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RecQL4 interacts with MRN complex to regulate DNA damage response

DNA 손상 기작 조절단계에서 RecQL4와 MRN complex의 상호 결합에 관한 연구

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홍 명 진

Abstract

RecQL4 interacts with MRN complex to regulate DNA damage

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DNA double strand breaks (DSBs) are a major threat to cell viability and thus needs to be repaired timely and accurately by DNA repair pathways. RecQL4, a member of the RecQ helicase family, known to be involved in the initiation of DNA replication, is evident to play a critical role in DNA repair in response to DNA DSBs. RecQL4 is found to interact with Mre11-Rad50-Nbs1 (MRN) complex in response to DNA damage, which is one of the early and major sensors of DNA DSBs. In this paper, the interaction between RecQL4 and MRN complex is investigated by immunoprecipitation and immunostaining. It is confirmed that RecQL4 interacts directly with Mre11 among the components of this complex. It is further proved that it is the C-terminus of RecQL4 that is responsible for this interaction. Having

proven that RecQL4 interacts with MRN complex, the role of RecQL4 in DNA repair is further questioned. It is found that RecQL4 is needed in Rad51 recruitment in response to DNA DSBs. Since mutations in the RecQL4 gene leads to the development of Rothmund-Thomson syndrome (RTS), this study will contribute to the understanding of maintenance of the genome integrity and DNA repair mechanisms.

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Keywords : RecQL4, Mre11, MRN complex, DNA repair, DNA damage response, Rothmund-Thomson Syndrome

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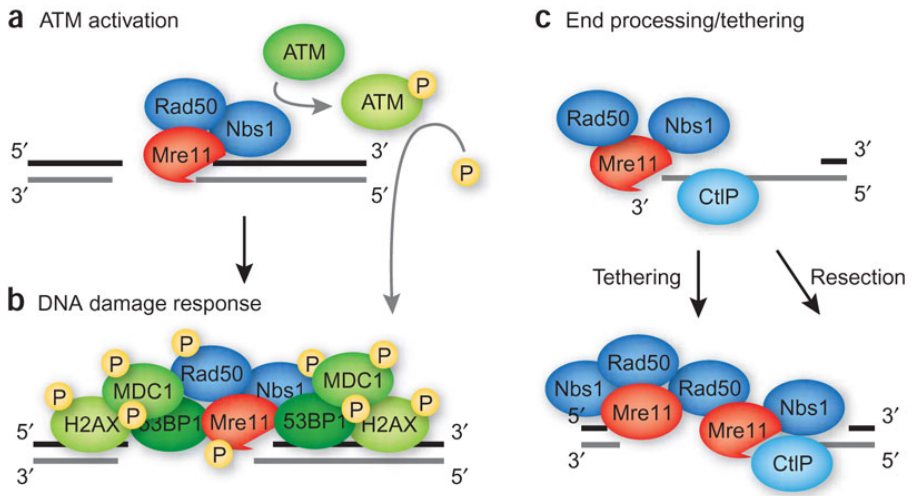
1. Introduction

1.1 Research Background

DNA double-strand breaks (DSBs), one of the most severe forms of DNA damage, can lead to mutagenesis, genomic instability and even cell death if left unrepaired or misrepaired (Rich et al., 2000; Helleday et al., 2007). Since DNA DSBs represent a major threat to cell viability it is important that they be recognized and repaired timely and accurately. Thus a complex and sophisticated network of DNA damage responses (DDR) occur in cells that encounter such damage.

Mre11-Rad50-Nbs1 (MRN) complex is one of the major sensors of DNA DSBs that has been known to be involved in both major mechanisms of DSB repair, DNA replication fork restart, telomere maintenance, meiosis and signalling to the cell cycle checkpoints (van den Bosch, Bree & Lowndes, 2003). It functions both in the recognition and processing of DNA DSBs. An early event in DNA damage response is the localization of MRN to the DNA DSB. The complex facilitates DNA unwinding and DNA resection, generating 5' resected ends (Fig. 1). This is faciliated by the endonuclease activity of Mre11. ATM is then recruited to the DNA DSB site, where it is phosphorylated and activated. As mentioned above, ATM is a central player in the DNA damage response, initiating signalling to a various cellular processes (Paull, 2015). Activated MRN complex by ATM in turn recruits and activates ATM at the site of damage for these consequent processes to occur.



