이학박사학위논문

Studies on the role of RecQL4 in cellular response to DNA damages in human cells

인간 세포내 DNA 손상반응에서 RecQL4의 역할에 관한 연구

2017년 2월

서울대학교 대학원
과학교육과 생물전공

박 순 영

이학박사학위논문

Studies on the role of RecQL4 in cellular response to DNA damages in human cells

인간 세포내 DNA 손상반응에서 RecQL4의 역할에 관한 연구

2017년 2월

서울대학교 대학원
과학교육과 생물전공

박 순 영

Abstract

Rothmund-Thomson Syndrome (RTS) is an autosomal recessive disorder related with mutation in hu*RecQL4* gene, which is a member of the RecQ helicase family. The clinical features of RTS are genome instability, growth deficiency, skeletal defects, signs of premature aging, and increased risk of osteosarcoma. It is well established that RecQL4 is involved in DNA replication initiation. However, the role of RecQL4 in cellular response to DNA Double strand breaks (DSBs) is less clear. In this study, I showed that RecQL4 regulates Homologous Recombination (HR) repair by mediating the stability of MRE11-Rad50-Nbs1 (MRN) complex which is primary sensor for DNA DSBs. Depletion of RecQL4 reduced the protein level of MRN complex and downstream signaling pathway involving ATM activation and HR. MRN complex is ubiquitinated by Skp2 E3 ligase and degraded in proteasome. Also, MRN complex interacts with USP28, deubiquitinating enzyme by the assistance of RecQL4. Thus, USP28 deubiquitinates MRN complex and protects from degradation until finishing the HR completely. The helicase activity of RecQL4 is required for the stability of MRN complex and HR repair. Finally, MRN complex is

prematurely degraded in RTS fibroblast. These results suggest that RecQL4 might be a key factor plays a critical role in the DNA repair mechanism as well as maintenance of genome integrity in the cell.

Keywords : **DSBs, HR repair, RecQL4, RTS syndrome, MRN complex, USP28**

Student Number: 2011-31083

Table of Contents

LIST OF FIGURES

Introduction

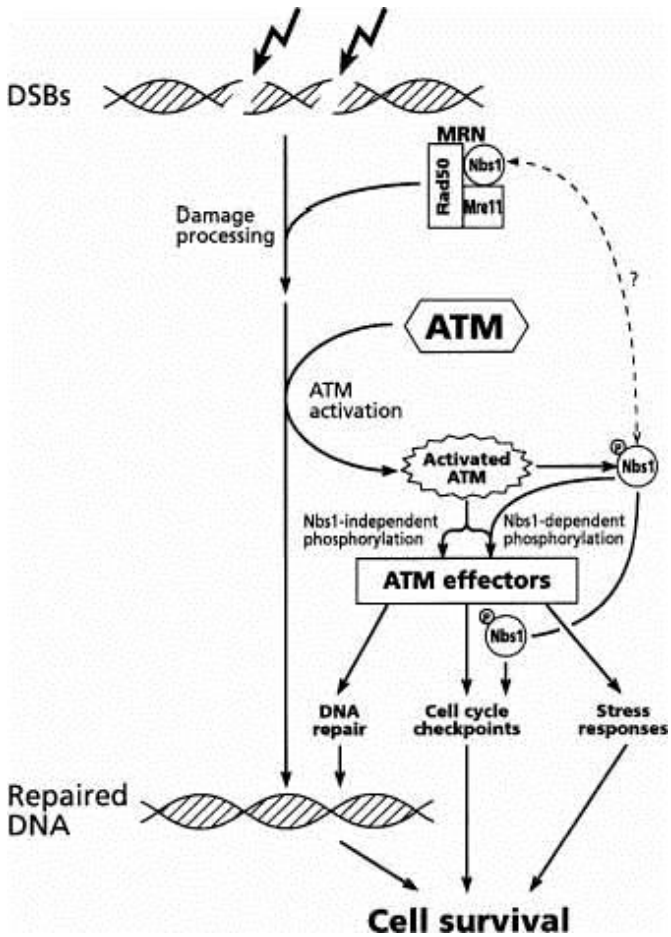
Research Background

DNA double-strand breaks (DSBs), one of the most severe forms of DNA damage, can lead to embryonic lethality, developmental disorders, immune deficiencies and predisposition to disease and cancer (Ciccia and Elledge, 2010; Jackson and Bartek, 2010). It is important that they be recognized and repaired timely and accurately. The nuclear protein kinase, ATM (Ataxia-telangiectasia-mutated protein) is regarded as the primary activator of this network (Shiloh, 2003). ATM is recruited and phosphorylated at DNA DSBs by the facilitation with Nbs1, a member of MRN complex (Lee and Paull, 2005). ATM is enhanced by auto-phosphorylation and it phosphorylates key proteins in numerous signaling pathway for cell survival (Bakkenist and Kastan, 2003, Uziel et al., 2003) (Figure 1).

DSBs are repaired in eukaryotes by the concerted action of mechanism based on non-homologous end joining (NHEJ) and homologous

Figure 1. Early events in the cellular response to DSBs.

The MRN complex is essential for the initial damage processing. Processed DNA lesions lead to the recruitment and activation of ATM, which in turn phosphorylated its substrates including Nbs1. Phosphorylated Nbs1 facilitates the phosphorylation of certain ATM substrates and plays a role in DNA repair, cell cycle checkpoint and stress responses.



(Uziel et al., 2003)

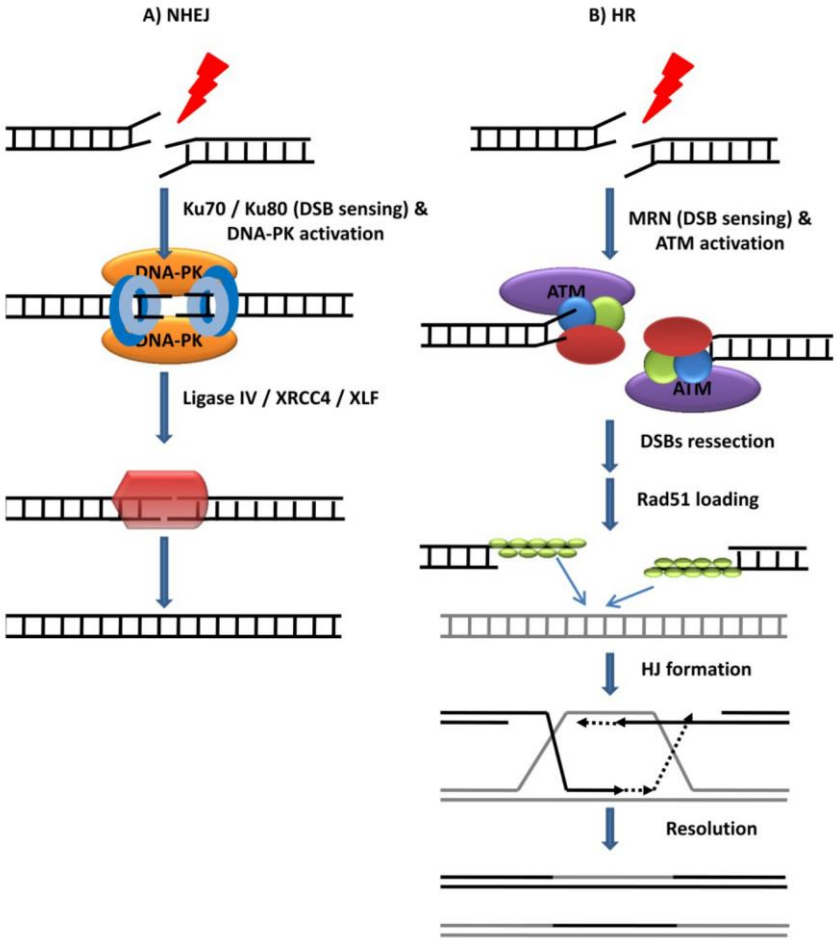
recombination (HR) (Figure 2). The choice of the DNA repair system is determined by the early step of DNA end resection which is regulated by many accessory factors (Daley and Sung, 2014, Li and Xu, 2016). DNA end resection inhibits NHEJ and triggers homology-directed DSB repair (Radhakrishnan et al., 2014, Yang et al., 2016). During NHEJ, the Ku70/80 heterodimers bind to DNA ends (Mari et al., 2006, Uematsu et al., 2007). And they help to recruit and stabilize DNA-PK at the DNA DSB sites. DNA-PK, in turn activates its own catalytic subunit (DNA-PKcs) and endonuclease Artemis removes excess single-strand DNA (ssDNA). And DNA ligase VI ligates the broken ends with XRCC4 (X-ray cross complementing protein 4) and XLF (XRCC4-like factor).

Ku70/80 complex binds to the DNA damage sites within seconds in all cell cycle phases (Mari et al., 2006, Britton et al., 2013, Shao et al., 2012). Ku forms a ring-shaped protein which slides onto the ends of broken DNA and has an extremely high affinity for dsDNA (Downs and Jackson, 2004, Walker et al., 2001). Similar to Ku70/80, assembly of DNA-PKcs to DSBs happens within seconds (Uematsu et al., 2007). Binding of DNA-PKcs to the DNA-Ku70/80 complex results in translocation of the Ku

Figure 2. DSBs repair mechanisms

A) During NHEJ, DSBs are sensed by Ku70/80 which then stabilizes the two DNA ends and recruits DNA-PK. Next, DNA-PK phosphorylates and activates the NHEJ effector complex (ligase Ⅵ/XRCC4/XLF) that finally religates the broken DNA.

B) During HR, the broken DNA ends are recognized by MRN complex and repair-factors including Exo1 and CtIP are recruited. They resect DNA to generate 3' overhang single-stranded DNA. RPA coated single strand DNA is displaced by RAD51 protein to facilitate DNA strand invasion and leads to Holliday junction formation.


A) NHEJ
B) HR
Ku70 / Ku80 (DSB sensing) &
DNA-PK activation
DNA-PK
DNA-PK
Ligase IV / XRCC4 / XLF
MRN (DSB sensing) &
ATM activation
ATM
ATM
DSBs ressection
Rad51 loading
HJ formation
Resolution

heterodimer inward on the dsDNA strand and results in activation of the DNA-PKcs (Yoo and Dynan, 1999, Calsou et al., 1999). The complex formed at the DSB consisting of DNA, Ku70/80 and DNS-PKcs is referred to as DNA-PK (Gottlieb and Jackson, 1993). DNA-PKcs is a member of PIKK (phosphatidylinositol-3 kinase-like kinase family), which also includes the two DNA damage responsive proteins, ATM and ATR (ATM and Rad3-related protein) (Bakkenist and Kastan, 2004). As well as Ku serves as a scaffold to recruit the NHEJ machinery to the DNA lesions, it maintains the stability of the ends of the broken DNA. It protects them from non-specific processing. Specifically, Ku has been shown to block DNA end processing enzymes including exonuclease 1 and the Mre11/Rad50/Nbs1 (MRN) complex in vitro (Sun et al., 2012). It is important for blocking non-specific processing of a DSB, because it protects against chromosomal aberrations and genomic instability.

HR functions in the S and G2 phases. The DSB ends are recognized primarily by MRN complex, which is an assembly of two Rad50 subunits, two Mre11 subunits, and Nbs1 proteins (Williams et al., 2010). Rad50 is recruited to the DNA ends of the DSBs (Kinoshita et al., 2015). Mre11 has

single-stranded DNA (ssDNA) endonucleases and 3’ to 5’ exonuclease activities which are significant for the initiation of DNA end resection (Kanaar and Wyman, 2008). And, Nbs1 has FHA domain and BRCT domain which are responsible for protein-protein interaction for DDR. (Lee and Paull, 2005; Jazayeri et al., 2008). MRN complex recruits CtIP which interacts with BRCA1 at DSBs (Huen et al., 2009) and resects ssDNA together with Exo1, BLM and DNA2 (Sartori et al., 2007; Bolderson et al., 2010). They generate a long stretch of 3’-overhang ssDNA that are coated with RPA (Chen et al., 2013). RPA coated ssDNA is displaced by RAD51 protein. The formed Rad51 nucleoprotein filament facilitates DNA strand invasion and leads to Holliday junction formation (Suwaki et al., 2011).

MRN complex is involved in checkpoint pathway as well as HR repair (Uziel et al., 2003, Lavin et al., 2015b). Nbs1 interacts with ATM through its C-terminal domain, which promotes the binding of ATM to DSBs (Lee and Paull, 2007). As recruited to DSBs, inactive dimer ATM is dissociated and auto-phosphorylated on multiple residues which are important for ATM activation (Bakkenist and Kastan, 2003). Activated ATM recruits and phosphorylates ATM substrates at DSBs for DDR amplification.

The most well-known substrate is Chk2 and p53 (Zhou and Elledge, 2000). Even though MRN complex plays important roles in acute DNA damage response, it is not known well how is regulated in any way.

According to previous studies, Mre11-Rad50-Xrs2 (MRX) complex in yeast is associated with DSBs within seconds and displaced from the DSB sites upon completion of its initial recognition and processing role (Williams et al., 2007). In fact, DNA damage-dependent nuclear localization of MRN complex is dynamic. Irradiated cells first show many small Mre11-foci and as cells repaired, the number of foci decreases and the remaining unrepaired foci get larger (Mirzoeva and Petrini, 2001, Petrini and Stracker, 2003). However, how MRN complex is regulated is unknown well. According to Stracker's study, MRN complex can be regulated by proteasome-mediated degradation by Adenovirus infection (Stracker et al., 2002). However, which E3 ubiquitin ligase acting on MRN complex remains to be identified. The Skp2 is the F-box protein, a components of the SCF (Skp1-Cul1-F box) which is the largest family of E3 ubiquitin-ligases (Lee and Lim, 2016). One of the most important proteins recognized by Skp2 is cyclin-dependent kinase inhibitor p27, which negatively regulates

the cell cycle progression in the G1 phase (Wang et al., 2012). Wu proposed Skp2 E3 ligase ubiquitinates Nbs1 for activating HR (Wu et al., 2012). However, these ubiquitination is for interaction with ATM not for degradation. One of the deubiquitinating enzymes (DUBs), ubiquitin specific protease 28 (Usp28) is involved in the stability of Nbs1 protein, even exact mechanism is not known. In the absence of Usp28, three groups of checkpoint proteins become unstable in response to ionizing irradiation. They are checkpoint mediators (53BP1, Mdc1, TopBP1 and Claspin), PIKK specificity factors (Nbs1 and ATRIP), and a checkpoint kinase (Chk2) (Zhang et al., 2006). Even it has been studied that Usp28 affects degradation of Myc by associating with Fbw7, tumor suppressor gene (Popov and Eilers, 2007), mechanism of Usp28 in controlling the stability of other checkpoint proteins needs to be studied.

RecQL4 is a member of the RecQ helicase family which is a highly conserved group of DNA helicase (Larsen and Hickson, 2013). It performs important roles in maintaining genome integrity including DNA replication, DNA repair and Autophagy (Chu and Hickson, 2009, Duan et al., 2016). Mutations in this gene is known to be related with Rothmund-Thomson

Syndrome (RTS), which results in growth deficiency, skeletal defects, signs of premature aging, and increased risk of osteosarcoma (Kitao et al., 1999). The *RECQL 4* gene encodes a protein of 1208 amino acids long (133 KDa), and contains the conserved Sld2 domain in its N-terminal region and 3' to 5' helicase domain in its center. The Sld2 domain is indispensable for the initiation of DNA replication and cell viability (Sangrithi et al., 2005; Abe et al., 2011). RecQL4 interacts with other replication factors (Im et al., 2009; Im et al., 2015; Kliszczak et al., 2015) and the frequency of DNA replication initiation is reduced in RecQL4-depleted cell (Thangavel et al., 2010).

However, most mutations of RecQL4 discovered in RTS patients are nonsense or frameshift mutation which result in a truncated helicase domain (Kitao et al., 1999; Hoki et al., 2003; Mann et al., 2005). In addition, RecQL4 gene-knockout cells complemented with N-terminal region of RecQL4 lacking the helicase domain showed relatively high sensitivity to DNA damaging agents (Abe et al., 2011). It suggests helicase activity of RecQL4 is important for genome instability. RTS fibroblasts are sensitive to ionizing radiation (Smith and Paterson, 1982). And recent studies suggest RecQL4 is involved in DNA repair process. RecQL4 is recruited to laser-

induced DSBs (Singh et al., 2010) and function in NER by interacting with a major NER factor, XPA when irradiated with UV in human cell (Fan and Luo, 2008). Shamanna et al. demonstrated that RecQL4 co-immunoprecipitates with Ku70/80, a component of NHEJ pathway (Shamanna et al., 2014). Other report suggested that RecQL4 co-localized and interacts with RAD51 in cells exposed to etoposide (Petkovic et al., 2005). And recent study suggests that RecQL4 promotes DNA end resection during HR repair in response to ionizing radiation. It show RecQL4 interacts with MRN complex and helicase activity of RecQL4 is important for HR repair (Lu et al., 2016a).

Purpose of Research

Although many studies suggest the role of RecQL4 in cellular response to DNA damages, the exact role of RecQL4 protein in these processes are not fully understood. First, RecQL4 reacts with diverse DNA damage and is involved in diverse DNA repair system by co-localizing and interacting with diverse repair factor. I wondered that RecQL4 mainly

responds which kinds of DNA damage and have roles in which repair system. In my study, I suggest that RecQL4 responds to DNA-DSBs inducing reagent and mainly involved in HR repair by interacting with MRN complex at DNA DSBs. Second, it is not known well how RecQL4 affect specific proteins in specific repair pathway. My results suggest that RecQL4 regulates the stability of MRN complex via mediating the ubiquitinating system. Finally, even though there are lots of suggestions to understand the RTS syndrome, it is difficult to know the fundamentals exactly. If the roles of RecQL4 are uncovered and confirmed in RTS-patient cells, it could be contributed to understand and cure the disease, RTS syndrome. I suggest that premature degradation of MRN complex upon absence of RecQL4 may cause the genome instability and disease, RTS syndrome.

Materials and Methods

Cell culture and reagents

Human U2OS osteosarcoma cells were cultured in Dulbecco's modified Eagle's medium (DMEM; Welgene, Korea) supplemented with 10% Fetal Bovine Serum (FBS) and antibiotics. The U2OS pTet ON cells expressing RecQL4 mutants, wildtype (WT), Helicase truncation (HT), walker 'A' mutants (A), walker 'B' mutants (B) were previously described (An Yoon young, 2013). The mutant proteins were induced with doxycycline (0.1 mg/ml). They were maintained in DMEM, 10% Tet-Free FBS, with the supplements described above. RTS-deficient skin primary fibroblasts (AG18371, AG17524) cell lines from Coriell Cell Repositories (NJ, USA) were used in the study. AG18371 shows mutation in g.2746del11 (exon8) of RecQL4 and AG1754 in g.2626G>A(exon8) g.4644delAT(exon15). The cells maintained in Alpha-MEM (Welgene, Korea), 15% FBS and antibiotics. Cells were in 5% CO_2 humidified incubator at 37°C

For Double strand breaks induction, cells were incubated with 200

ng/ml Neocarzinostatin (NCS; Sigma Aldrich) for 15 min and released in fresh medium for 0.5 or 1 hour or treated with 20uM Etoposide (Sigma Aldrich) for 3 hours.

