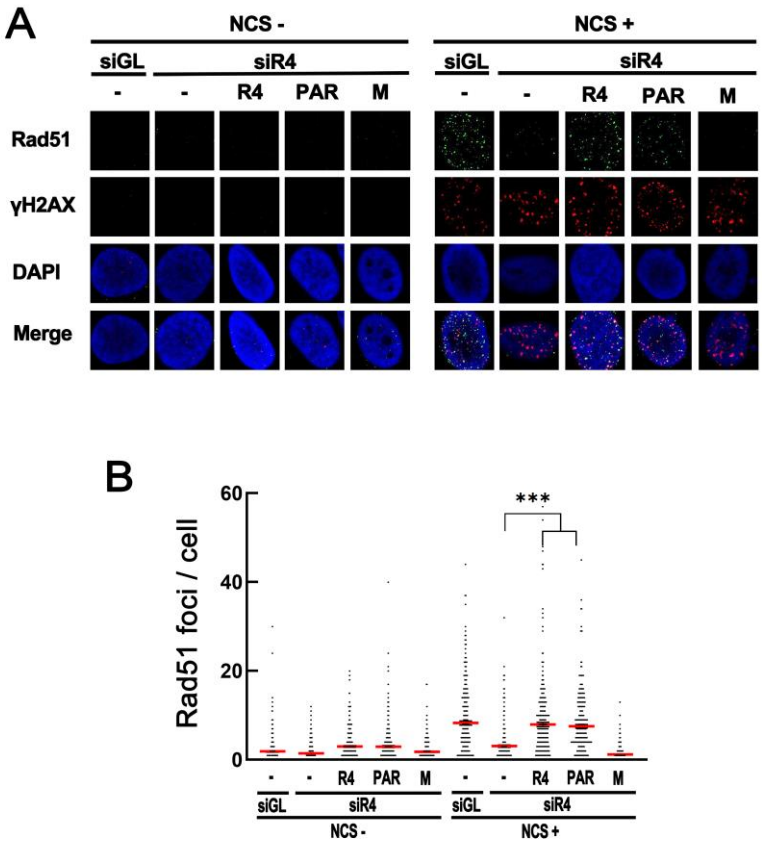

Figure 6. The necessity of RECQL4's PBM for the recruitment of Rad51 during the cellular response to DSBs

(A) The images show immunostaining of RAD51 and γH2AX within the nuclei of cells following knockdown of RECQL4 using siRNA with expression of RECQL4, RECQL4-hnPAR, and RECQL4-hnPAR-A^m. siGL2 was used as a control.
(B) The graph represents the quantification of the number of RAD51 within the nuclei(n=310). The red line indicates the mean value, and the error bars represent the standard error. $p < 0.001$.
R4 : RECQL4, PAR : RECQL4-hnPAR, M : RECQL4-hnPAR-A^m

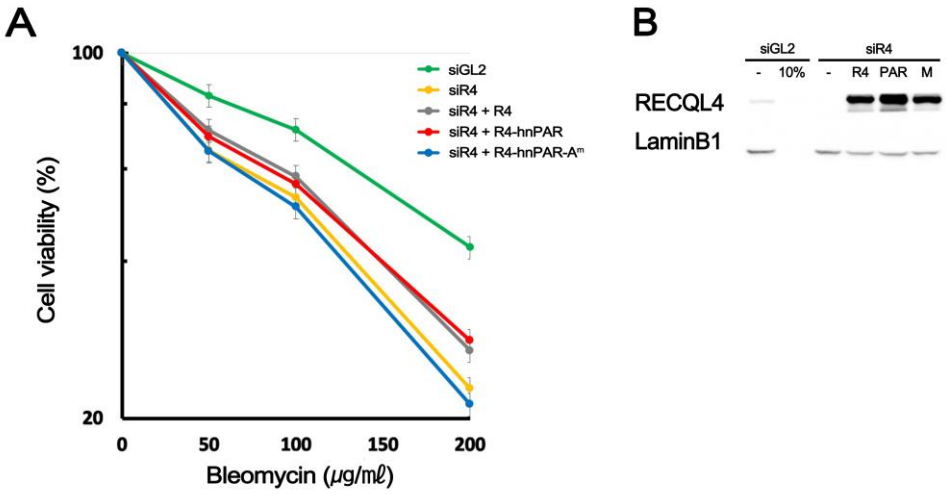

Figure 7. The impact of putative PBM of RECQL4 on cell viability

U2OS cells transfected with empty, wild type RECQL4, RECQL4-hnPAR, or RECQL4-hnPAR-A^m plasmids were treated with various concentrations of bleomycin for 12 hours.