(Zha, Boboila & Alt, 2009)

Figure 1. The multiple functions of Mre11.

Mre11 interacts with Nbs1 and Rad50 to form the MRN complex, which activates ATM kinase, participates in the DNA damage response with other ATM substrates and also can function to tether broken ends, and may function with CtIP and potentially other factors to resect the broken ends.

There are two major DNA DSB repair pathways in mammalian cells – nonhomologous end-joining (NHEJ) and homologous recombination (HR) (Khanna and Jackson, 2001). NHEJ repairs DSBs that occur in the G1 phase, not utilizing sequence homology, but by a ligation-based mechanism. Homologous recombination happens during the late S an G2 phases of the cell cycle, when an intact sister chromatin is available for error-free repair (Johnson and Jasin, 2000). There are evidences to support that ATM plays a significant role in HR. Many HR factors, including H2AX, BRCA1, BLM, NBS1, MRE11 and CtIP, are ATM substrates (Shiloh, 2003). Depletion of ATM using small molecule inhibitor or siRNA was followed by reduction in phosphorylation and activation of such substrates (Serrano et al., 2013).

The RecQ family is a highly conserved group of DNA helicases, which maintain chromosomal stability and suppress tumorigenesis (Larsen & Hickson, 2013). RecQL4, a member of the RecQ helicase family, is essential in maintaining the genome integrity. Mutations in this gene are known to be associated with Rothmund-Thomson Syndrome (RTS), which leads to developmental abnormalities, signs of premature aging, and high incidences of osteosarcoma (Larizza et al., 2006). The *RECQL 4* gene encodes a protein of 1208 amino acids long (133 kDa), and contains a conserved 3' to 5' helicase domain in its center (Kitao et al., 1999). Unlike other RecQ family proteins, RecQL4 has a distinct N-terminus showing certain homology to Sld2, an initiation factor in yeast, and this domain is known to be essential for DNA replication initiation and pre-RC formation in vertebrates (De et al., 2012). RecQL4 is also implicated in DNA repair, having seen that it interacts with proteins for base excision repair (Schurman et al., 2009) and co-localizes with Rad51 foci after induction of DSBs (Petkovic et

al., 2005). Cells from patients with RTS have a truncated helicase domain by either frameshift or nonsense mutations, but there is little known about the function of the helicase domain. Thus, it is postulated that RecQL4 may be involved in DSB repair by HR.

1.2 Purpose of Research

The results from the previous researches point to a role of RecQL4 in the repair of DSBs, but additional studies are required to further decipher the RecQL4 protein's role and molecular mechanisms of action. It is evident that the interaction between RecQL4 and the MRN complex is essential for DNA repair upon DNA DSB, and that this pathway involves ATM for futher DNA damage response to occur (not published). The question to answer from this finding would be whether RecQL4 and the MRN complex interacts directly rather than indirectly, and if so, via which protein of the MRN complex. Ultimately, this study aims to contribute in understanding of the maintenance of the human genomic stability, such as DNA damage response, cell cycle control, tumorigenesis, and ageing related to RecQL4, and contribute to the understanding of the molecular mechanism of the genetic disease Rothmund-Thomson Syndrome.

In this study, the protein of MRN complex responsible for the interaction with RecQL4 upon DNA damage is investigated first, and checked for the directness. Then, the cloned domain mutants of RecQL4 are used to investigate which domain specifically is required for the interaction between RecQL4 and MRN complex upon DNA damage. The proteins of interest are overexpressed in cells by transfection of DNA plasmids, and so all the mutant DNA plasmids required in this study have been cloned beforehand. RecQL4 mutants have a single flag tag attached at the N-terminus, whereas Mre11 has a myc tag, and Nbs1 a HA tag.

2. Materials and Methods

Cell culture and reagents

Cells were grown in 5% CO2 humidified incubator at 37° C. Human osteosarcoma U2OS osteosarcoma cells were cultured in Dulbecco's modified Eagle's medium (DMEM; Welgene) supplemented with 10% Fetal Bovine Serum (FBS) and antibiotics. The U2OS-RecQL4 doxycycline inducible cell line (U2OS Tet-RecQL4) was developed using parental U2OS cells and the Tet-On system (Invitrogen) according to the manufacturer's protocol. Cells were maintained in DMEM, 10% Tet-Free FBS, with the supplements described above.. Doxycline (2mg/ml) was added to the medium for doxycycline-dependent induction.. Neocarzinostatin (NCS) was purchased from Sigma Aldrich. For DSB induction, cells were incubated with 200 ng/ml of NCS for 15 min and released in fresh medium for one hour.