For Single strand breaks induction, cell were treated with 0.01% Methanesulfonate (MMS; Sigma Aldrich) or 1uM Benzopyrene (BP; Sigma Aldrich) for 15 minutes and incubated in fresh medium for indicated different times.

Plasmid preparation and transfection

All RecQL4 mutants including WT, HT, A, B, CD1 to CD3 (C-terminus deletion 1, 2, and 3), ND1 to ND3 (N-terminus deletion 1, 2, and 3) and C1 to C3 (C-terminal 1, 2 and 3) were previously described (An Yoon young, 2013, Hong Myung gin, 2016). They were cloned into a mammalian expression vector pcDNA3.1 (-) with a double Flag-tag at the N-terminus. . Mre11 and Nbs1 were cloned with a single Myc-tag and a double HA-tag, respectively (Hong Myung gin, 2016). USP28 (WT, C171A) cloned with a Flag-tag were obtained from the laboratory of

professor, Back Sung hee (Seoul National University, Seoul). The DNA constructs were co-transfected and overexpressed in U2OS cells using Polyfect transfection reagent (Qiagen) according to the manufacturer's instructions. Cells were then incubated for 48 hours until harvest.

For siRNA-mediated interference, siRNA duplex oligoribonu cleotides were synthesized from ST Pharm (Seoul, Korea). Transfections of siRNA duplexes were performed by electrophorator (Neon® transfection system; Invitrogen) according to the manufacturer's instructions. Experiments were performed 48 hours after transfection. The sense sequences were as follows:

GL siRNA	:	5'-AACGUACGCAUACUUCGATT-3'
RecQL4 siRNA	:	5'- GACUGAGGACCUGGGCAAATT-3'
Skp2 siRNA	:	5'-GAUAGUGUCAUGCUAAAGAAUTT-3'
Cdc34 siRNA	:	5'-GGAAGUGGAAAGAGAGAGCAATT-3'
Ubc5 siRNA	:	5'-GAGAAUGGACUCAGAAAUATT-3'
Ubc13 siRNA	:	5'-CCAGAUGAUCCAUUAGCAATT-3'
USP28 siRNA	:	5'-CUGCAUUCACCUUAUCAUU-3'

Nbs1 siRNA : 5’-CCAACUAAAUUGCCAAGUAUU-3’

Mre11 siRNA : 5’-GGAGGUACGUCGUUUCAGATT-3’

KU70 siRNA : 5’-GGAAGAGATAGTTTGATTT-3’

HR repair assay

To determine the effect of RecQL4 on HR repair, the fluorescence-based HR repair was performed. U2OS cells integrated HR reporter DR-GFP were obtained from the laboratory of professor, Jeremy M. Stark (Institute of the City of Hope, CA, USA). The stable pDR-GFP cells transfected with either control siRNA or RecQL4 for 1 days were further transfected with either I-Scel-expressing vector or a control vector and RecQL4 mutants. Forty-eight hours later, the cells were subjected to the analysis of GFP-positive cells using flow cytometer (Becton Dickinson) with CellQuest software.

Immunoblot analysis

Cells were harvested, washed with PBS and lysed in RIPA buffer (50 mM Tris-HCl, pH 7.5, 1 mM EDTA, 150 mM NaCl, 1% NP-40, 0.25% Na· deoxycholate) with protease inhibitors (5 ug/ml leupeptin, 1mM benzamidine, 5 ug/ml pepstatin A, 1 mM PMSF) and phosphase inhibitors (10 mM sodium fluoride, 10 uM sodium Orthovanadate). The proteins were quantified using the Bradford assay and whole cell lysates were mixed with 5X SDS sample buffer and boiled at 95℃ for 5 minutes. And cells were disrupted by sonication and separated by 8-12% SDS-PAGE and transferred to nitrocellulose membranes. Membranes were blocked for 30 min with 5% fat-free milk in TBS, and probed with primary antibodies in 3% bovine serum albumin in TBS. After washing three times with TBS containing 0.04% Tween 20 (TBST), the membranes were incubated with secondary antibodies and detected by using ECL immunoblot reagents. Antibodies were purchased from various companies respectively: anti-RecQL4 (purified from rabbit), anti-HA (purified from mouse), anti-Mre11, anti-pATM, anti-pATR, anti-pChk2, anti-pChk1 (Cell Signaling), anti-Rad50, anti-Flag, anti-ATR, anti-Chk1, anti-p53, anti-BRCA1, anti-Skp2 (Sigma

Aldrich), anti-ATM, anti-chk2 (Abcam), anti-CtIP, anti-USP28 (Bethyl), λ H2AX (Upstate), anti-Myc (Millipore), and anti-Nbs1 (Novus and Genetax).

Immunoprecipitation

Cells were harvested and washed once with cold PBS. Protein extracts were prepared with lysis buffer (40 mM Tris-HCl, 150 mM NaCl, 2.5 mM MgCl2, 1 mM DTT, 5% glycerol, 0.2% NP40), protease inhibitors and phosphatase inhibitors. After sonication, benzonase (90 Unit/ml) was added and incubated for 4 hours at 4 °C. They were then centrifuged at 13000 rpm, at 4°C for 10 minutes. The supernatants were mixed with 5 µl of Protein G or A beads for bead clearing for an hour at 4 °C. The protein extracts were incubated with a respective primary antibody overnight at 4°C. Then protein G or A beads bind with the extracts for the next hour at 4°C. They were centrifuged at 2500 rpm at 4°C, and the beads were washed three times with the same buffer with 0.02% NP40. 1.5X SDS sample buffer was added and boiled for 5 min, for subsequent gel electrophoresis and Western

blot analysis.

Immunostaining and microscopy

Cells grown on cover slips were washed once with cold phosphate buffered saline (PBS) and either untreated or treated with pre-extraction by pre-extraction buffer (0.5% Triton X-100 diluted in PBS) on ice for 5 min. And cells fixed with 4% paraformaldehyde/PBS at room temperature for 10 min. Fixed cells were permeabilized with working buffer (0.3% Triton X-100 diluted in PBS) on ice for 5 minutes. Blocking was carried out with blocking buffer (3% BSA diluted in working buffer) at room temperature for 30 minutes. The cells were incubated for an hour with primary antibodies diluted in blocking buffer. After being washed three times with washing buffer (0.1% Triton X-100 diluted in PBS), the cells were incubated for 30 min with secondary anti-mouse Alexa-fluoro 594 and anti-rabbit Alexa-fluoro 488 (Invitrogen) at a dilution of 1:1000. The nuclei were counterstained with 4'-6'-diamidino-2-phenylindole (DAPI). Slides were mounted and immunofluorescence was observed with the fluoroscence

microscope (Zeiss AX10).

RESULTS

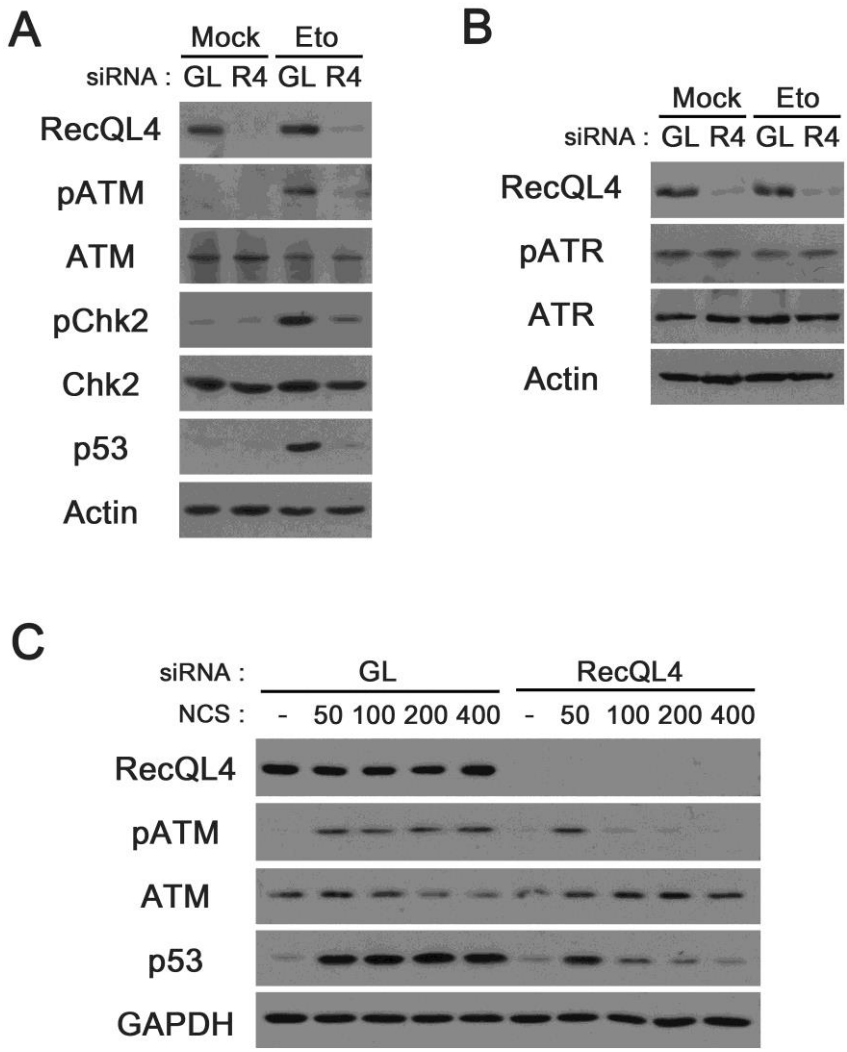
The helicase activity of RecQL4 is required for ATM activation and Homologous recombination repair.

In order to examine the function of RecQL4 in cellular response to DNA DSBs, I depleted RecQL4 protein by siRNA from U2OS cells and examined checkpoint activation after treatment with Etoposide, topo-isomerase inhibitor. While the activation of ATR-signaling pathway was not affected by Etoposide treatment, the phosphorylation of ATM and Chk2 and the protein level of p53 were significantly reduced in RecQL4 depleted cells (Figure 3A and B). Activation of ATM signaling pathway was also reduced when RecQL4-depleted cells were treated with other kinds of DNA DSBs-inducing reagent, NCS. However, ATM activation was still observed when RecQL4-depleted cells were treated with low concentration of NCS (Figure 3C). It suggests that other factors are also involved in this reduction of ATM activation. In addition, the number and intensity of phosphor-ATM foci were reduced when RecQL4-depleted cells are treated with NCS or bleomycin

Figure 3. RecQL4 is required for ATM activation.

A. and B. U2OS cells transfected with GL2* or RecQL4 (R4) siRNA and incubated for 2 days were either untreated (Mock) or treated with 20uM Etoposide (Eto) for 3hrs. Checkpoint activation was examined by Western blot analysis using phosphor-specific antibodies. *GL2 siRNA, targets Luciferase, was used as positive control siRNA.

C. U2OS cells transfected with GL or RecQL4 siRNA and incubated for 2 days were either untreated (Mock) or treated with indicated concentration of NCS for 15 minutes. After incubation in fresh medium for 30 minutes, checkpoint activation was examined by Western blot analysis.


A
Mock
Eto
siRNA : GL R4 GL R4
RecQL4
pATM
ATM
pChk2
Chk2
p53
Actin
B
Mock
Eto
siRNA : GL R4 GL R4
RecQL4
pATR
ATR
Actin
C
siRNA :
GL
RecQL4
NCS : - 50 100 200 400 - 50 100 200 400
RecQL4
pATM
ATM
p53
GAPDH

(Figure 4). Collectively, these results suggest that RecQL4 is required for activation of ATM signaling pathway in response to DNA DSBs.

To determine whether the helicase activity of RecQL4 is required for ATM activation, RecQL4 proteins deleted with helicase domain (helicase truncation) or mutated in helicase motifs (Walker A or Walker B mutant) were expressed in endogenous RecQL4-depleted cells (Figure 5A) and ATM activation was observed after NCS treatment. As shown in Figure 3B, ATM phosphorylation and p53 stabilization were significantly reduced in these cells (Figure 5B). These data suggests that helicase activity of RecQL4 protein is required for ATM activation after DSBs.

I also studied whether RecQL4 play an important role in DNA DSB repair pathway. For this purpose, I examined the foci formation of BRCA1 and 53BP1, the key factor of HR pathway and NHEJ pathway, respectively. The number of BRCA1-foci was significantly reduced in RecQ4-depleted cells, and it was recovered by expressing wild type RecQL4 proteins, but not by expressing helicase mutants. On the other hand, 53BP1-foci were not affected by RecQL4 depletion (Figure 6A). It is correlated with previous data that 53BP1-foci are maintained until 10 hours when RTS fibroblast

Figure 4. RecQL4 is required for ATM-foci formation.

U2OS cells transfected with the GL or RecQL4 siRNA for 2 days were either untreated (Mock) or treated with NCS (200ng/ml) for 15 minutes and incubated in fresh medium for an hour. Immunostaining with anti-phosphor ATM was performed. A cell containing 20 or more foci was considered as a foci-positive cell. The percentage of phosphor ATM foci-positive cells was plotted. The intensity of phosphor ATM foci-positive cell was measured by AxioVS40. The average of GL siRNA (Mock) for fluorescence was regarded as 1 for comparison.

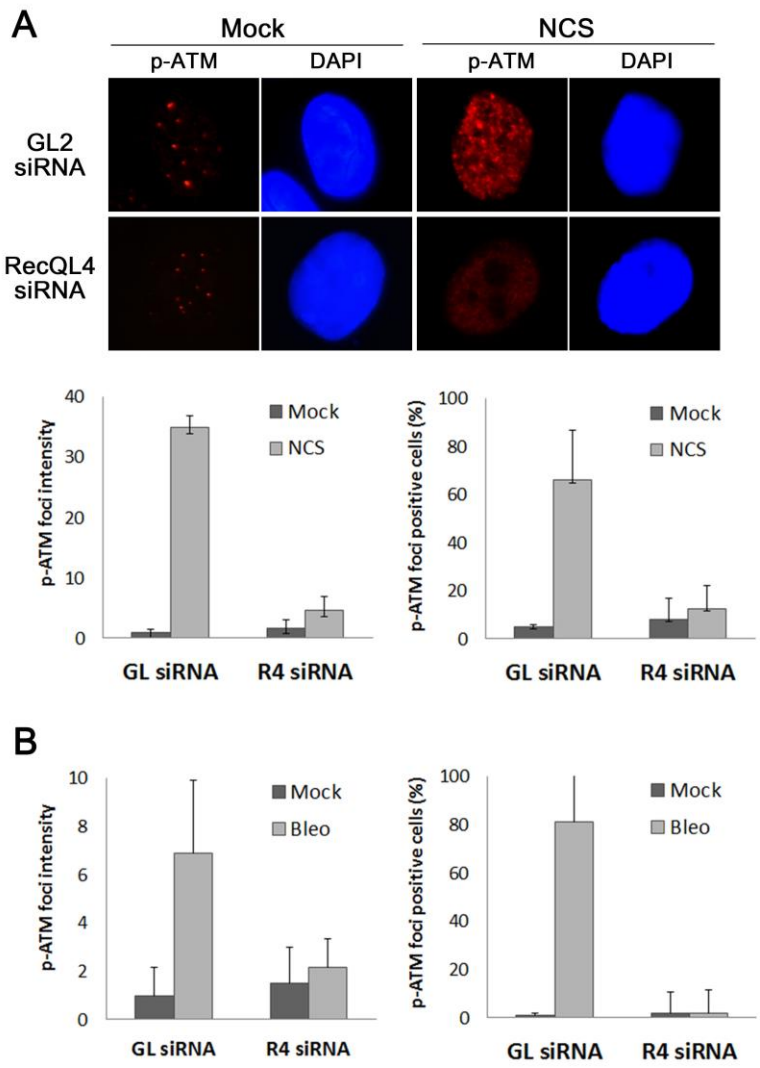
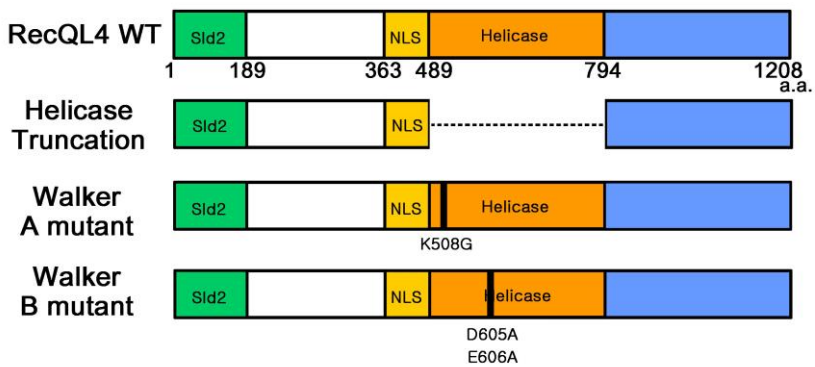

A
Mock
NCS
p-ATM
DAPI
p-ATM
DAPI
GL2 siRNA
RecQL4 siRNA
p-ATM foci intensity
40
30
20
10
0
Mock
NCS
GL siRNA
R4 siRNA
p-ATM foci positive cells (%)
100
80
60
40
20
0
Mock
NCS
GL siRNA
R4 siRNA
B
p-ATM foci intensity
10
8
6
4
2
0
Mock
Bleo
GL siRNA
R4 siRNA
p-ATM foci positive cells (%)
100
80
60
40
20
0
Mock
Bleo
GL siRNA
R4 siRNA

Figure 5. The helicase activity of RecQL4 is required for ATM activation after DNA damage.

A. Diagram of plasmids encoding wildtype (WT) or deletion mutants (Helicase Truncation, Walker 'A' mutant, and Walker 'B' mutant) of RecQL4 are shown. Different domains of RecQL4 can be distinguished by color. The amino acid numbers are labelled below each diagram.

B. U2OS cells depleted with GL or RecQL4 siRNA and transfected RecQL4 mutant plasmids were treated with 20uM Etoposide (Eto) for 3hrs or not. Expression levels of each protein were analyzed by immunoblotting. WT, wildtype; HT, helicase truncation; A, walker 'A' motif mutant; B, walker 'B' motif mutant.