(A) 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.

(B) The Western blot presented here illustrates the detection of siRNA presence or absence and the protein expression levels.

R4 : RECQL4, PAR : RECQL4-hnPAR, M : RECQL4-hnPAR-A^m

Chapter 4. Discussion

In this study, I demonstrated that RECQL4's PAR biding motif (PBM) is crucial for its PAR dependent recruitment to the DSB sites and its involvement in the DNA damage response. This finding suggests that the putative PBM indeed functions as a functional PAR binding motif.

Most studies investigating the recruitment of RECQL4 to DSB sites primarily utilize micro-irradiation. Due to the rapid and transient recruitment of RECQL4, its visualization through immunofluorescence staining has limitations, making micro-irradiation the preferred method. However, even when using micro-irradiation as a condition for DSB, it is challenging to achieve homogeneous damage sites (Dinant et al., 2007, Saquilabon Cruz et al. 2016, Bekker-Jensen et al., 2006). Moreover, RECQL4 has various biological roles, so even a slight amount of single-strand breaks within the micro-irradiation sites could lead to the recruitment of RECQL4. As a result, it becomes difficult to determine whether RECQL4 is specifically recruited to DSBs. However, my data comparing the PARylation-dependent recruitment form and its mutant forms of RECQL4 have confirmed that PARylation-dependent recruitment of RECQL4 to micro-irradiation sites affects DSB repair. This finding is significant as it supports the assertion that the recruitment of RECQL4 to micro-irradiation sites corresponds to its recruitment to DSB sites.

The typical PAR binding motif consists of approximately 20 amino acids and contains conserved regions. The conserved pattern involves an alternating arrangement of hydrophobic and basic amino acids, known as $[ACGVILMFYW]_1-X_2-[KRH]_3-X_4-[ACGVILMFYW]_5-[ACGVILMFYW]_6-[KRH]_7-[KRH]_8-[ACGVILMFYW]_9-[ACGVILMFYW]_{10}-[KRH]_{11}$. Additionally, there is a cluster of amino acids rich in positive charges located at the N-terminal side of the motif (Gagné et al., 2003). The putative amino acid sequence (361-372) of RECQL4's PBM is $L_1-R_2-S_3-R_4-L_5-L_6-R_7-K_8-Q_9-A_{10}-$

W_{11}–K_{12} (Figure 1. A). While the 3rd and 4th amino acid residues do not match exactly match the consensus PAR binding motif amino acid sequence (they are not completely mismatched but have swapped positions), and there is an additional amino acid inserted between the 8th and 9th amino acid residues. However, the overall pattern characterized by the alternating arrangement of hydrophobic and basic amino acids remains consistent. Additionally, the front portion of this sequence contains a cluster of positively charged basic amino acids (**KH**YV**R**G**R**A ; Positions in **bold** are basic amino acids). Based solely on the characteristics of the amino acid arrangement alone, it is highly probable that the 361–372 region of RECQL4 represents a PAR–binding motif. However, due to the lack of complete matching, the 361–372 region of RECQL4 remained unclear as a typical PAR binding motif until it was experimentally tested. The results of the micro–irradiation experiment demonstrated that wild–type RECQL4 was recruited to DSB sites much more strongly compared to when it was replaced with the hnRNP A2 PBM. This suggests that wild–type RECQL4 interacts more effectively with PAR, and at least the surrounding region, including this specific portion, exhibits characteristics of a PAR binding motif.

As observed in micro–irradiation experiments (Figure 3), RECQL4 rapidly recruits to DNA damage sites but dissociates within a few minutes. However, HR repair and end resection are time–consuming processes, raising questions about how the recruitment of RECQL4 to PAR–dependent DSB sites influence HR repair. Several possible hypotheses have been proposed regarding the relationship between the rapid and transient PAR–dependent recruitment of RECQL4 and its influence on long–lasting HR repair.

First, it is possible that even after the PAR–dependent recruitment subsides, a fraction of RECQL4 protein remains associated with the vicinity of DNA double–strand breaks (DSBs) for an extended period. Although the PAR–dependent recruitment may dissipate, specific regions of RECQL4 could continue to bind to DSBs, allowing for long–term interaction with HR repair processes.