Plasmid preparation and transfection

RecQL4 was 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. 2flag-RecQL4 and 2HA-Nbs1, or 2flag-RecQL4 and myc-Mre11 were co-transfected and overexpressed in U2OS cells. Plasmid transfections were performed using Polyfect transfection reagent (Qiagen). For a 60mm dish, 100 μl of serum-free media, 8 μl of Polyfect reagent, and plasmid DNA were mixed and left at room temperature for 10 min. The mixture was added to the cells in dropwise manner. Cells were then incubated for 48 hours until harvest.

siRNA-mediated interference

siRNA duplex oligoribonucleotides were synthesized by ST Pharm (Seoul, Korea). Transfections of siRNA duplexes were performed using the Neon® transfection system (Invitrogen) according to the manufacturer's instructions. Experiments were performed 48 hours after transfection. The sense sequences were as follows: control siRNA (GL), 5'-AACGUACGCAUACUUCGATT-3'; and RecQL4 siRNA , 5'-GACUGAGGACCUGGGCAAATT-3'.

Immunoblot analysis

Cells were harvested, washed with PBS and lysed in NP40 buffer (50mM Tris-HCl(pH7.5), 0.5% NP-40, 50mM potassium acetate, 1mM EDTA(pH8.0)) with protease inhibitors (5mg/ml leupeptin, 1mM benzamidine, 5mg/ml pepstatin A, 1mM PMSF) and phosphase inhibitors (10mM sodium fluoride, 1mM sodium vanadate). Cells were disrupted by sonication and the proteins were quantified using the Bradford assay. Whole-cell lysates were mixed with SDS sample buffer, 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-Mre11 (Cell Signaling), anti-Nbs1 (Novus), anti-RecQL4(), anti-Myc (Millipore), and anti-Flag (Sigma Aldrich).

Immunoprecipitation

Cells were harvested and washed once with cold PBS. Protein extracts were prepared with lysis buffer containing 40mM Tris-HCl, 150-175mM NaCl, 2mM MgCl2, 1mM DTT, 5% glycerol, 0.2% NP40, 10mM NaF, 1mM Na2VO3, 1mM PMSF, 5mg/ml leupeptin, 1mM benzamidine, and 5mg/ml pepstatin A. After sonication, extracts were mixed with 5㎕ of Protein G beads for bead clearing for an hour at 4° C. They were then centrifuged at 13000 rpm, at 4° C for 20 min. Taking the supernatant, benzonase was added at a concentation of 0.25U/ul for 6 hours at 4° C. The protein concentrations were determined using the Bradford assay. The supernantants were incubated with a respective primary antibody for three hours at 4° C. Protein G beads were blocked in BSA solution containing 0.02% NP40 for an hour, then binded 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. For IP experiments using flag beads instead of Protein G beads, the methods are the same except there is no primary antibody binidng step. The extract iis incubated with pre-blocked flag beads for 3 hours at 4° C.

Immunostaining and microscopy

Cells grown on cover slips were washed once with cold phosphate buffered saline (PBS) and fixed with 4% paraformaldehyde/PBS for 10 min. Fixed cells were permeabiliezed with 0.2% Triton X-100/PBS on ice for 5 min. Blocking was carried out with 0.5% Triton X-100/BSA at room temperature for 30 min. The cells were incubated for an hour with primary antibodies: mouse

monoclonal anti-RPA antibody (Santa Cruz) at a dilution of 1:100; rabbit polyclonal anti-Rad51 antibody (Millipore) at a dilution of 1:50 ; mouse monoclonal anti-phospho-H2AX antibody (Upstate) at a dilution of 1:1000. After being washed three times with 0.1% Triton X-100/PBS, the cells were incubated for 30 min with secondary anti-mouse Alexa-fluoro594 and anti-rabbit Alexa-fluoro488 (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 Zeiss fluoroscence microscope.