A


RecQL4 WT
Sld2
NLS
Helicase
1
189
363
489
794
1208
a.a.
Helicase Truncation
Walker A mutant
K508G
Walker B mutant
D605A
E606A

B

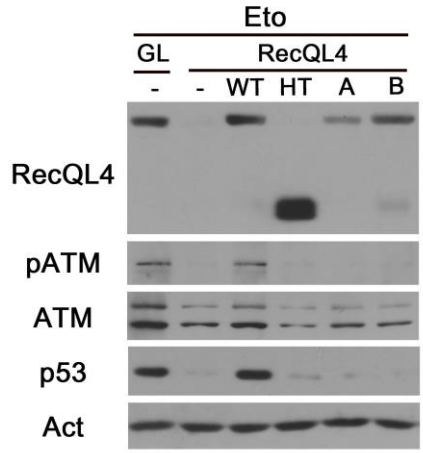

Eto
GL
RecQL4
-
-
WT
HT
A
B
RecQL4
pATM
ATM
p53
Act

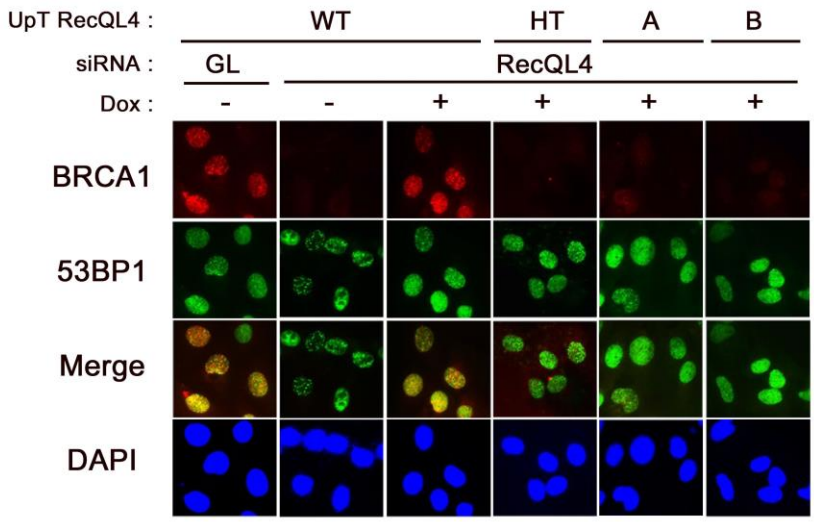
Figure 6. Helicase domain of RecQL4 is essential for BRCA1 foci formation after DNA double-strand breaks.

U2OS pTet On stable cell lines (UpT) expressing RecQL4 mutants (WT, HT, A, B) were silenced with the GL or RecQL4 siRNA and RecQL4 mutant proteins were induced with doxycycline (0.1 mg/ml) for 2 days. And they were treated with NCS (200ng/ml) for 15 minutes and incubated in fresh medium for an hour.

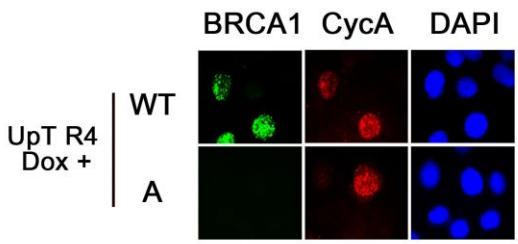
A. Immunostaining with anti-BRCA1 and anti-53BP1 was performed.

B. Immunostaining with anti-BRCA1 and anti-CycA was performed.

A


UpT RecQL4 :
WT
HT
A
B
siRNA :
GL
RecQL4
Dox :
-
-
+
+
+
+
BRCA1
53BP1
Merge
DAPI

B


BRCA1
CycA
DAPI
UpT R4
Dox +
WT
A

were irradiated (Singh et al., 2010).The BRCA1-foci were observed in only S and G2 phase cells and were disappeared in RecQL4 helicase mutant cells (Figure 6B). These result suggested that HR repair pathway might be affected by the depletion of RecQL4. Therefore, I tested whether RecQL4 play any role in HR repair pathway using I-SceI reporter assay for HR. I found that RecQL4 depletion led to a threefold reduction in HR frequency compared to control siRNA. This reduction was recovered by expressing wild type RecQL4, but not by expressing walker 'A' mutant (Figure 7). Also, Rad51 foci that indicated the completion of end resection for HR, were not observed in the RecQL4 walker 'A' mutant cell (Figure 8). Collectively, these results suggest that RecQL4 plays an important role in homologous recombination repair, and its helicase activity is critical.

Figure 7. Helicase domain of RecQL4 is required for Homologous recombination repair.

DR-GFP-integrated U2OS cells silenced with GL or RecQL4 siRNA were transfected with I-Sce1 along with RecQL4 wild-type or walker 'A' mutants. After 2 days, cells were harvested and analyzed by flow cytometry assay. RecQL4 'walker A mutant' cells resulted in a twofold reduction in the number of GFP-positive cells compared to RecQL4 wild-type cells.

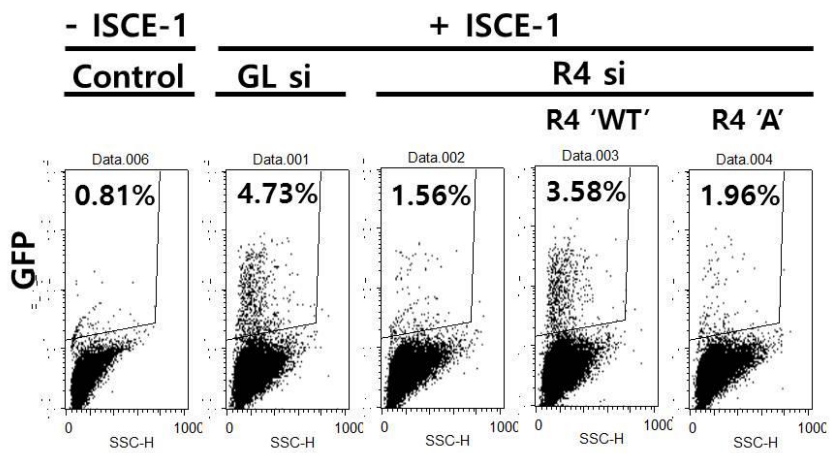
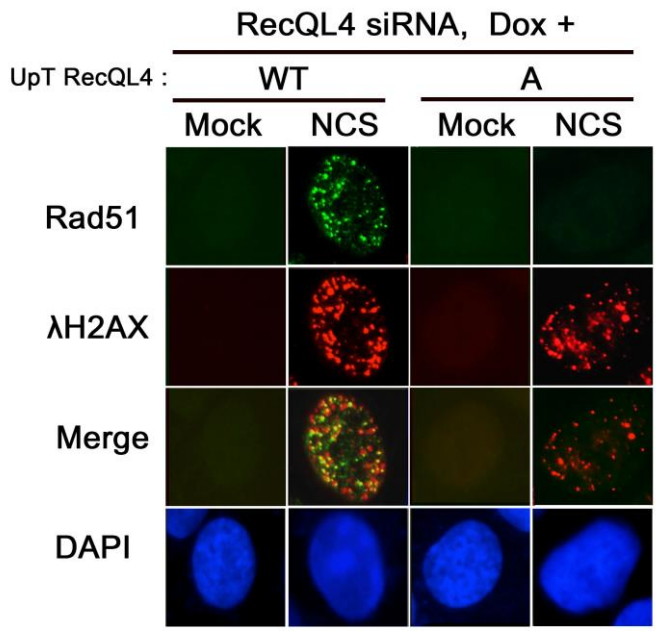
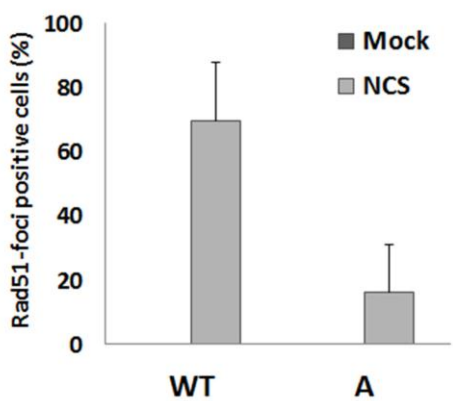

- ISCE-1
+ ISCE-1
Control
GL si
R4 si
R4 'WT'
R4 'A'
Data.006
Data.001
Data.002
Data.003
Data.004
0.81%
4.73%
1.56%
3.58%
1.96%
GFP
SSC-H
0
1000

Figure 8. Helicase domain of RecQL4 is essential for recruiting Rad51 at DNA double-strand break sites.

U2OS stable cell lines transfected with RecQL4 siRNA and treated with doxycycline (0.1ug/ml) for 2 days were either untreated (Mock) or treated with NCS (200ng/ml) for 15 minutes and incubated in fresh medium for an hour. Immunostaining with anti-Rad51 and anti-λH2AX was performed. Merged image of green and red signals shows co-localization between Rad51 and γH2AX. A cell containing 20 or more foci was considered as a foci-positive cell. The percentage of Rad51-foci positive cells was plotted.


RecQL4 siRNA, Dox +
UpT RecQL4 :
WT
A
Mock
NCS
Mock
NCS
Rad51
λH2AX
Merge
DAPI


Rad51-foci positive cells (%)
100
80
60
40
20
0
Mock
NCS
WT
A

Helicase activity of RecQL4 is required for stability of MRN complex after DNA DSBs.

Since RecQL4 is required for both ATM activation and HR, I reasoned that the upstream factors involved in both processes might be affected. Indeed, the protein levels of Nbs1 and Mre11, components of MRN complex playing essential roles in ATM activation and HR, were significantly reduced when RecQL4-depleted cells were treated with NCS or λ-radiation (Figure 9A and B). In order to investigate whether the stability of MRN complex is changed upon the intensity of DNA DSBs in RecQL4-depleted cells, I treated NCS with dose and time differently. The degradation of Nbs1 and Mre11 increases in time- and concentration-dependent manner (Figure 9C). These result correlates with the change of ATM activation upon intensity of DNA DSBs (Figure 3C). Also, the number of Nbs1-foci was significantly reduced when RecQL4- depleted cells were treated with NCS (Figure 10). These data suggests that RecQL4 protein is required for the maintenance of MRN complex.

However, other kinds of DNA damage inducing reagents involving

Figure 9. RecQL4 is required for stability of MRN complex after DNA DSBs.

U2OS cells were transfected with GL or RecQL4 siRNA for 2 days.

A. They were either untreated (-) or treated with NCS (200ng/ml) for 15 minutes and incubated in fresh medium for indicated different times.

B. They were ionizing-radiated at 20Gy or not (-) and incubated for indicated different times.

C. They were either untreated (-) or treated with various doses NCS (from 50 to 400 ng/ml) for 15 minutes and incubated in fresh medium for indicated different times. Expression levels of each protein were analyzed by immunoblotting.

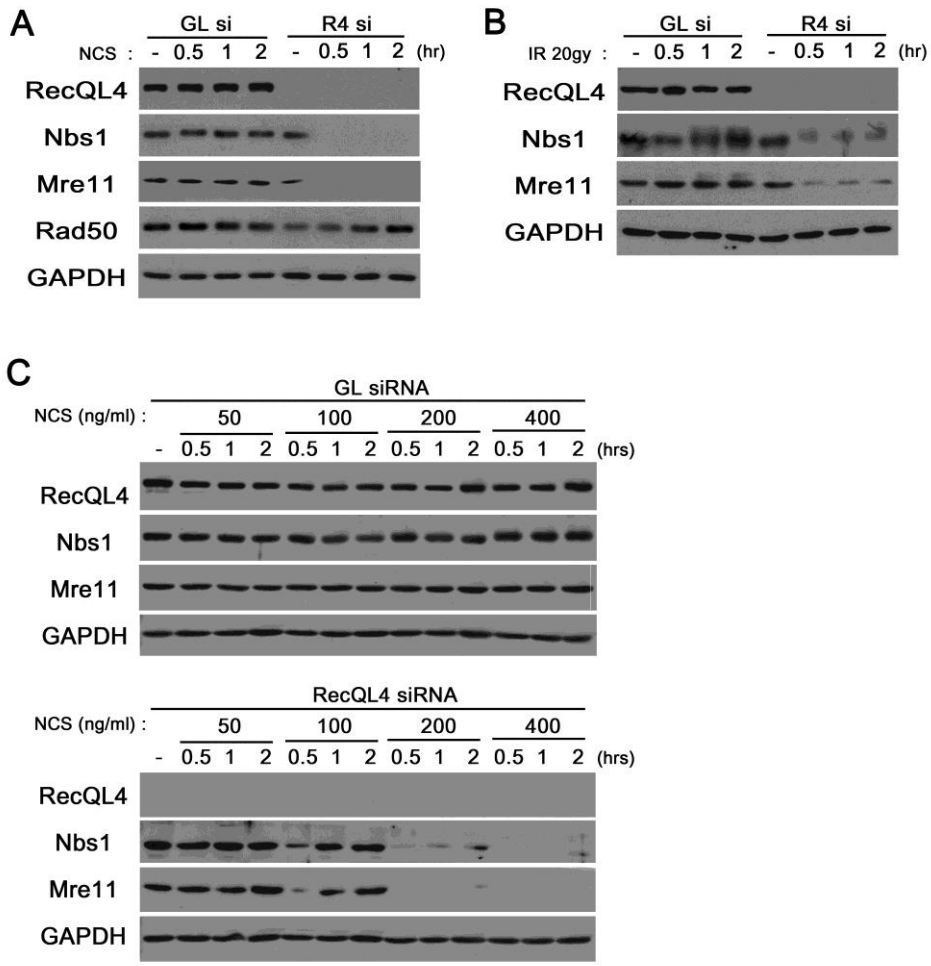
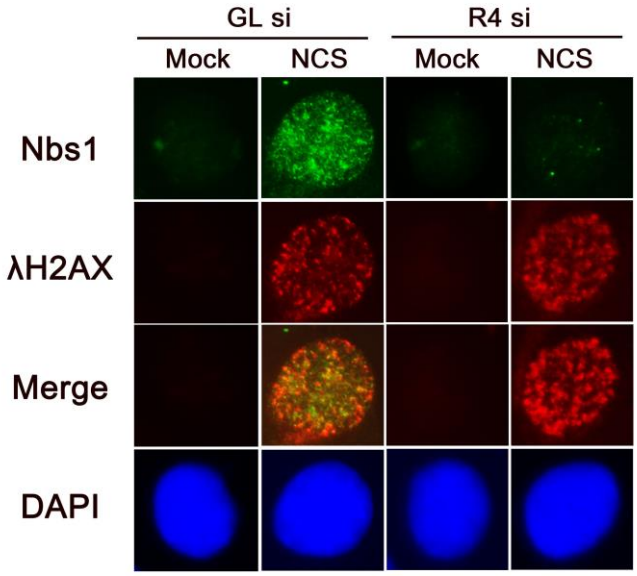
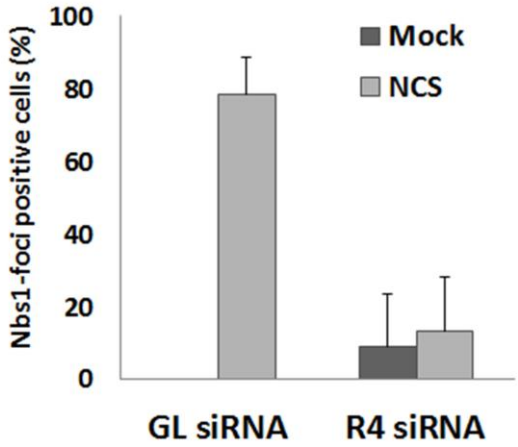

A
GL si
R4 si
NCS : - 0.5 1 2 - 0.5 1 2 (hr)
RecQL4
Nbs1
Mre11
Rad50
GAPDH
B
GL si
R4 si
IR 20gy : - 0.5 1 2 - 0.5 1 2 (hr)
RecQL4
Nbs1
Mre11
GAPDH
C
GL siRNA
NCS (ng/ml) : 50 100 200 400
- 0.5 1 2 0.5 1 2 0.5 1 2 0.5 1 2 (hrs)
RecQL4
Nbs1
Mre11
GAPDH
RecQL4 siRNA
NCS (ng/ml) : 50 100 200 400
- 0.5 1 2 0.5 1 2 0.5 1 2 0.5 1 2 (hrs)
RecQL4
Nbs1
Mre11
GAPDH

Figure 10. RecQL4 deficiency caused a reduction in Nbs1 foci.

U2OS cells transfected with GL or RecQL4 siRNA for 2 days were either untreated (Mock) or treated with NCS (200ng/ml) for 15 minutes and incubated in fresh medium for an hour. Immunostaining with anti-Nbs1 and anti-λH2AX was performed. Merged image of green and red signals shows co-localization of two proteins. A cell containing 20 or more foci was considered as a foci-positive cell. The percentage of Nbs1-foci positive cells was plotted.


GL si
R4 si
Mock
NCS
Mock
NCS
Nbs1
λH2AX
Merge
DAPI


Nbs1-foci positive cells (%)
100
80
60
40
20
0
Mock
NCS
GL siRNA
R4 siRNA

MMS, UV and Benzopyrene didn't affect the maintenance of MRN complex (Figure 11). It indicates that the protein level of MRN complex is decreased in only response to DNA double strands breaks.

I tested whether the helicase activity of RecQL4 is important for the maintenance of MRN complex. The cells expressing wild-type RecQL4 and RecQL4 mutants involving helicase domain (CD3) were completely recovered from the reduced protein of Nbs1 and Mre11. On the other hand, the cells expressing RecQL4 helicase mutant failed to recover the reduced MRN complex (Figure 12). These data suggests that the helicase activity of RecQL4 is critical for the maintenance of MRN complex.

Figure 11. Single-strand breaks don't affect the stability of MRN complex.

U2OS cells were transfected with GL or RecQL4 siRNA for 2 days.

A. They were either untreated (-) or treated with UV ($20J/m^2$) and incubated for indicated different times.

B. They were either untreated (-) or treated with 0.01% Methanesulfonate (MMS) for 15 minutes and incubated in fresh medium for indicated different times.

C. They were either untreated (-) or treated with 1uM Benzopyrene (BP) for 15 minutes and incubated in fresh medium for indicated different times. Expression levels of each protein were analyzed by immunoblotting.

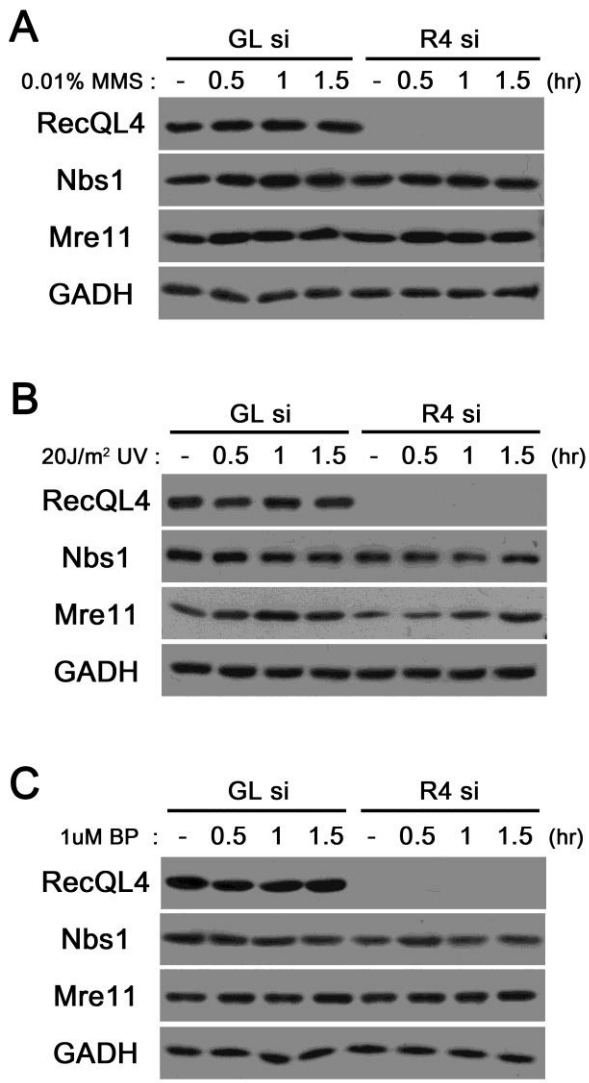
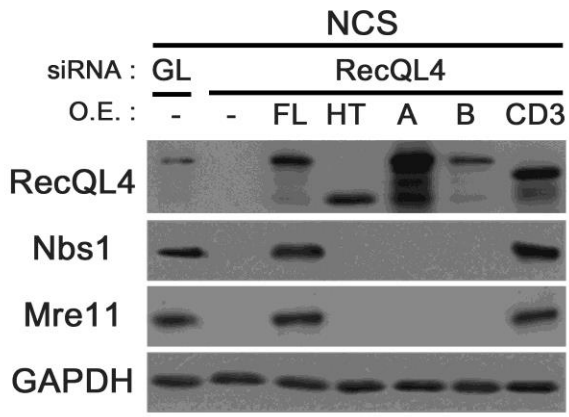

A
GL si
R4 si
0.01% MMS : - 0.5 1 1.5 - 0.5 1 1.5 (hr)
RecQL4
Nbs1
Mre11
GADH
B
GL si
R4 si
20J/m² UV : - 0.5 1 1.5 - 0.5 1 1.5 (hr)
RecQL4
Nbs1
Mre11
GADH
C
GL si
R4 si
1uM BP : - 0.5 1 1.5 - 0.5 1 1.5 (hr)
RecQL4
Nbs1
Mre11
GADH

Figure 12. The helicase domain of RecQL4 is required for the stability of MRN complex.