In this scenario, RECQL4 may plays a role in DNA structure remodeling or protein recruitment at the DSB site over an extended period.

Secondly, RECQL4 possesses helicase activity (Xu & Liu, 2009), which can potentially influence protein recruitment through its involvement in various processes such as chromatin structure, DNA structure, and chromatin remodeling. By modulating these aspects, RECQL4 can indirectly impact the assembly of protein complexes at DNA damage sites. Its helicase activity allows it to unwind DNA duplexes, resolve DNA structures, and remodel chromatin, all of which can affect the accessibility of DNA and the recruitment of proteins involved in repair and other DNA metabolic processes.

Additionally, after being recruited to DSBs, RECQL4 is anticipated to facilitate the recruitment of proteins involved in the HR repair pathway. Even if RECQL4 dissociates from the site, the HR repair proteins are expected to continue performing their roles. Therefore, the transient recruitment of RECQL4 provides an opportunity for HR repair proteins to remain at the DSB site and actively participate in the DNA repair process.

PARylation is a primary event catalyzed by PARPs in response to DSBs within a few seconds after IR. It occurs at the adjacent regions of DSBs and facilitates the accumulation of DNA damage response proteins to the break sites via their PAR binding domains(Liu et al., 2017). Among the proteins involved in DSB response, APTX and PNKP interact with PAR through their FHA domains, while BRACA1, Ligase4, XRCC1, and NBS1 bind to PAR through their BRCT domains (Li & Yu, 2013) (Masson et al., 1998). Additionally, CHFR and APLF interact with PAR through their PBZ domains (Li et al., 2010). This study has revealed RECQL4 as a novel protein that binds to PAR through its PBM, leading to recruitment to DSB sites and playing a role in repair.

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Abstract in Korean

RECQL4 단백질은 RecQ 헬리케이즈 계열에 속하며 유전체 안정성 유지에 중요한 역할을 한다. 특히, RECQL4 단백질은 DNA 이중 가닥 절단 발생 시 ATM을 활성화시켜 DNA 손상 반응이 일어나게 하고, MRN의 안정성 유지를 통해 상동 재조합 수선이 잘 일어나도록 한다. 이전 연구를 통해 RECQL4는 폴리 ADP 라이보실화 (PARylation) 의존적인 방식으로 DNA 이중 가닥 절단 부위에 모집된다고 알려졌다. 최근 연구는 RECQL4의 아미노산 360-437 영역만으로도 PARylation 의존적인 방식으로 DNA 이중 가닥 절단 부위에 모집될 수 있고, 이 영역이 in vitro에서 폴리 ADP 라이보스 (PAR)와 직접적으로 상호작용한다는 것을 보여 주었다. 이는 RECQL4의 아미노산 360-437 영역에 PAR 결합 모티프 (PBM)가 있을 가능성을 암시한다. 하지만 RECQL4의 PBM으로 추정되는 영역이 실제 DNA 이중 가닥 절단 부위로의 모집에 중요한지, 이러한 PARylation 의존적 모집이 DNA 이중 가닥 절단 반응에서 중요한 역할을 하는지는 여부는 아직 알려지지 않았다.

본 연구에서 RECQL4의 PBM으로 추정되는 아미노산 부위를 잘 알려진 hnRNP A2의 PBM과 그것의 돌연변이형으로 교체한 RECQL4 변이 단백질을 이용하여 DNA 이중 가닥 절단 부위로의 모집과 DNA 이중 가닥 절단이 대한 세포 반응에 PAR와의 상호작용이 중요한지 확인하는 실험을 진행하였다. 그 결과 RECQL4의 PBM으로 추정되는 영역이 PARylation 의존적으로 DNA 이중 가닥 절단 부위에 모집되는데 중요하고, ATM 활성화, MRN 안정성 유지, HR repair가 진행되도록 하는데 관여한다는 것을 확인하였다. 이는 PBM을 통한 RECQL4와 PAR와의 결합이 DNA 이중 가닥 절단 반응에 중요한 역할을 한다는 것을 밝혀냈다는 점에서 의의가 있다.

주요어 : RECQL4, 폴리 ADP 라이보스, 폴리 ADP 라이보실화, PAR 결합 모티프, DNA 이중 가닥 절단, ATM, MRN, 상동 재조합 수선

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