3. Results

3.1 Interaction between RecQL4 and Mre11

RecQL4 co-localizes with Mre11 to the damage sites. The foci of Mre11 and RecQL4 was observed to be co-localized, by the presence of the yellow signal in the merged photographs,, when treated with neocarzinostatin (NCS), a chemical known to cause DNA double strand breaks (Fig. 2). This result is further confirmed by immunoprecipitation, where the physical interaction between RecQL4 and MRN complex is observed again (Fig. 3). When immunoprecipitated with anti-RecQL4, all three proteins of MRN complex is blotted. The same phenomenon is observed when immunoprecipitated reversely.

3.2 Mre11-interacting domain of RecQL4

After having confirmed the endogeneous interaction of RecQL4 and MRN complex, each plasmid was overexpressed to check for the possible direct interaction between RecQL4 and a factor of MRN complex. When the lysate of overexpressed Nbs1 and RecQL4 was pulled with flag beads, Nbs1 was not detected. Also, when this same lysate was pulled with anti-Nbs1 antibody, no flag band was observed (Fig. 4A). Since flag is tagged at RecQL4, this means that RecQL4 and Nbs1 are not physically interacting. However, when the lysate of overexpressed Mre11 was immunoprecipitated with flag beads, Mre11 band was blotted clearly. Similarly, when pulled with anti-Mre11 instead, flag band was observed (Fig. 4B).

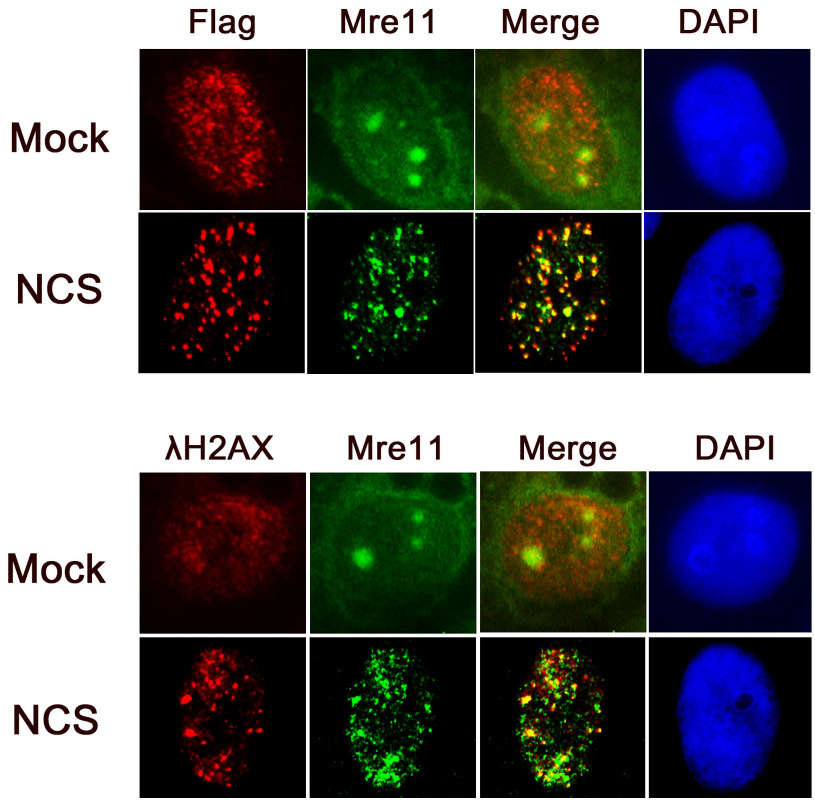


Figure 2. Flag-RecQL4 is co-localized with Mre11 at the damage site.
U2OS cells transfected with the indicated DNA constructs for 2 days were either untreated or treated with neocarzinostatin (200ng/ml) for 15 minutes and incubated in fresh medicum for an hour. Immunostaining with anti-flag and anti-Mre11 antibody was performed. γH2AX is used as double strand break marker. A cell containing 20 or more foci was considered as a foci-positive cell.