U2OS cells depleted with RecQL4 or not (GL) were transfected with the plasmids of RecQL4 WT, Helicase truncation (HT), walker 'A' motif mutant (A), walker 'B' motif mutant (B) and Carboxyl-deletion (CD3) mutant. After 24hrs, they were treated with NCS (200ng/ml) for 15min and incubated in fresh medium for 30 minutes. O.E. means overexpression.


NCS
siRNA : GL
RecQL4
O.E. : - - FL HT A B CD3
RecQL4
Nbs1
Mre11
GAPDH

RecQL4 co-localizes and interacts with MRN complex

As RecQL4 was required for the maintenance of MRN complex in response to DNA Double Strand Breaks, I examined whether RecQL4 is co-localized with MRN complex. Immunostaining of RecQL4 and Mre11 in NCS-treated cells showed that RecQL4 was co-localized with MRN complex at DNA DSBs site (Figure 13). To test whether RecQL4 could bind to MRN complex, I performed reciprocal co-immunoprecipitation experiments with antibodies against RecQL4 or Nbs1. While the interaction between endogenous RecQL4 and MRN complex was barely detected in non-damaged cells, the interactions between those proteins were significantly increased when NCS treated (Figure 14). To test the requirement of phosphorylation for this interaction, I treated λ-phosphatase in extracts for immunoprecipitation. Treatment of λ-phosphatase completely abolished the interaction between RecQL4 and MRN complex, and the addition of phosphatase inhibitor recovered their interaction (Figure 15). These results suggested that interactions between RecQL4 and MRN complex depend on DNA damages and the phosphorylation was responsible

Figure 13. Flag-RecQL4 is co-localized with Mre11 at DNA damage sites.

U2OS cells transfected with the flag-tagged RecQL4 plasmids for 2 days were either untreated (Mock) or treated with NCS (200ng/ml) for 15 minutes and incubated in fresh medium for an hour. Immunostaining with anti-Mre11 and anti-flag or anti-γH2AX antibody was performed. γH2AX is used as DNA DSBs marker.

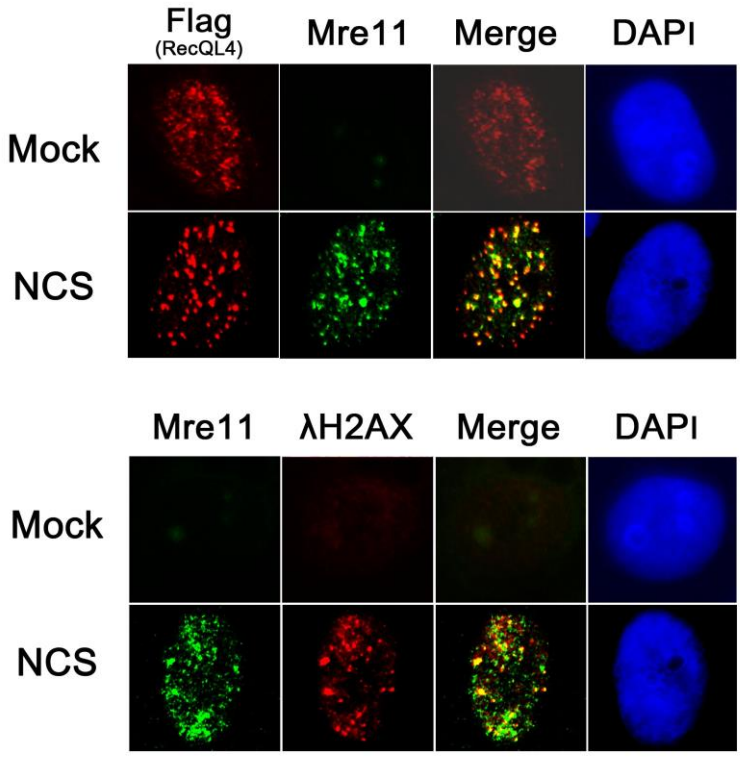
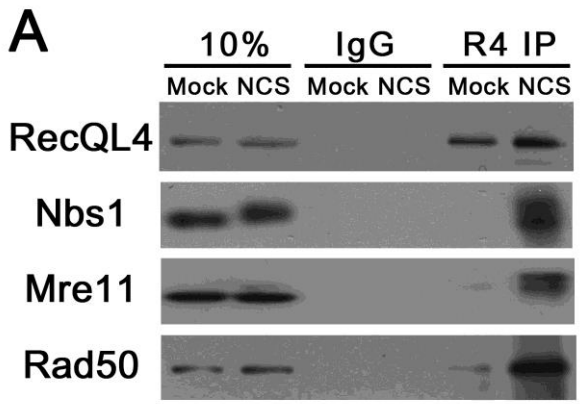

Flag
(RecQL4)
Mre11
Merge
DAPI
Mock
NCS
Mre11
λH2AX
Merge
DAPI
Mock
NCS

Figure 14. Endogenous RecQL4 interacts with MRN complex.

Cell lysates treated with NCS or not (Mock) were subjected to immunoprecipitation by (A) anti-RecQL4 antibody and (B) anti-Nbs1 antibody. The immunoprecipitates were blotted as indicated. 10% : 10% Input


A
10%
IgG
R4 IP
Mock NCS
Mock NCS
Mock NCS
RecQL4
Nbs1
Mre11
Rad50

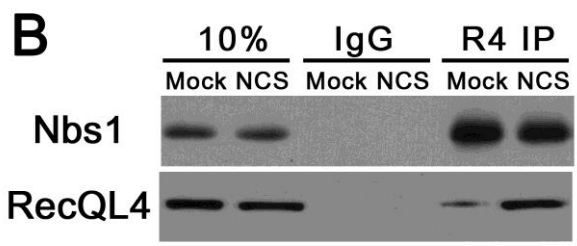
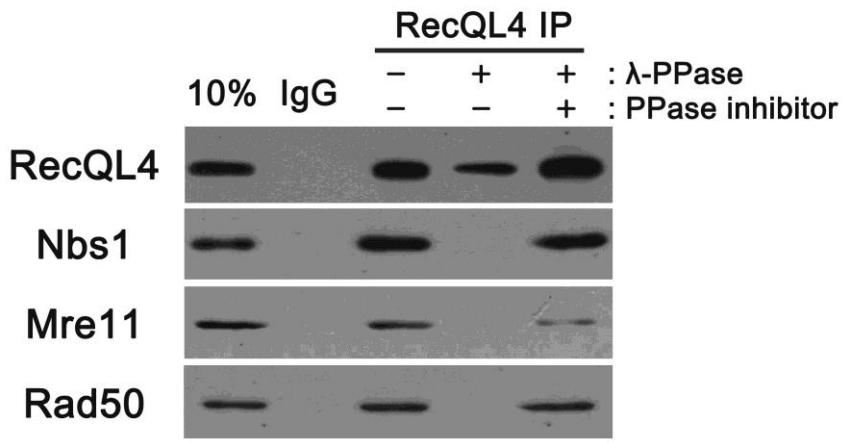

B
10%
IgG
R4 IP
Mock NCS
Mock NCS
Mock NCS
Nbs1
RecQL4

Figure 15. RecQL4 interacts with MRN complex in a phosphorylation-dependent manner.

Cell lysates prepared from U2OS cells treated with NCS were subjected to immunoprecipitation by anti-RecQL4 antibody. The immunoprecipitates were either untreated or treated with λ-phosphatase (400unit/ul) and phosphastase inhibitors (50mM NaF, 1mM $NaVO_4$) for 30 minutes at 30℃. Samples were analyzed by immunoblotting.


RecQL4 IP
10%
IgG
−
+
+
: λ-PPase
−
−
+
: PPase inhibitor
RecQL4
Nbs1
Mre11
Rad50

for those interactions.

In order to determine which conmponent of MRN complex directly interacts with RecQL4, U2OS cells were transfected with RecQL4 and Mre11 or RecQL4 and Nbs1 and immunoprecipitated reciprocally. I found that RecQL4 directly interacts with Mre11 (Figure 16). In addition, to identify the specific region of RecQL4 which are responsible for the RecQL4-Mre11 interaction, I generated deletion mutants of RecQL4 (Figure 17A). C-terminal region of RecQL4 interacts with Mre11 directly (Figures 17B). I divided the C-terminal region into three parts. C3 mutants (residues 1079-1208) abolished the binding of RecQL4 with Mre11 (Figure 17C). I concluded that residue 807-1084 of RecQL4 (C-terminal) is responsible for the interaction with Mre11.

Figure 16. RecQL4 interacts with Mre11 directly.

2flag-RecQL4 and 2HA-Nbs1 or 2flag-RecQL4 and Myc-Mre11 were co-expressed in U2OS cells for 48 hours. Cells were treated with 200ng/ml NCS for 15 minutes, and incubated in fresh medium for an hour before harvest. Cell lysates were subjected to immunoprecipitation by indicated antibodies.

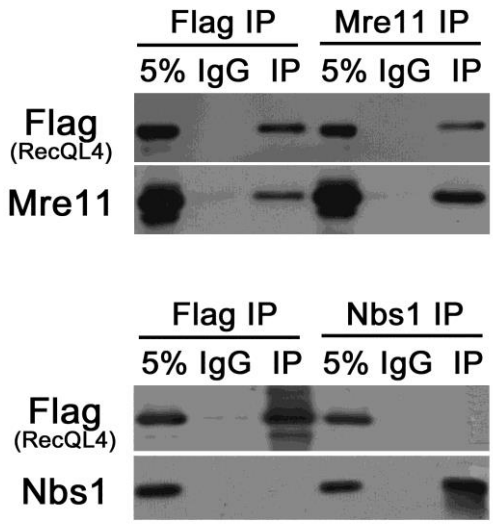
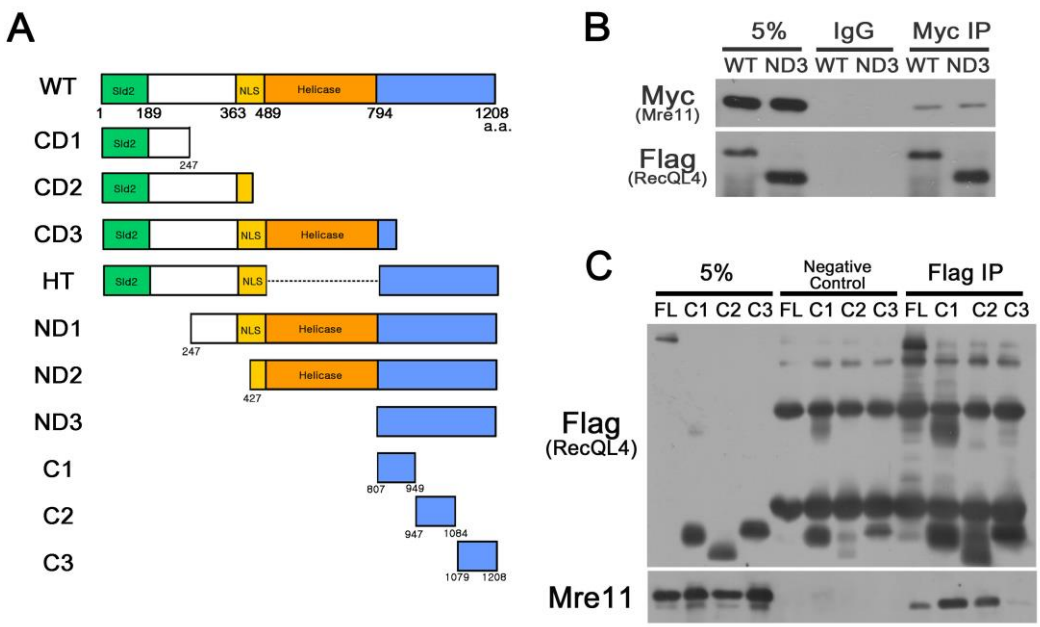

Flag IP
Mre11 IP
5% IgG IP
5% IgG IP
Flag
(RecQL4)
Mre11
Flag IP
Nbs1 IP
5% IgG IP
5% IgG IP
Flag
(RecQL4)
Nbs1

Figure 17. C-terminal region of RecQL4 interacts with Mre11 directly.

A. Diagram of plasmids encoding full-length or deletion mutants of RecQL4 was shown. Different domains of RecQL4 can be distinguished by color. The amino acid numbers are labelled below each diagram. WT, wildtype; CD1~3, C-terminus deletion 1~3; HT, helicase truncation; ND1~3, N-terminus deletion 1~3; C1~3, C-terminal 1~3.

B. and C. U2OS cells co-transfected with Myc-tagged Mre11 and 2flag-tagged RecQL4 mutants for 2 days were treated with NCS (200 ng/ml) for 15 minutes, and incubated in fresh medium for an hour. The lysates were immunoprecipitates with anti-Myc antibody and anti-flag agarose beads.


A
WT
Sld2
NLS
Helicase
1
189
363
489
794
1208
a.a.
CD1
247
CD2
CD3
HT
ND1
ND2
427
ND3
C1
807
949
C2
947
1084
C3
1079
1208
B
5%
IgG
Myc IP
WT ND3 WT ND3 WT ND3
Myc
(Mre11)
Flag
(RecQL4)
C
5%
Negative
Control
Flag IP
FL C1 C2 C3 FL C1 C2 C3 FL C1 C2 C3
Flag
(RecQL4)
Mre11

RecQL4 mediates ubiquinination of MRN complex and proteasome-dependent degradation.

To understand why the protein level of MRN complex is reduced in RecQL4 deficient cells, RecQL4-depleted cells were blocked with protein synthesis by cycloheximide (CHX). The protein level of Nbs1 and Mre11 became decreased within 15 minuates (Figure 18). I guess those proteins may be ubiquitinated and started to be degraded by proteasome. Indeed, degraded Nbs1 and Mre11 protein are recovered by adding the proteasome inhibitor MG132 in DSBs damaged RecQL4-depleted cell (Figure 19A). Also, poly-ubiquitination of Nbs1 and Mre11 was observed in response to NCS in RecQL4-depleted cells by in vivo ubiquitination assay (Figure 19B and C). Collectively, these results suggest that Nbs1 and Mre11 proteins are ubiquitinated and degraded by proteasome in RecQL4-deficient cells damaged by DBSs.

In a recent report, Skp2 E3 ligase ubiquitylates Nbs1 protein with Ubc13 E2 enzyme when cells were gamma-irradiated and the ubiquitination of Nbs1 is used for interaction with ATM (Wu et al., 2012). Because Skp2

Figure 18. Cycloheximide (CHX) treatment of RecQL4-deficient cells shows active degradation of MRN complex.

Cells were incubated with 50 ug/ml CHX and NCS (200ng/ml) after 2 days of GL or RecQL4 siRNA transfection and released into NCS-free medium for indicated different time. Expression levels of each protein were analyzed by immunoblotting right. Quantification of proteins is shown as graph left.

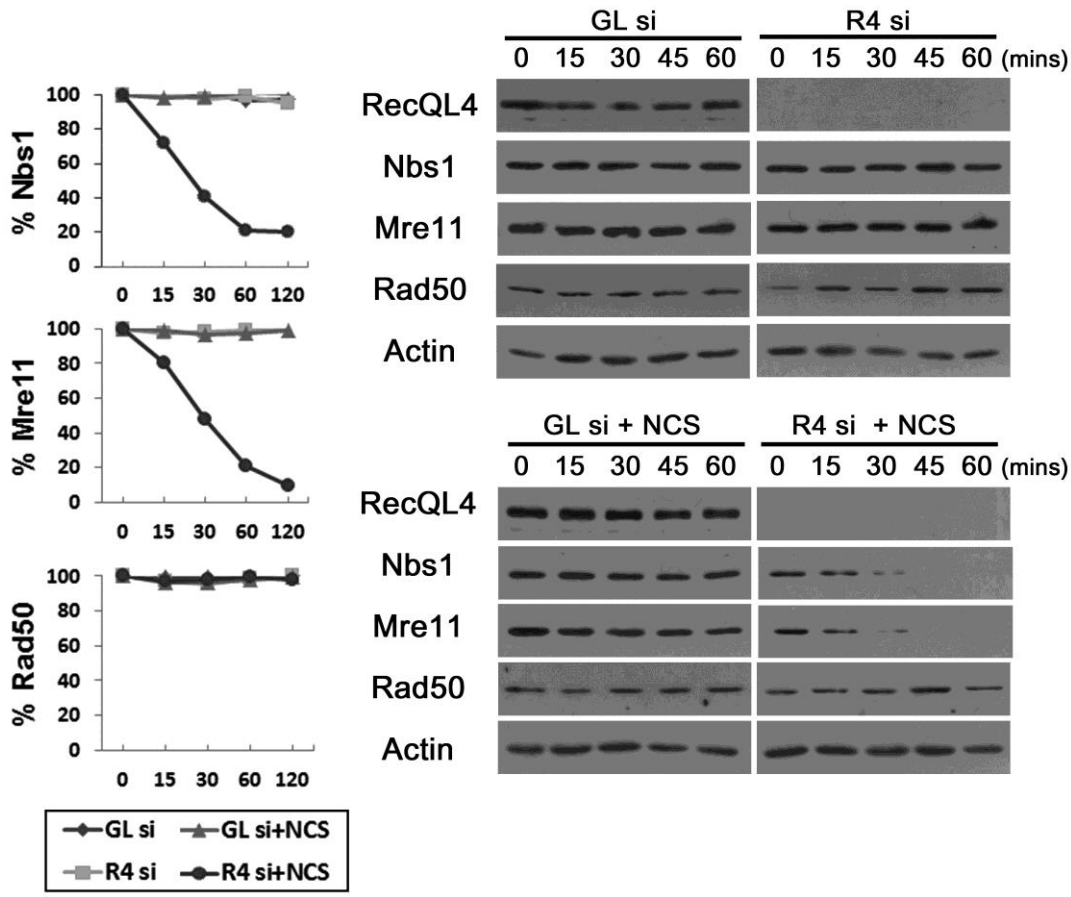
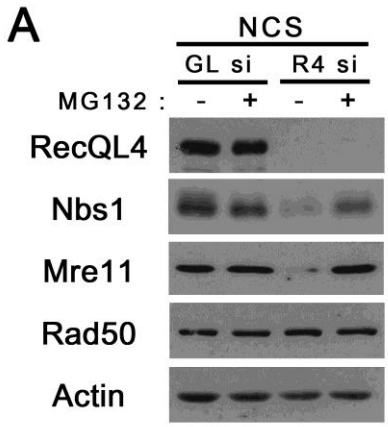
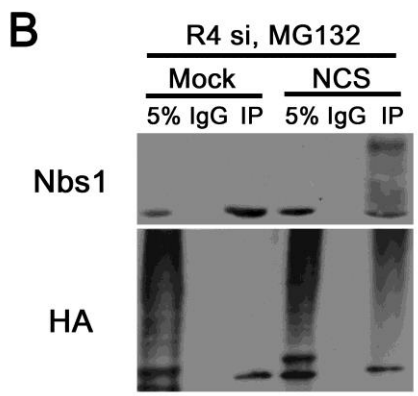

% Nbs1
% Mre11
% Rad50
100
80
60
40
20
0
0 15 30 60 120
GL si
GL si+NCS
R4 si
R4 si+NCS
GL si
R4 si
0 15 30 45 60 0 15 30 45 60 (mins)
RecQL4
Nbs1
Mre11
Rad50
Actin
GL si + NCS
R4 si + NCS
0 15 30 45 60 0 15 30 45 60 (mins)
RecQL4
Nbs1
Mre11
Rad50
Actin

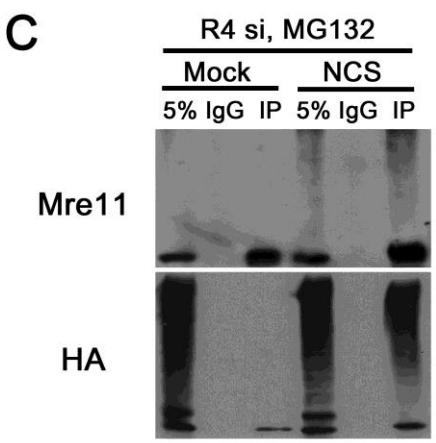
Figure 19. MRN complex is ubiquitylated and degraded by the proteasome.