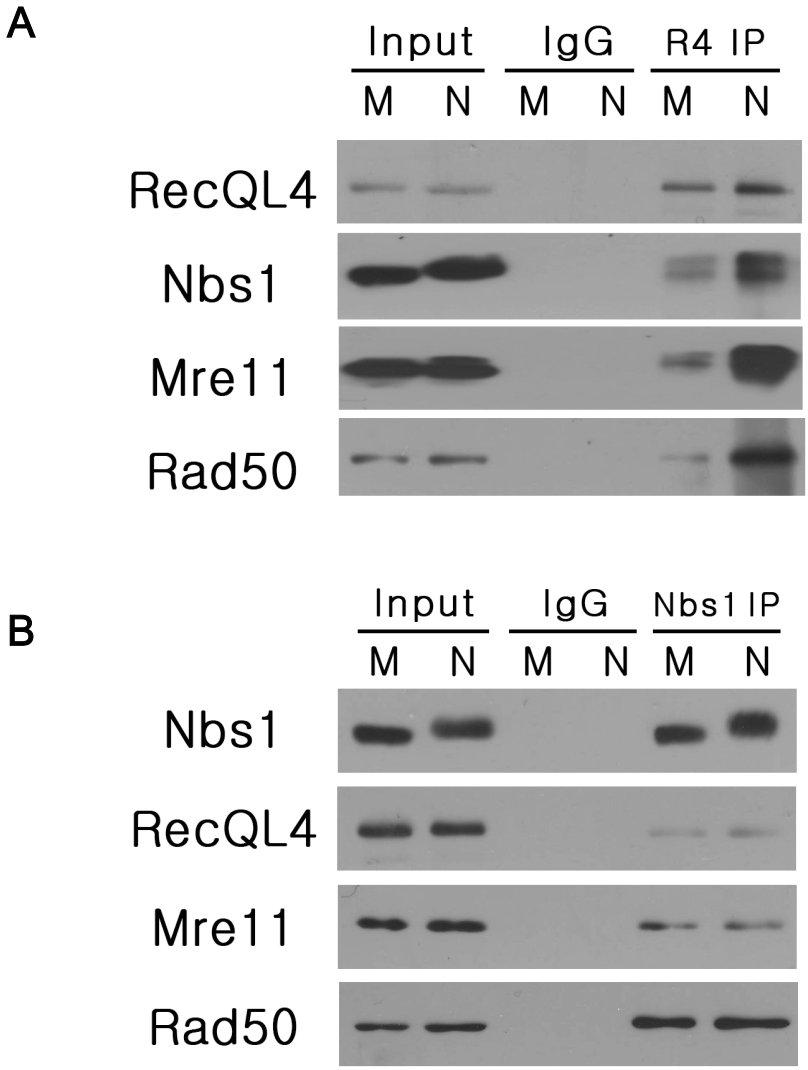


Figure 3. Endogenous RecQL4 interacts with MRN complex.

Cell lysates prepared from U2OS cells either treated with mock(M) or neocarzinostatin(N) were subjected to immunoprecipitation by (A) anti-RecQL4 antibody and (B) anti-Nbs1 antibody. The immunoprecipitates were blotted as indicated.

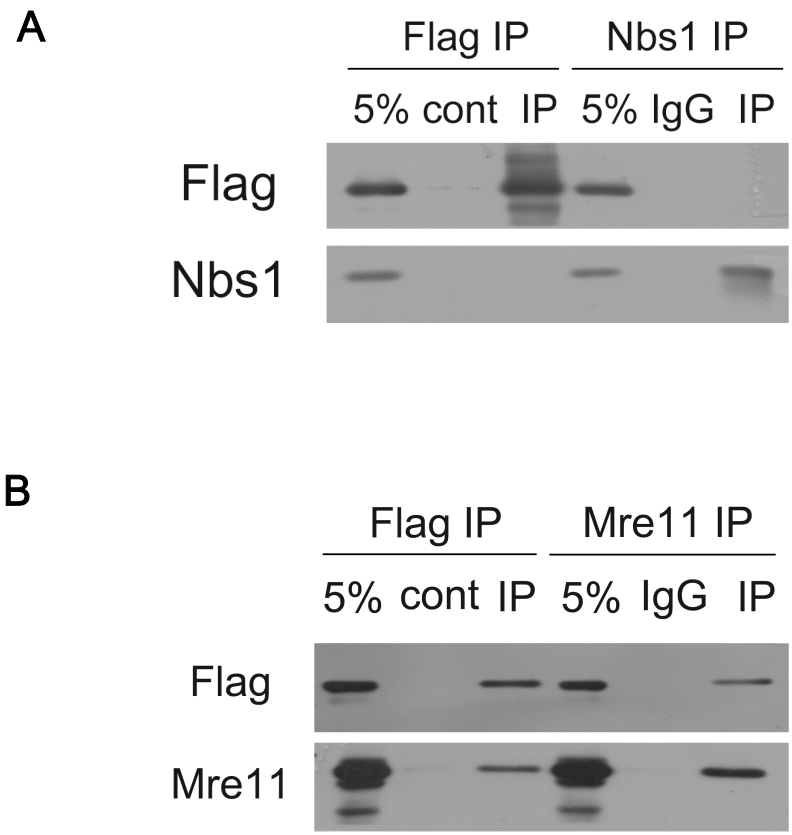


Figure 4. RecQL4 interacts with Mre11, but not Nbs1, in response to DSBs when overexpressed.

2flag-RecQL4 and 2HA-Nbs1 (A), or 2flag-RecQL4 and myc-Mre11 (B) were co-expressed in U2OS cells for 48 hours. Cells were treated with 200ng/ml neocarzinostatin for 15 minutes, and incubated in fresh medium for an hour before harvest. 5%, 5% input; cont, control IP; IgG, IgG IP. Flag IP was done using flag beads. Nbs1 IP and Mre11 IP were done with Protein G beads.

After having confirmed the direct interaction between RecQL4 and Mre11 among the MRN complex, the domain mutants of RecQL4 were cloned for futher investigation of which RecQL4 domain interacts with Mre11. Same as the wildtype RecQL4, all the other domain mutants were cloned into a mammalian expression vector with 2 flag tags at the N-terminus (Fig. 5).

Myc-Mre11 and each of these RecQL4 domain mutants were co-expressed in U2OS cells as before, and whole cell extracts were prepared for immunoprecipitation. When pulled with anti-Mre11, clear interaction with wildtype and helicase-truncated RecQL4 domain with Mre11 were observed, except for mutants with deleted C-terminus (Fig. 6A). N-terminus deleted mutants also showed strong interaction with Mre11 (Fig. 6B).

Since both RecQL4 and Mre11 have DNA binding domains, it was necessary to check if the observed interaction is not mediated by DNA. This was done by adding benzonase to the cell extracts, an endonuclease degrading all forms of DNA and RNA. When immunoprecipitated with anti-Mre11 antibody, it was observed again that flag-tagged wildtype, helicase truncated, and N-terminus deleted mutants of RecQL4 are pulled along. C-terminus deleted mutant did not seem to interact with Mre11 (Fig. 7A). Cloned full length C-terminus domain of RecQL4 (ND3) (Fig. 5) is also confirmed to interact directly with Mre11 (Fig. 7B). From these results, it can be concluded that the C-terminus of RecQL4 is responsiblefor the direct interaction between RecQL4 and Mre11 when DSBs are caused by NCS, without DNA mediation.