A. The proteasome inhibitor MG132 (50 ug/ml) was added to RecQL4-depleted cells for an hour. And they were treated with NCS (200 ng/ml) for 15 minutes and incubated in fresh medium for 30 minutes.

B. and C. RecQL4-depleted cells transfected with HA-tagged ubiquitin plasmids were treated with MG132 (50 ug/ml) for an hour and either untreated (Mock) or treated with NCS (200ng/ml) for 15 minutes and incubated in fresh medium for 30 minutes. They were harvested for immunoprecipitation assay with IgG and anti-Nbs1 or anti-Mre11 and followed by immunoblot analysis.


A
NCS
GL si
R4 si
MG132 : - + - +
RecQL4
Nbs1
Mre11
Rad50
Actin


B
R4 si, MG132
Mock
NCS
5% IgG IP 5% IgG IP
Nbs1
HA


C
R4 si, MG132
Mock
NCS
5% IgG IP 5% IgG IP
Mre11
HA

mainly acts a role in ubiquitin-dependent proteasomal degradation (Chan et al., 2010), I postulated that Skp2 may play other roles with another E2 enzyme for regulating the stability the Nbs1 protein in RecQL4-deficient cell. When co-depleted with RecQL4 and Skp2 proteins, Nbs1 and Mre11 were not degraded (Figure 20A). Only Cdc34 among several SCF-bound E2 enzyme stabilized the protein level of Nbs1 and Mre11 (Figure 20B). These results suggest that RecQL4 is required for ubiquitylation of MRN complex by Skp2 E3 ligase and Cdc34 E2 enzyme.

To determine which kinase affects the stability of MRN complex, I treated DNA-PK or ATM inhibitor in RecQL4-deficient cells and induced DNA DSBs. I guess ATM may be involved in regulating the stability of MRN complex because MRN complex is phosphorylated by ATM (Lavin et al., 2015a). However, Nbs1 and Mre11 proteins were not degraded only when Ku70 was depleted or DNA-PK inhibited. ATM inhibitor doesn't affect the protein level (Figure 21). These data suggest that the stability of MRN complex is dependent on DNA-PK.

Figure 20. RecQL4 is required for ubiquitylation of MRN complex by Skp2-Cdc34 complex.

U2OS cells were co-depleted with and indicated siRNA and treated with NCS (200ng/ml) for 15 minutes and incubated in fresh medium for 30 minutes. Expression levels of each protein were analyzed by immunoblotting.

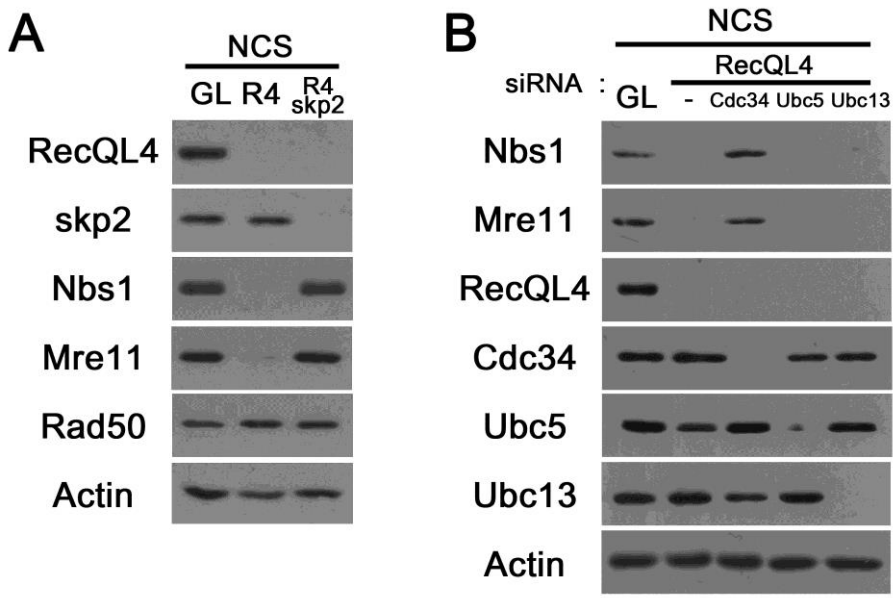
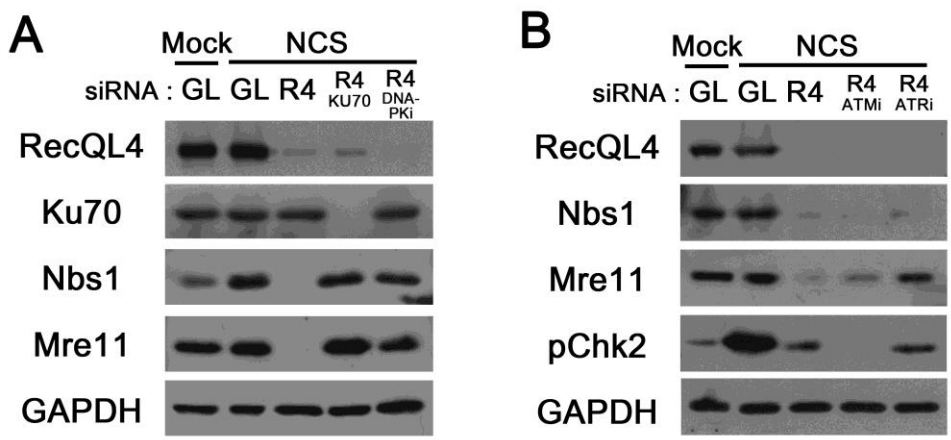

A
NCS
GL R4 R4 skp2
RecQL4
skp2
Nbs1
Mre11
Rad50
Actin
B
NCS
RecQL4
siRNA : GL - Cdc34 Ubc5 Ubc13
Nbs1
Mre11
RecQL4
Cdc34
Ubc5
Ubc13
Actin

Figure 21. Degradation of MRN complex is dependent on DNA-PK kinase.

A. U2OS cells transfected with the indicated siRNA or treated with 10 uM DNA-PK inhibitor (NU7441) were either untreated or treated with Neocarzinostatin (200ng/ml) for 15 minutes and incubated in fresh medium for 30 minutes.

B. U2OS cells transfected with the indicated siRNA or treated with 10uM ATM inhibitor (KU55933) or 10uM ATR inhibitor (VE821) were either untreated (Mock) or treated with NCS (200ng/ml) for 15 minutes and incubated in fresh medium for 30 minutes.

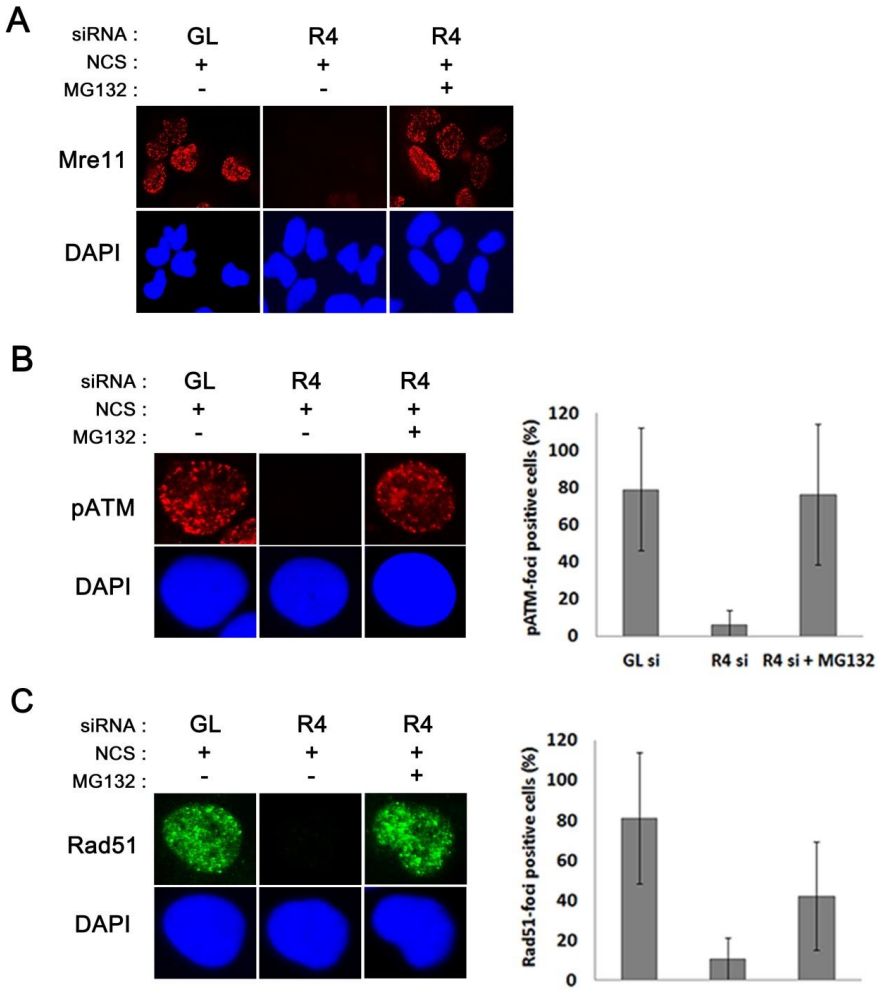

A
Mock
NCS
siRNA : GL GL R4 R4 KU70 R4 DNA-PKi
RecQL4
Ku70
Nbs1
Mre11
GAPDH
B
Mock
NCS
siRNA : GL GL R4 R4 ATMi R4 ATRi
RecQL4
Nbs1
Mre11
pChk2
GAPDH

RecQL4 regulates MRN complex through mediating the interaction with deubiquitinating enzyme.

As I shown before, the level of MRN complex-foci is decreased in RecQL4-depleted cells. Thus I wonder whether MRN complex is degraded by proteasome and cannot localize at DNA damage sites or whether MRN complex cannot be recruited at DNA damage sites when RecQL4 is absent. To examine MRN complex-foci is formed normally even RecQL4 is deficient, I treated MG132 to block the degradation of MRN complex. Interestingly, Mre11-foci was formed normally even RecQL4 is absent (Figure 22A). It indicates that MRN complex can localized at DNA damage sites independently on RecQL4 and I guess degradation of MRN complex begins after foci-formation. And I examined the cellular response in DNA checkpoint and repair when MG132 treated to RecQL4-depleted cells. Phosphor ATM –foci was recovered almost and Rad51-foci was recovered as a half level (Figure 22 B and C). It indicates that If MRN complex is not degraded by RecQL4 depletion, ATM activation and Homologous

Figure 22. Defects in DNA checkpoint and HR are caused by the degradation of MRN complex in RecQL4-depleted cells.

U2OS cells depleted by GL or RecQL4 siRNA were pre-treated with MG132 (50 ug/ml) for an hour and treated with NCS (200ng/ml) for 15 minutes, and incubated in fresh medium for an hour. Immunostaining with (A) anti-Mre11, (B) anti-pATM and (C) anti-Rad51 antibody was performed respectively. A cell containing 20 or more foci was considered as a foci-positive cell. The percentage of pATM and Rad51 foci-positive cells was plotted.


A
siRNA :
NCS :
MG132 :
GL
R4
R4
+
+
+
-
-
+
Mre11
DAPI
B
siRNA :
NCS :
MG132 :
GL
R4
R4
+
+
+
-
-
+
pATM
DAPI
pATM-foci positive cells (%)
120
100
80
60
40
20
0
GL si
R4 si
R4 si + MG132
C
siRNA :
NCS :
MG132 :
GL
R4
R4
+
+
+
-
-
+
Rad51
DAPI
Rad51-foci positive cells (%)
120
100
80
60
40
20
0

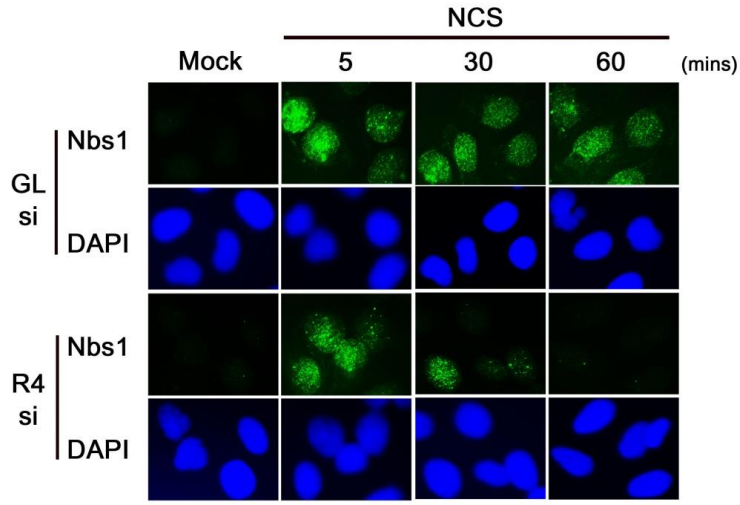
recombination is processed normally. Collectively, these data suggest that MRN complex might be recruited at DNA double strand break sites without RecQL4 protein and RecL4 is involved in DNA checkpoint and repair pathway by mediating the stability of MRN complex.

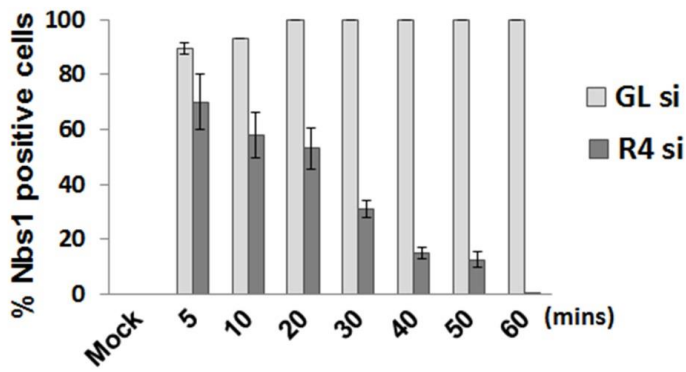
As MRN complex is recruited at DNA damage sites normally in RecQL4-depleted cell, I examined when MRN complex become disappear. As correlated with previous data, Nbs1 foci were formed normally at early time point after DNA damage even RecQL4 is absent. However, the foci begin to disappear totally within one hour even though it is maintained in normal cells (Figure 23). These data suggest that MRN complex might be recruited at DNA damage site without the assist of RecQL4 and starts to degrade prematurely when RecQL4 is deficient.

A study reported that the protein level of Nbs1 showed significant reduction when USP28-depleted cells were ionizing-radiated (Zhang et al., 2006). In order to know whether USP28 affects the stability of MRN complex in fact, I tested the stability of MRN complex after transfecting with $USP28^{CI}$ (catalytic inactive form, C171A) or USP28 siRNA in response

Figure 23. MRN complex on DNA damage foci is disappeared prematurely in RecQL4-deficient cells.

U2OS cells depleted by GL or RecQL4 siRNA were either untreated (Mock) or treated with NCS (200ng/ml) for 15 minutes, and incubated in fresh medium for indicated different time points. Immunostaining with anti-Nbs1 antibody was performed. A cell containing 20 or more foci was considered as a foci-positive cell. The percentage of Nbs1 foci-positive cells was plotted.


NCS
Mock
5
30
60
(mins)
GL si
Nbs1
DAPI
R4 si
Nbs1
DAPI


% Nbs1 positive cells
100
80
60
40
20
0
Mock
5
10
20
30
40
50
60
(mins)
GL si
R4 si

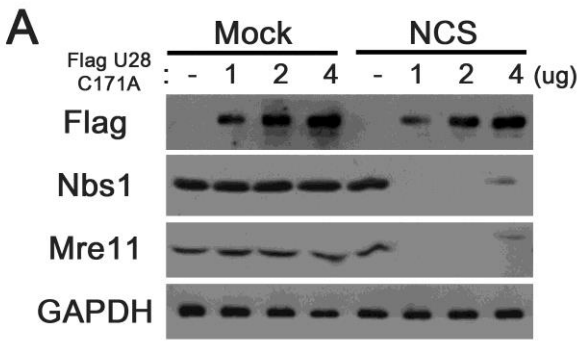
to NCS. The protein levels of Nbs1 and Mre11 were significantly reduced in USP28-inactive cells or USP28-depleted cell treated with NCS (Figure 24). These results suggest that USP28 is required for the stability of MRN complex. In turn, I wondered whether wild-type USP28 affects the stability of MRN complex. The number of Mre11-foci is decreased in normal cell, however, the majority of Mre11-foi is maintained overtime in USP28-expressed cells (Figure 25). It means that DNA breakage remains without being repaired because USP28 deubiquitinates the MRN complex and protects from degradation. Collectively, these results indicate that USP28 might be involved in regulating the stability of MRN complex.

To know how MRN complex is regulated, I first examined physical association between RecQL4, USP28 and MRN complex upon DNA DSBs. Interestingly, flag-tagged USP28 interacts with Nbs1 and RecQL4 and these interactions are increased by DNA DSBs (Figure 26A). While the interaction between USP28, RecQL4 and MRN complex was not changed upon treatment with ATM inhibitor, inhibition of DNA-PK showed a stronger reduction of those interactions (Figure 26B). Collectively, these data suggest RecQL4 interacts with USP28 and MRN complex and these

Figure 24. USP28 helps to stabilize the MRN complex.

A. U2OS cells overexpressed with USP28 C171A constructs were treated either untreated (Mock) or treated with NCS (200ng/ml) for 15 minutes and incubated in fresh medium for 30 minutes.

B. U2OS cells silenced with GL or USP28 siRNA were treated either untreated (Mock) or treated with NCS (200ng/ml) for 15 minutes and incubated in fresh medium for 30 minutes. Expression levels of each protein were analyzed by immunoblotting.


A
Mock
NCS
Flag U28 C171A : - 1 2 4 - 1 2 4 (ug)
Flag
Nbs1
Mre11
GAPDH

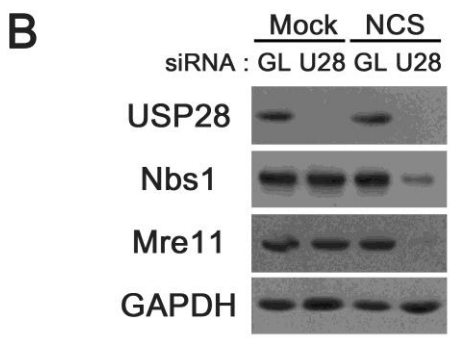

B
Mock
NCS
siRNA : GL U28 GL U28
USP28
Nbs1
Mre11
GAPDH

Figure 25. Overexpression of USP28 interferes with degradation of MRN complex at DNA damage site.

U2OS cells transfected with either vector or USP28 WT DNA constructs for 2 days were treated with NCS (200ng/ml) for 15 minutes and incubated in fresh medium for 30 minutes (0hr). And the cells on coverslips were harvested and fixed at indicated time points (1, 3, 5 hrs). Immunostaining with anti-Mre11 antibody was performed. A cell containing 20 or more foci was considered as a foci-positive cell. The relative percentage of Mre11 foci-positive cells was plotted.

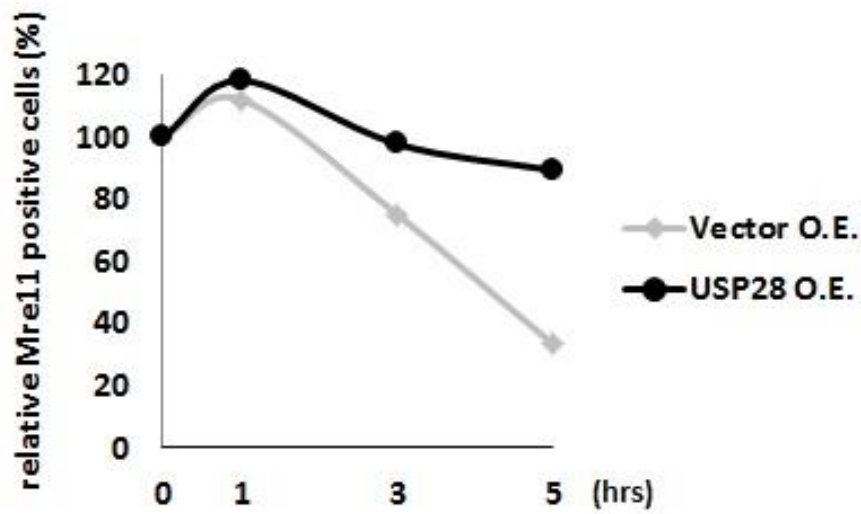
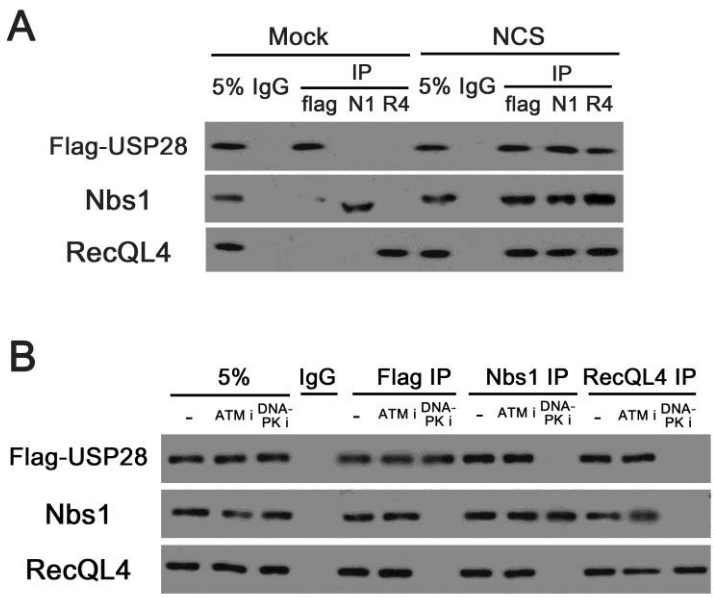

relative Mre11 positive cells (%)
120
100
80
60
40
20
0
0
1
3
5
(hrs)
Vector O.E.
USP28 O.E.

Figure 26. Interactions between RecQL4, USP28 and MRN complex are dependent on DNA-PK.

A. U2OS cells transfected with Flag-USP28 construct were either untreated (Mock) or treated with NCS (200ng/ml) for 15 minutes and incubated in fresh medium for an hour. Cell lysates were immunoprecipitated with anti-flag agarose bead, anti-nbs1, and anti-RecQL4 antibody respectively.

B. U2OS cells transfected with Flag-USP28 construct were treated 10 uM Ku55933 (ATM inhibitors) or 10 uM NU7441 (DNA-PK inhibitors) for an hour and treated with NCS (200ng/ml) for 15 minutes, and incubated in fresh medium for an hour. Cell lysates were immunoprecipitated with anti-flag agarose bead, anti-nbs1, anti-RecQL4 antibody respectively.


A
Mock
NCS
5% IgG
IP
flag N1 R4
5% IgG
IP
flag N1 R4
Flag-USP28
Nbs1
RecQL4
B
5%
IgG
Flag IP
Nbs1 IP
RecQL4 IP
-
ATM i
DNA-PK i
Flag-USP28
Nbs1
RecQL4

interactions are dependent on DNA-PK kinase.

And I examine which protein mediates those interactions. The deletion of protein Nbs1 or USP28 by siRNA does not affect the interaction of RecQL4 and USP28 or RecQL4 and MRN complex respectively (Figure 27 B and C). However, USP28 interacts with MRN complex only when RecQL4 protein exists (Figure 27A). It suggests that USP28 cannot bind to MRN complex when RecQL4 is absent and remained ubiquitin of MRN complex may cause degradation. And I tested whether the helicase domain of RecQL4 is required for the interaction of USP28 and MRN complex. Interestingly, the association level of USP28 and MRN complex is reduced when RecQL4 walker 'A' mutant expressing cells (Figure 28). It suggests that helicase domain is essential for the interaction between USP28 and MRN complex. However, how the helicase domain affect the interaction is remained unknown. Collectively, these data suggest that RecQL4 regulates the stability of MRN complex through mediating the binding of USP28 to MRN complex.

In addition, I tested the interaction between skp2 and MRN complex is changed upon presence of RecQL4. The association between skp2 and

Figure 27. Interaction between USP28 and MRN complex is dependent on RecQL4.

U2OS cells silenced with indicated siRNA were overexpressed with flag-USP28 construct and pre-treated with or without MG132 (50 ug/ml) for an hour. And they were treated with or without NCS (200ng/ml) for 15 minutes and incubated in fresh medium for 30 minutes. Cell lysates were immunoprecipitates with (A and B) anti-flag agarose beads, (C) anti-RecQL4 antibody respectively.

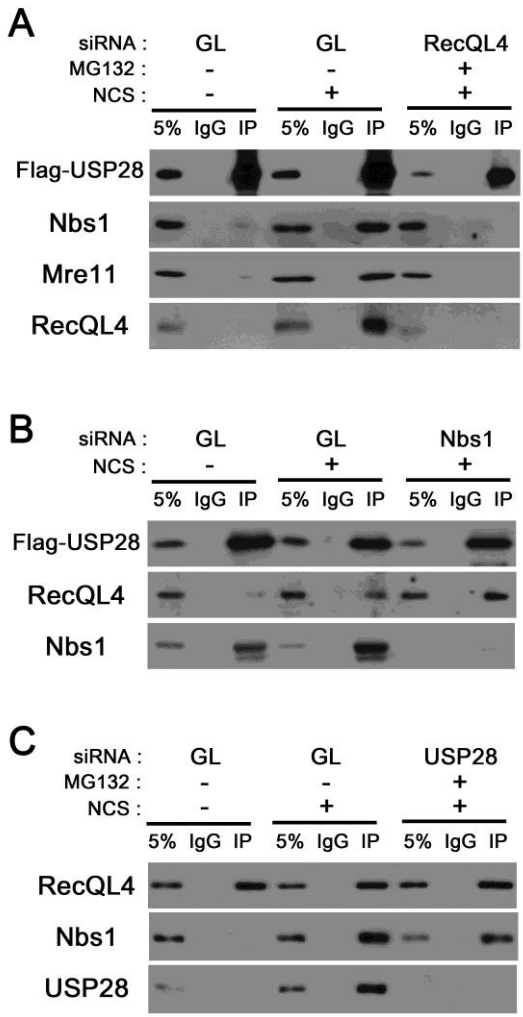
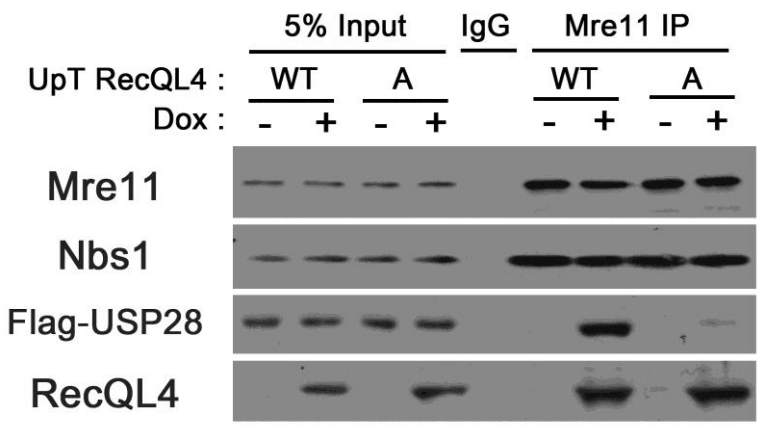

A
siRNA :
MG132 :
NCS :
GL
GL
RecQL4
-
-
+
-
+
+
5% IgG IP
5% IgG IP
5% IgG IP
Flag-USP28
Nbs1
Mre11
RecQL4
B
siRNA :
NCS :
GL
GL
Nbs1
-
+
+
5% IgG IP
5% IgG IP
5% IgG IP
Flag-USP28
RecQL4
Nbs1
C
siRNA :
MG132 :
NCS :
GL
GL
USP28
-
-
+
-
+
+
5% IgG IP
5% IgG IP
5% IgG IP
RecQL4
Nbs1
USP28

Figure 28. The helicase domain of RecQL4 is required for interaction between USP28 and MRN complex.

U2OS pTet On stable cell lines (UpT) expressing RecQL4 WT and walker 'A' mutants were silenced with RecQL4 siRNA and induced with doxycycline (0.1 mg/ml) or not for 2 days. And they were treated with NCS (200ng/ml) for 15 minutes and incubated in fresh medium for an hour. Cell lysates were immunoprecipitates with anti-Mre11 antibody.


5% Input
IgG
Mre11 IP
UpT RecQL4 :
WT
A
WT
A
Dox :
- + - +
- + - +
Mre11
Nbs1
Flag-USP28
RecQL4

MRN complex is observed even RecQL4 depleted (Figure 29A). It indicates that Skp2 always interacts with and ubiquitinates the MRN complex independently on RecQL4. Also, these interactions are required for the two kinase activity, ATM and DNA-PK (Figure 29B). It suggests that phosphorylation of MRN complex by ATM and DNA-PK might be recognized by and associated with Skp2 because Skp2 could recognize phosphorylation of target protein for degradation (Wang et al., 2012). Also, I postulated that MRN complex will be degraded after finishing the HR repair because Skp2 always interacts with and ubiquitinates MRN complex. I observed Mre11-foci after inducing DNA DSBs in MG132-pretreated cell. Interestingly, even 5 hours passed after occurring DNA damage, majority of Mre11-foci was maintained when proteasome was blocked (Figure 30). Collectively, these data suggests that ubiquitinated MRN complex by Skp2 might be degraded in proteasome after HR repair is finished successfully.

Figure 29. Interaction between Skp2 and MRN complex is dependent on ATM and DNA-PK.

A. U2OS cells depleted by GL or RecQL4 siRNA were pre-treated with MG132 (50 ug/ml) for an hour and treated with NCS (200ng/ml) for 15 minutes, and incubated in fresh medium for an hour. Cell lysates were immunoprecipitates with anti-Mre11 antibody.

B. U2OS cells were treated with either 10 uM Ku55933 (ATM inhibitors) or 10 uM NU7441 (DNA-PK inhibitors) or both for an hour and treated with NCS (200ng/ml) for 15 minutes, and incubated in fresh medium for an hour. Cell lysates were immunoprecipitated with aniti-Skp2 antibody.

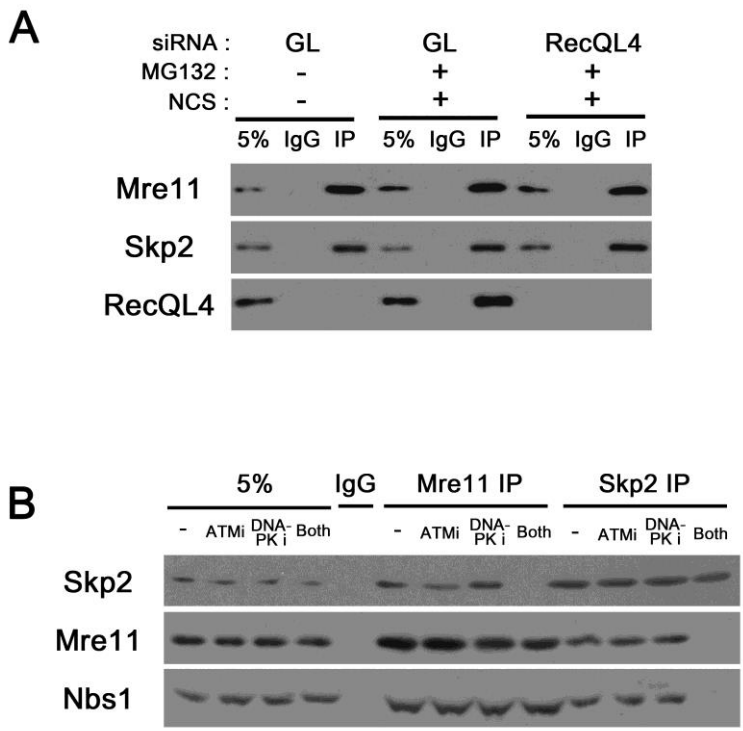
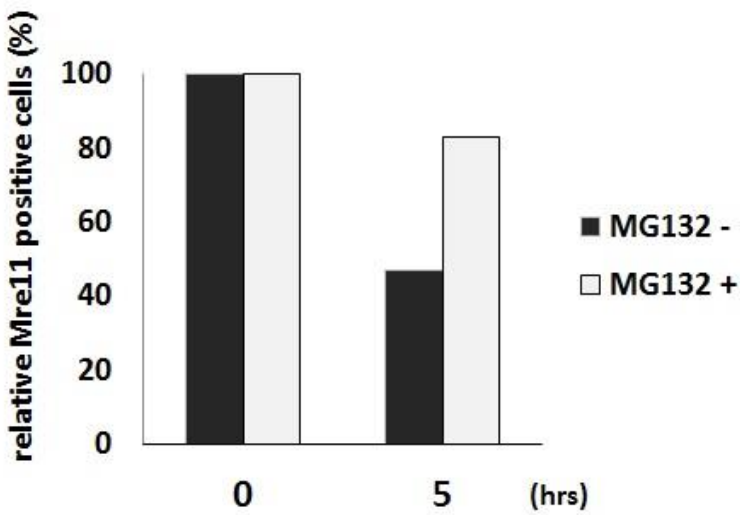

A
siRNA :
MG132 :
NCS :
GL
GL
RecQL4
-
+
+
-
+
+
5% IgG IP
5% IgG IP
5% IgG IP
Mre11
Skp2
RecQL4
B
5%
IgG
Mre11 IP
Skp2 IP
- ATMi DNA-PK i Both
- ATMi DNA-PK i Both
- ATMi DNA-PK i Both
Skp2
Mre11
Nbs1

Figure 30. MRN complex is degraded in proteasome in Normal cell.

U2OS cells were pre-treated with either MG132 (50ug/ml) or not for an hour and treated with NCS (200ng/ml) for 15 minutes and incubated in fresh medium for 30 minutes (0hr). And the cells on coverslips were harvested and fixed after 5 hrs. Immunostaining with anti-Mre11 antibody was performed. A cell containing 20 or more foci was considered as a foci-positive cell. The relative percentage of Mre11 foci-positive cells was plotted.


relative Mre11 positive cells (%)
100
80
60
40
20
0
0
5
(hrs)
MG132 -
MG132 +

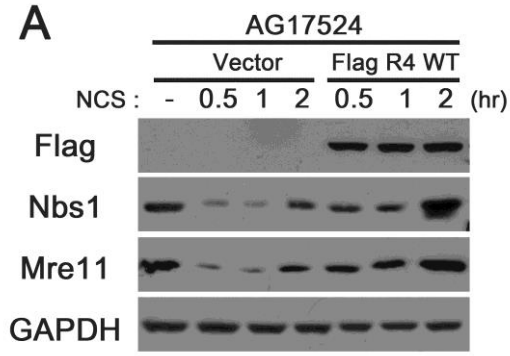
MRN complex is prematurely degraded in RTS fibroblast upon DNA DSBs.

Considering the data so far, RecQL4 protein, especially helicase activity of RecQL4 is critical for the cellular response to DNA double strand breaks. RecQL4 affects ATM activation and Homologous recombination through mediating the stability of MRN complex. I examined the stability of MRN complex using RTS fibroblasts (AG17524 and AG18371) obtained from two different patients.

The proteins level of MRN complex are reduced within 2 hours after NCS treatment and maintained when RecQL4 is expressed in RTS cells (Figure 31). In addition, phosphor-ATM and Rad51-foci were observed when wild type RecQL4 is expressed or MG132 is treated to NCS-treated RTS cells (Figure 32 and 33). These results suggest that genome instability observed in RTS patients might be caused by premature degradation of MRN complex.

Figure 31. MRN complex is prematurely degraded in RTS fibroblast upon DNA DSBs.

RTS fibroblast (AG17524, AG18371) transfected with pCMV-vector or flagged-RecQL4 WT were treated either untreated (-) or treated with NCS (200ng/ml) for 15 minutes and incubated in fresh medium for indicated time. The levels of protein were analyzed by immunoblotting.


A
AG17524
Vector
Flag R4 WT
NCS : - 0.5 1 2 0.5 1 2 (hr)
Flag
Nbs1
Mre11
GAPDH

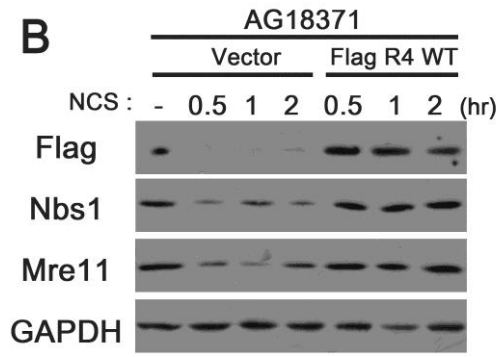
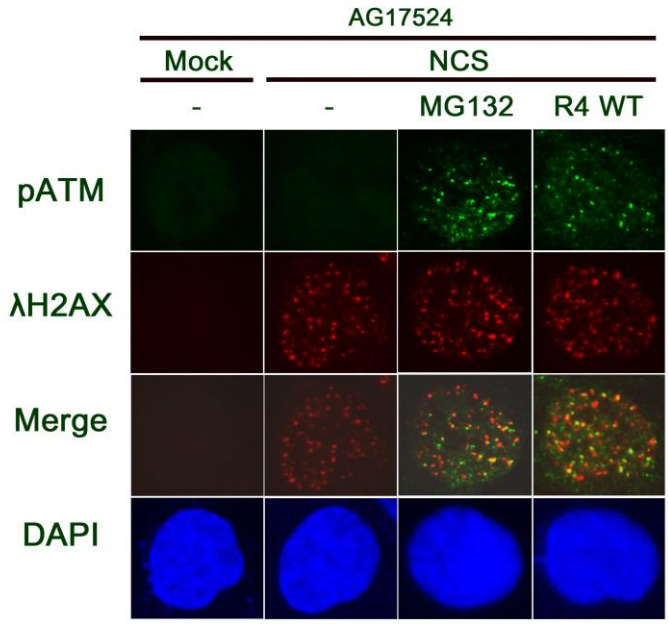

B
AG18371
Vector
Flag R4 WT
NCS : - 0.5 1 2 0.5 1 2 (hr)
Flag
Nbs1
Mre11
GAPDH

Figure 32. Defects in ATM activation are caused by the degradation of MRN complex in RTS fibroblasts.

RTS fibroblasts (AG17524) transfected with RecQL4 wildtype or pre-treated with MG132 (50 ug/ml) were treated either untreated (Mock) or treated with NCS (200ng/ml) for 15 minutes and incubated in fresh medium for 30 minutes. Immunostaining with anti-ATM and anti-λH2AX antibody was performed. A cell containing 20 or more foci was considered as a foci-positive cell. The percentage of pATM foci-positive cells was plotted.


AG17524
Mock
NCS
-
-
MG132
R4 WT
pATM
λH2AX
Merge
DAPI

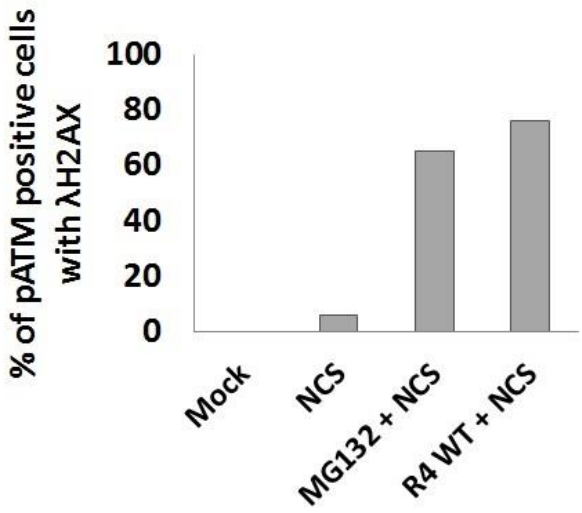
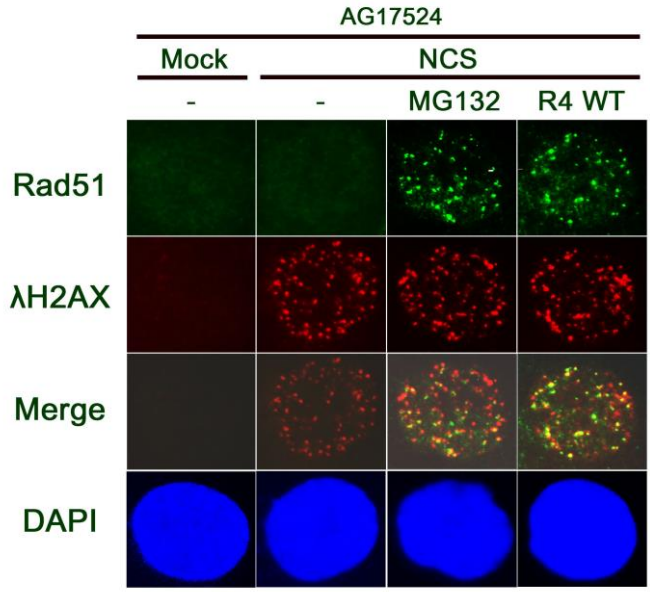

% of pATM positive cells with λH2AX
100
80
60
40
20
0
Mock
NCS
MG132 + NCS
R4 WT + NCS

Figure 33. Defects in Homologous recombination are caused by the degradation of MRN complex in RTS fibroblasts.

RTS fibroblasts (AG17524) transfected with or without flag-tagged RecQL4 were treated either untreated (Mock) or treated with NCS (200ng/ml) for 15 minutes and incubated in fresh medium for 30 minutes. Immunostaining with anti-Rad51 and anti-λH2AX antibody was performed.

A cell containing 20 or more foci was considered as a foci-positive cell. The percentage of Rad51 foci-positive cells was plotted.


AG17524
Mock
NCS
-
-
MG132
R4 WT
Rad51
λH2AX
Merge
DAPI

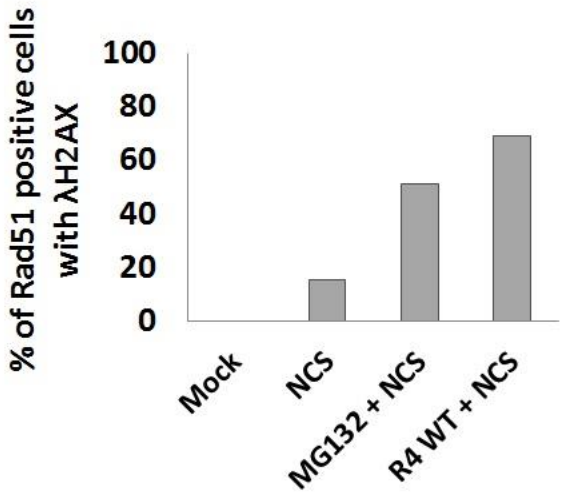
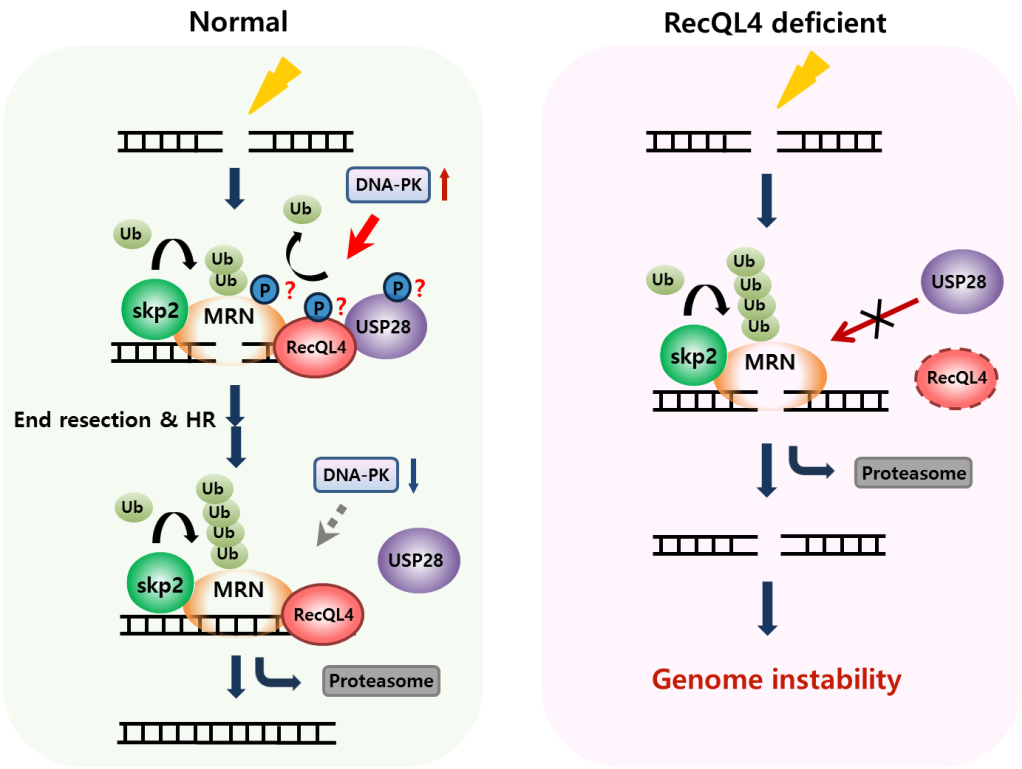

% of Rad51 positive cells with λH2AX
100
80
60
40
20
0
Mock
NCS
MG132 + NCS
R4 WT + NCS

Figure 34. Model presents RecQL4's role in HR repair

In normal cell, when DNA double strand breaks occurs, MRN complex recognizes and localizes at DNA damage sites to activate checkpoint and repair pathway. And skp2 E3 ubiquitin ligases interacts with and ubiquitinates MRN complex. At the same time, RecQL4 mediates the interaction between MRN complex and USP28 by the assist of DNA-PK. USP28 deuibiquitinates the MRN complex and protects from the degradation. After finishing the homologous recombination repair, USP28 is dissociated from RecQL4b by low kinase activity of DNA-PK and ubiquitinated MRN complex is degraded at proteasome. However, when RecQL4 is deficient, MRN complex is recruited to DNA damage sites normally. USP28 cannot interact with MRN complex and fail to de-ubiquitinate. So, ubiquitinated MRN complex by skp2 is degraded in proteasome and cannot complete Homologous recombination repair. These results could cause genome instability.


Normal
RecQL4 deficient
DNA-PK
Ub
skp2
MRN
RecQL4
USP28
P
?
End resection & HR
Proteasome
Genome instability

Discussion

In this study, I demonstrated RecQL4 is required for RecQL4 in Homologous recombination repair by regulating MRN complex. MRN complex is a primary sensor for DNA DSBs and activates downstream signaling pathway involving ATM activation and HR repair. Even MRN complex has critical role in DNA damage response, how regulated is unclear until now. I got the clues from the experiments using RecQL4-depleted cell.

When RecQL4 is depleted and exposed to DNA DSBs inducing reagent, protein level of MRN complex is reduced and downstream signaling pathway involving ATM activation and HR repair was blocked. Interestingly, degradation of MRN complex was dependent on intensity and time of DNA damages. From these data, I focused on why the protein level of MRN complex is changed upon DNA damage level and how MRN complex is disappear when RecQL4 is absent. I first tested MRN complex can be recruited on DNA DSBs sites without the help of RecQL4. MRN complex was localized at DNA damage site quickly without RecQL4 and started to be disappeared gradually. It indicates that MRN complex starts

disappeared after being recruited at DNA damage sites thus the level of degradation increases as the DNA damage increases. Also, it showed possibilities that MRN complex might be ubiquitinated and degraded as protein level. Indeed, MRN complex became unstable when protein synthesis was blocked and was not degraded when proteasome was inhibited. In addition, ubiquitination of Nbs1 and Mre11 was observed in experiments.

I tried to discover the E3 ubiquitin ligase targeting the MRN complex. An earlier study demonstrated that Skp2 E3 ligase triggers ubiquitination of Nbs1 which is K63-linked and important for interaction with ATM (Wu et al., 2012). However, I examined that Skp2 is involved in ubiquitination of MRN complex in RecQL4-depleted cell because all Skp2's substrates identified so far are known to take part in ubiquitin-dependent proteasomal degradation (Chan et al., 2010). Interestingly, Skp2 can be associated with MRN complex without RecQL4 and involved in stability of MRN complex. Also, interactions between MRN complex and Skp2 were required for ATM and DNA-PK activity. Phosphorylation by ATM and DNA-PK might be recognized by and bound with Skp2 because Skp2 is a

member of the F-box protein family containing leucine-rich repeats (LRR). Phosphorylation of the substrates on either serine or threonine is required for SCF-mediated protein degradation, which is mediated through LRR repeats (Wang et al., 2012). And I also found that Cdc34 protein is the involved ubiquitin conjugating enzyme (E2), which is known to be able to bind SCF and to attach a K48-linked ubiquitin chain to target protein (Petroski and Deshaies, 2005). Ubiquitin chains linked via k48 ubiquitin are recognized by proteasome (Thrower et al., 2000). The presence of RecQL4 may show differences of the role of Skp2 E3 ligase comparing with previous report (Wu et al., 2012). I guess Skp2 may choose to another E2 enzyme when RecQL4 is absent.

Which molecule is responsible for regulating the MRN complex dependent on RecQL4? I tested deubiquitinating enzyme, USP28 which is known for involved in DNA-damage response. It is responsible for the degradation of 53BP1, Mdc1, Nbs1 and so on (Zhang et al., 2006). When USP28 is inactive or USP28 is silenced and DNS DSBs occurs, degradation of MRN complex was observed as reported. In addition, Mre11-foci were observed even 5 hours passed after occurring DNA damage when wild-type

USP28 was overexpressed. These data indicate that USP28 is required for the maintenance of MRN complex. And I found RecQL4 interacts with USP28 and MRN complex each other upon DNA damage and those interactions are dependent on DNA-PK. However, the exact target by DNA-PK among RecQL4, USP28 and MRN complex is not identified yet. Notably, It is observed that RecQL4 mediates the interaction between MRN complex and USP28. Thus it could be explained why MRN complex is degraded when RecQL4-depleted cells were damaged. MRN complex is recruited at DNA DSBs sites and ubiquitinated by Skp2 E3 ligase and degraded in proteasome without interacting with USP28 when RecQL4 is absent.

In normal cell, I can guess that ubiquitinated MRN complex by Skp2 will be degraded after finishing the HR repair. Indeed, it is observed that Mre11-foci in DNA-damaged cell are maintained for a long time when proteasome is blocked. This result is correlated with previous data. The protein level of MRN complex was reduced in Adenovirus-infected Hela cells (Stracker et al., 2002; Carson et al., 2003; Araujo et al., 2005) and the degradation of MRN complex is mediated by MG132 (Stracker et al.,

2002).

Because most RTS patients have truncated helicase domain (Lu et al., 2016b), I tried to identify the function of helicase domain of RecQL4 to contribute to solve the disease. I found that the helicase domain of RecQL4 is responsible for the stability for MRN complex and downstream signaling pathway involving ATM activation and HR. It is correlated with previous reports which suggests that the helicase activity is required for DNA end resection (Lu et al., 2016a). In addition, it is observed that interaction between USP28 and MRN complex is decreased in helicase-mutant expressing cell line. However, how the helicase activity is involved in this interaction is not known exactly. The helicase mutants can be recruited at DSBs normally and interact with Mre11. The role of helicase activity is remained to discover.

I confirmed premature degradation of MRN complex in RTS fibroblasts. MRN complex was maintained and ATM activation and HR repair were normally processed when RTS fibroblasts were treated MG132. These results provide an understanding of RTS syndrome and role of RecQL4 proteins. In other words, RecQL4 is required for the stability of

MRN complex and defects of RecQL4 in RTS cells cause failure HR repair in response to DNA DSBs.

I suggested a model based on my data (Figure 34). In normal cell, when DNA double strand breaks occurs, MRN complex recognizes and localizes at DNA damage sites to activate checkpoint and repair pathway. And skp2 E3 ubiquitin ligases interacts with and ubiquitinates MRN complex. At the same time, RecQL4 mediates the interaction between MRN complex and USP28 by the assist of DNA-PK. USP28 deuibiquitinates the MRN complex and protects from the degradation. After finishing the homologous recombination repair, USP28 is dissociated from RecQL4b by low kinase activity of DNA-PK and ubiquitinated MRN complex is degraded at proteasome. However, when RecQL4 is deficient, MRN complex is recruited to DNA damage sites normally. USP28 cannot interact with MRN complex and fail to de-ubiquitinate. So, ubiquitinated MRN complex by skp2 is degraded in proteasome and cannot complete Homologous recombination repair. These results could cause genome instability.

To understand the importance of DNA repair mechanism, RTS

syndrome can be compared with genome instability disorders like A-T, ATLD and NBS. A-T (Ataxia-telangiectasia) is a rare autosomal recessive disorder caused by mutations in the *ATM* gene. The mutated gene responsible for ATLD (Ataxia-telangiectasia-like disorder) is *MRE11* gene. And NBS (Nijmegen breakage syndrome) is a rare autosomal recessive chromosome instability disorder caused by hypomorphic mutations in the *NBS* gene. Specific clinical features of each disease are different, however there are significant overlaps between disorders which are immunodeficiency, genomic instability, predisposition to lympho-reticular malignancies and sensitivity to ionizing radiation (Taylor et al., 2004, Antoccia et al., 2006, Czornak et al., 2008). Because RecQL4 regulates HR repair by mediating the MRN complex upon DNA DSBs, clinical features of RTS patients could be similar to those of A-T, ATLD and NBS. I suggest that RecQL4 might be a key factor plays a critical role in the DNA damage repair mechanism as well as maintenance of genome integrity in the cell. I expect that clues from my data are suggested to solve the disease, RTS syndrome and that it is finally contributed to the understanding of maintenance of the genome integrity and DNA repair mechanism.

REFERENCES

Abe, T., Yoshimura, A., Hosono, Y., Tada, S., Seki, M., and Enomoto, T. (2011). The N-terminal region of RECQL4 lacking the helicase domain is both essential and sufficient for the viability of vertebrate cells: Role of the N-terminal region of RECQL4 in cells. Biochim. Biophys. Acta - Mol. Cell Res. *1813*, 473–479.

Antoccia, a, Kobayashi, J., Tauchi, H., Matsuura, S., and Komatsu, K. (2006). Nijmegen breakage syndrome and functions of the responsible protein, NBS1. Genome Dyn. *1*, 191–205.

Araujo, F.D., Stracker, T.H., Carson, C.T., Lee, D. V, and Weitzman, M.D. (2005). Adenovirus Type 5 E4orf3 Protein Targets the Mre11 Complex to Cytoplasmic Aggresomes Adenovirus Type 5 E4orf3 Protein Targets the Mre11 Complex to Cytoplasmic Aggresomes. *79*, 11382–11391.

Bakkenist, C.J., and Kastan, M.B. (2003). DNA damage activates ATM through intermolecular autophosphorylation and dimer dissociation. Nature *421*, 499–506.

Bakkenist, C.J., and Kastan, M.B. (2004). Initiating cellular stress responses. Cell *118*, 9–17.

Bolderson, E., Tomimatsu, N., Richard, D.J., Boucher, D., Kumar, R., Pandita, T.K., Burma, S., and Khanna, K.K. (2010). Phosphorylation of Exo1 modulates homologous recombination repair of DNA double-strand breaks. Nucleic Acids Res. *38*, 1821–1831.

Britton, S., Coates, J., and Jackson, S.P. (2013). A new method for high-resolution imaging of Ku foci to decipher mechanisms of DNA double-strand break repair. J. Cell Biol. *202*, 579–595.

Calsou, P., Frit, P., Humbert, O., Muller, C., Chen, D.J., and Salles, B. (1999). The DNA-dependent protein kinase catalytic activity regulates DNA end processing by means of Ku entry into DNA. J. Biol. Chem. *274*, 7848–7856.

Carson, C.T., Schwartz, R. a, Stracker, T.H., Lilley, C.E., Lee, D. V, and Weitzman, M.D. (2003). The Mre11 complex is required for ATM activation and the G 2 / M checkpoint. EMBO J. *22*.

Chan, C.-H., Lee, S.-W., Wang, J., and Lin, H.-K. (2010). Regulation of Skp2 expression and activity and its role in cancer progression. ScientificWorldJournal. *10*, 1001–1015.

Chen, H., Lisby, M., and Symington, L.S. (2013). RPA Coordinates DNA

End Resection and Prevents Formation of DNA Hairpins. Mol. Cell *50*, 589–600.

Chu, W.K., and Hickson, I.D. (2009). RecQ helicases: multifunctional genome caretakers. Nat. Rev. Cancer *9*, 644–654.

Ciccia, A., and Elledge, S.J. (2010). The DNA damage response: making it safe to play with knives. Mol. Cell *40*, 179–204.

Czornak, K., Chughtai, S., and Chrzanowska, K.H. (2008). Mystery of DNA repair: the role of the MRN complex and ATM kinase in DNA damage repair. J. Appl. Genet. *49*, 383–396.

Daley, J.M., and Sung, P. (2014). 53BP1, BRCA1, and the Choice between Recombination and End Joining at DNA Double-Strand Breaks. Mol. Cell. Biol. *34*, 1380–1388.

Downs, J. a, and Jackson, S.P. (2004). A means to a DNA end: the many roles of Ku. Nat. Rev. Mol. Cell Biol. *5*, 367–378.

Duan, Y., Fang, H., and Medicine, P. (2016). Title : RecQL4 regulates autophagy and apoptosis in U2OS cells. *559*, 1–21.

Fan, W., and Luo, J. (2008). RecQ4 facilitates UV light-induced DNA damage repair through interaction with nucleotide excision repair factor xeroderma pigmentosum group A (XPA). J. Biol. Chem. *283*, 29037–29044.

Gottlieb, T.M., and Jackson, P. (1993). The DNA-Dependent Protein Ki for DNA Ends and AssocWbn with Ku Ant. *72*, 131–142.

Hoki, Y., Araki, R., Fujimori, A., Ohhata, T., Koseki, H., Fukumura, R., Nakamura, M., Takahashi, H., Noda, Y., Kito, S., et al. (2003). Growth retardation and skin abnormalities of the Recql4-deficient mouse. Hum. Mol. Genet. *12*, 2293–2299.

Huen, M.S., Sy, S.M., and Chen, J. (2009). BRCA1 and its toolbox for the maintenance of genome integrity. Nat. Rev. Mol. Cell Biol. *11*.

Im, J.-S., Ki, S.-H., Farina, A., Jung, D.-S., Hurwitz, J., and Lee, J.-K. (2009). Assembly of the Cdc45-Mcm2-7-GINS complex in human cells requires the Ctf4/And-1, RecQL4, and Mcm10 proteins. Proc. Natl. Acad. Sci. U. S. A. *106*, 15628–15632.

Im, J.-S., Park, S.-Y., Cho, W.-H., Bae, S.-H., Hurwitz, J., and Lee, J.-K. (2015). RecQL4 is required for the association of Mcm10 and Ctf4 with replication origins in human cells. Cell Cycle *14*, 1001–1009.

Jackson, S.P., and Bartek, J. (2009). The DNA-damage response in human biology and disease. Nature 461(7267), 1071-8.

Jazayeri, A., Balestrini, A., Garner, E., Haber, J.E., and Costanzo, V. (2008). Mre11-Rad50-Nbs1-dependent processing of DNA breaks generates oligonucleotides that stimulate ATM activity. Embo J *27*, 1953–1962.

Jin, W., Liu, H., Zhang, Y., Otta, S.K., Plon, S.E., and Wang, L.L. (2008). Sensitivity of RECQL4-deficient fibroblasts from Rothmund-Thomson syndrome patients to genotoxic agents. Hum Genet *123*, 643–653.

Kanaar, R., and Wyman, C. (2008). DNA Repair by the MRN Complex: Break It to Make It. Cell *135*, 14–16.

Kinoshita, E., Van Rossum-Fikkert, S., Sanchez, H., Kertokalio, A., and Wyman, C. (2015). Human RAD50 makes a functional DNA-binding complex. Biochimie *113*, 47–53.

Kitao, S., Shimamoto, a, Goto, M., Miller, R.W., Smithson, W. a, Lindor, N.M., and Furuichi, Y. (1999). Mutations in RECQL4 cause a subset of cases of Rothmund-Thomson syndrome. Nat. Genet. *22*, 82–84.

Kliszczak, M., Sedlackova, H., Pitchai, G.P., Streicher, W.W., Krejci, L.,

and Hickson, I.D. (2015). Interaction of RECQ4 and MCM10 is important for efficient DNA replication origin firing in human cells. Oncotarget *6*.

Larsen, N.B., and Hickson, I.D. (2013). RecQ Helicases: Conserved Guardians of Genomic Integrity. Adv. Exp. Med. Biol. *767*, 161–184.

Lavin, M., Kozlov, S., Gatei, M., and Kijas, A. (2015a). ATM-Dependent Phosphorylation of All Three Members of the MRN Complex: From Sensor to Adaptor. Biomolecules *5*, 2877–2902.

Lavin, M.F., Kozlov, S., Gatei, M., and Kijas, A.W. (2015b). ATM-Dependent Phosphorylation of All Three Members of the MRN Complex: From Sensor to Adaptor. Biomolecules *5*, 2877–2902.

Lee, J.-H., and Paull, T.T. (2005). ATM activation by DNA double-strand breaks through the Mre11-Rad50-Nbs1 complex. Sci. (New York, NY) *308*, 551–554.

Lee, J.-H., and Paull, T.T. (2007). Activation and regulation of ATM kinase activity in response to DNA double-strand breaks. Oncogene *26*, 7741–7748.

Lee, Y., and Lim, H. (2016). Skp2 Inhibitors: Novel Anticancer Strategies. 2363–2379.

Li, J., and Xu, X. (2016). DNA double-strand break repair: a tale of pathway choices. Acta Biochim. Biophys. Sin. (Shanghai).

Lu, H., Shamanna, R.A., Keijzers, G., Anand, R., Rasmussen, L.J., Cejka, P., Croteau, D.L., and Bohr, V.A. (2016a). RECQL4 Promotes DNA End Resection in Repair of DNA Double-Strand Breaks. Cell Rep. *16*, 161–173.

Lu, L., Jin, W., and Wang, L.L. (2016b). Title: Aging in Rothmund-Thomson Syndrome and Related RECQL4 Genetic Disorders Aging in Rothmund-Thomson Syndrome and Related RECQL4 Genetic Disorders. Ageing Res. Rev.

Mann, M.B., Hodges, C.A., Barnes, E., Vogel, H., Hassold, T.J., and Luo, G. (2005). Defective sister-chromatid cohesion, aneuploidy and cancer predisposition in a mouse model of type II Rothmund-Thomson syndrome. Hum. Mol. Genet. *14*, 813–825.

Mari, P.-O., Florea, B.I., Persengiev, S.P., Verkaik, N.S., Brüggenwirth, H.T., Modesti, M., Giglia-Mari, G., Bezstarosti, K., Demmers, J.A.A., Luider, T.M., et al. (2006). Dynamic assembly of end-joining complexes requires interaction between Ku70/80 and XRCC4. Proc. Natl. Acad. Sci. U. S. A. *103*, 18597–18602.

Mirzoeva, O.K., and Petrini, J.H. (2001). DNA damage-dependent nuclear dynamics of the Mre11 complex. Mol. Cell. Biol. *21*, 281–288.

Petkovic, M., Dietschy, T., Freire, R., Jiao, R., and Stagljar, I. (2005). The human Rothmund-Thomson syndrome gene product, RECQL4, localizes to distinct nuclear foci that coincide with proteins involved in the maintenance of genome stability. J Cell Sci *118*, 4261–4269.

Petrini, J.H.J., and Stracker, T.H. (2003). The cellular response to DNA double-strand breaks: Defining the sensors and mediators. Trends Cell Biol. *13*, 458–462.

Petroski, M.D., and Deshaies, R.J. (2005). Mechanism of lysine 48-linked ubiquitin-chain synthesis by the cullin-RING ubiquitin-ligase complex SCF-Cdc34. Cell *123*, 1107–1120.

Popov, N., and Eilers*, S.H.M.L.C.S.M. (2007). Fbw7 and Usp28 Regulate Myc Protein Stability in Response to DNA Damage. *6*, 1449–1454.

Radhakrishnan, S.K., Jette, N., and Lees-Miller, S.P. (2014). Non-homologous end joining: Emerging themes and unanswered questions. DNA Repair (Amst). *17*, 2–8.

Sangrithi, M.N., Bernal, J.A., Madine, M., Philpott, A., Lee, J., Dunphy, W.G., and Venkitaraman, A.R. (2005). Initiation of DNA replication requires the RECQL4 protein mutated in Rothmund-Thomson syndrome. Cell *121*, 887–898.

Sartori, A.A., Lukas, C., Coates, J., Mistrik, M., Fu, S., Bartek, J., Baer, R., Lukas, J., and Jackson, S.P. (2007). Human CtIP promotes DNA end resection. Nature *450*, 509–514.

Shamanna, R.A., Singh, D.K., Lu, H., Mirey, G., Keijzers, G., Salles, B., Croteau, D.L., and Bohr, V.A. (2014). RECQ helicase RECQL4 participates in non-homologous end joining and interacts with the Ku complex. Carcinogenesis *35*, 2415–2424.

Shao, Z., Davis, A.J., Fattah, K.R., So, S., Sun, J., Lee, K.J., Harrison, L., Yang, J., and Chen, D.J. (2012). Persistently bound Ku at DNA ends attenuates DNA end resection and homologous recombination. DNA Repair (Amst). *11*, 310–316.

Shiloh, Y. (2003). ATM and related protein kinases: safeguarding genome integrity. Nat. Rev. Cancer *3*, 155–168.

Singh, D.K., Karmakar, P., Aamann, M., Schurman, S.H., May, A., Croteau,

D.L., Burks, L., Plon, S.E., and Bohr, V. a. (2010). The involvement of human RECQL4 in DNA double-strand break repair. Aging Cell *9*, 358–371.

Smith, P.J., and Paterson, M.C. (1982). Enhanced radiosensitivity and defective DNA repair in cultured fibroblasts derived from Rothmund Thomson syndrome patients. Mutat Res *94*, 213–228.

Stracker, T.H., Carson, C.T., and Weitzman, M.D. (2002). Adenovirus oncoproteins inactivate the Mre11–Rad50–NBS1 DNA repair complex. Nature *418*, 348–352.

Sun, J., Lee, K.-J., Davis, A.J., and Chen, D.J. (2012). Human Ku70/80 protein blocks exonuclease 1-mediated DNA resection in the presence of human Mre11 or Mre11/Rad50 protein complex. J. Biol. Chem. *287*, 4936–4945.

Suwaki, N., Klare, K., and Tarsounas, M. (2011). RAD51 paralogs: Roles in DNA damage signalling, recombinational repair and tumorigenesis. Semin. Cell Dev. Biol. *22*, 898–905.

Taylor, A.M.R., Groom, A., and Byrd, P.J. (2004). Ataxia-telangiectasia-like disorder (ATLD) - Its clinical presentation and molecular basis. DNA Repair (Amst). *3*, 1219–1225.

Thangavel, S., Mendoza-Maldonado, R., Tissino, E., Sidorova, J.M., Yin, J., Wang, W., Monnat, R.J., Falaschi, A., and Vindigni, A. (2010). Human RECQ1 and RECQ4 helicases play distinct roles in DNA replication initiation. Mol. Cell. Biol. *30*, 1382–1396.

Thrower, J.S., Hoffman, L., Rechsteiner, M., and Pickart, C.M. (2000). Recognition of the polyubiquitin proteolytic signal. EMBO J. *19*, 94–102.

Uematsu, N., Weterings, E., Yano, K.I., Morotomi-Yano, K., Jakob, B., Taucher-Scholz, G., Mari, P.O., Van Gent, D.C., Chen, B.P.C., and Chen, D.J. (2007). Autophosphorylation of DNA-PKCS regulates its dynamics at DNA double-strand breaks. J. Cell Biol. *177*, 219–229.

Uziel, T., Lerenthal, Y., Moyal, L., Andegeko, Y., Mittelman, L., Shiloh, Y., Abraham, R., Abraham, R., Andegeko, Y., Moyal, L., et al. (2003). Requirement of the MRN complex for ATM activation by DNA damage. EMBO J. *22*, 5612–5621.

Walker, J.R., Corpina, R.A., and Goldberg, J. (2001). Structure of the Ku heterodimer bound to DNA and its implications for double-strand break repair. Nat. Www.nature.com *412*.

Wang, G., Chan, C.-H., Gao, Y., and Lin, H.-K. (2012). Novel roles of Skp2

E3 ligase in cellular senescence, cancer progression, and metastasis. Chin. J. Cancer *31*, 169–177.

Williams, G.J., Lees-Miller, S.P., and Tainer, J.A. (2010). Mre11-Rad50-Nbs1 conformations and the control of sensing, signaling, and effector responses at DNA double-strand breaks. DNA Repair (Amst). *9*, 1299–1306.

Williams, R.S., Williams, J.S., Tainer, J.A., Williams, R.S., Williams, J.S., and Tainer, J.A. (2007). Mre11–Rad50–Nbs1 is a keystone complex connecting DNA repair machinery, double-strand break signaling, and the chromatin template. Biochem. Cell Biol *85*, 509–520.

Wu, J., Zhang, X., Zhang, L., Wu, C.Y., Rezaeian, A.H., Chan, C.H., Li, J.M., Wang, J., Gao, Y., Han, F., et al. (2012). Skp2 E3 ligase integrates ATM activation and homologous recombination repair by ubiquitinating NBS1. Mol Cell *46*, 351–361.

Yang, K., Guo, R., and Xu, D. (2016). Non-homologous end joining: advances and frontiers. Acta Biochim. Biophys. Sin. (Shanghai). *48*, 632–640.

Zhang, D., Zaugg, K., Mak, T.W., and Elledge, S.J. (2006). A Role for the Deubiquitinating Enzyme USP28 in Control of the DNA-Damage Response.

Zhou, B.-B.S., and Elledge[2], S.J. (2000). The DNA damage response: putting checkpoints in perspective. Nat. Www.nature.com *408*.

국문초록

서울대학교 사범대학

생물전공 박순영

RecQL4 단백질은 RecQL4 helicase family에 속하며, 유전체 안정성을 유지하는데 매우 중요한 역할을 하는 것으로 알려져 있다. RecQL4 유전자에 변이가 일어나면 RTS 신드롬 (Rothmund-Thomson Syndrome)이 발생한다. 이 질병은 성장발달 지연, 뼈 발달 결함, 조기 노화, 골육종암 발생율 증가와 같은 증상을 일으킨다. RTS 신드롬 환자로부터 RecQL4 유전자의 변이 양상을 분석해보면, 대부분 유전자 중앙에 위치한 helicase domain의 결실 등이 발견된다. RecQL4 유전자의 N-terminal 부분은 yeast부터 보존되어온 domain으로써, DNA 복제 개시에 더 중요한 역할을 한다. 세포나 쥐를 이용하여 RecQL4 단백질 발현을 거의 줄이고, RecQL4 단백질의 N-terminal 부분만 발현시키는 경우에, DNA 손상을 유발하면 매우 민감한 반응을 보인다. RTS 환자로부터 세포를 떼어낸 후, laser 등을 이용하여 세포에 damage를 유발해도 마찬가지의 민감한 반응을 확인할 수 있다. 이런 정황으로 보아 RecQL4의 helicase domain이 세포손상반응에서 매우 중요한 역할을 하는 것으로 추정할 수 있지만, 정확한 역할에 대해서는

밝혀진 바가 적다. 흥미로운 점은 정상적인 RecQL4는 DNA double-strand breaks (DSBs)를 유발할 경우, DNA breakage site로 모이고, HR repair 에 중요한 역할은 하는 Rad51 단백질과 상호작용한다는 점이다. 이를 근거로 RecQL4 단백질이 DNA DSBs repair 기작에서 중요한 역할을 하는 것을 예상해 볼 수 있다.

세포 내 DNA DSBs가 생기면, 제일 먼저 MRN complex (Mre11-Rad50-Nbs1)가 그것을 인지하고 그곳으로 모이게 된다. 그리고 MRN complex는 ATM과 수많은 repair 관련 인자들을 DSBs로 모은다. ATM이 활성화되면 다시 MRN complex를 활성화시키고, ATM의 다양한 하위인자들을 활성화하여 DDR pathway의 신호전달이 증폭된다. MRN complex가 repair와 checkpoint 기작에서 매우 중요한 역할을 함에도 불구하고, MRN complex가 어떻게 조절이 되는 것인지 밝혀진 바는 거의 없다.

RecQL4가 ATM 활성화에 영향을 끼치는 실험결과를 확인한 후, ATM의 상위인자인 MRN complex에 어떤 영향을 끼치는지 알아보았다. 그 결과, RecQL4 단백질은 DNA DSBs를 유발하였을 때, MRN complex와 damage site에서 co-localization 하며, 특히 Mre11과 직접적으로 상호작용하는 것을 관찰하였다. 또한, 세포 내 RecQL4를 제거하고 DSBs를 유발한 경우, MRN complex가 사라지는 것을 관찰하였다. 이를 통해 RecQL4가 MRN complex의 유지에 중요한 역할을 하는 것으로 추정할 수 있었다. RecQL4가 어떻게 MRN complex를 조절하는지 연구해본 결과, post-translational modification

중 하나인 ubiquitination 시스템을 이용하는 것을 확인하였다. 특히, E3 ubiquitin ligase인 skp2와 특정deubiquitinating enzyme (DUB)인 USP28이 MRN complex의 안정성을 직접적으로 조절하는 것을 밝혔다.

마지막으로 실제 RTS syndorme을 앓고 있는 환자로부터 얻은 세포를 이용하여 MRN complex의 단백질 수준을 확인하였다. DNA DSBs를 유발한 결과, MRN complex의 단백질 양은 매우 감소하였고, RecQL4 야생형을 주입한 결과 MRN complex가 다시 원래 수준으로 회복하는 것을 관찰할 수 있었다. 이것은 RecQL4의 DNA 손상반응에서 역할을 밝힘으로써, RTS syndrome을 치료할 수 있는 단서를 제공했다는 점에서 매우 의미가 크다고 볼 수 있다. 뿐만 아니라, 이 연구내용은 RecQL4 단백질의 DNA repair 조절기작을 구체적으로 밝혀냈다는 점에서 매우 새롭고 중요한 결과라고 할 수 있다.