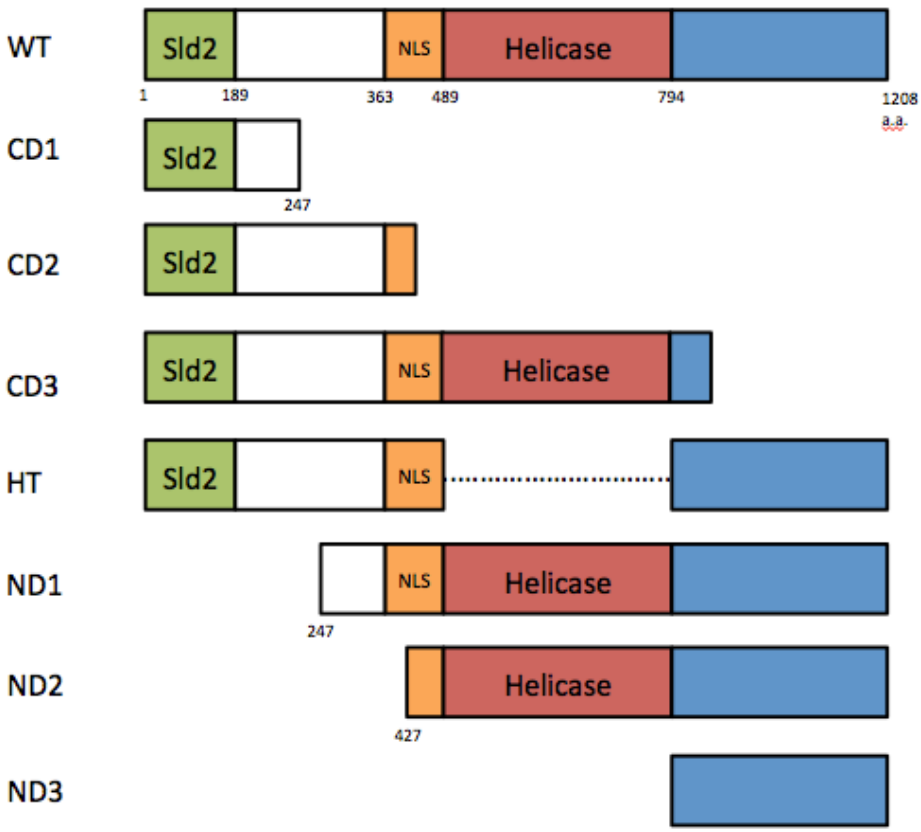


Figure 5. Diagrams of plasmids encoding full length or deletion mutants of RecQL4

Different domains of RecQL4 can be distinguished by color. The amino acid numbers are labelled below each diagram. WT, wildtype; CD1, C-terminus deletion 1; CD2, C-terminus deletion 2; CD3, C-termnius deletion 3; HT, helicase truncation; ND1, N-terminus deletion 1; ND2, N-terminus deletion 2; ND3, N-terminus deletion 3.

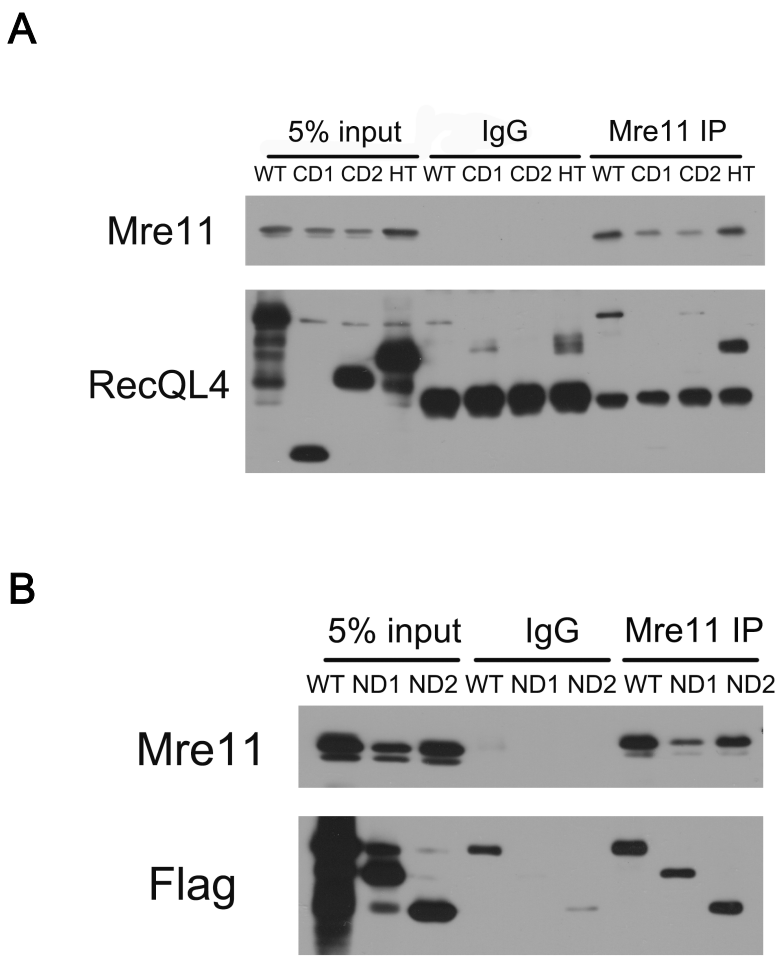


Figure 6. Mre11 interacts with the C-terminus of RecQL4

Immunoprecipitated proteins were immunoblotted for the stated proteins and/or tags. (A) Cell extracts were immunoprecipitated with α-Mre11 antibody. (B) Cell extracts were immunoprecipitated with flag-tagged agarose beads. 5% input, 5% of the whole input; IgG, negative control; Mre11 IP, immunoprecipitated sample with Mre11 antibody; flag IP, immunoprecipitated sample with flag beads.

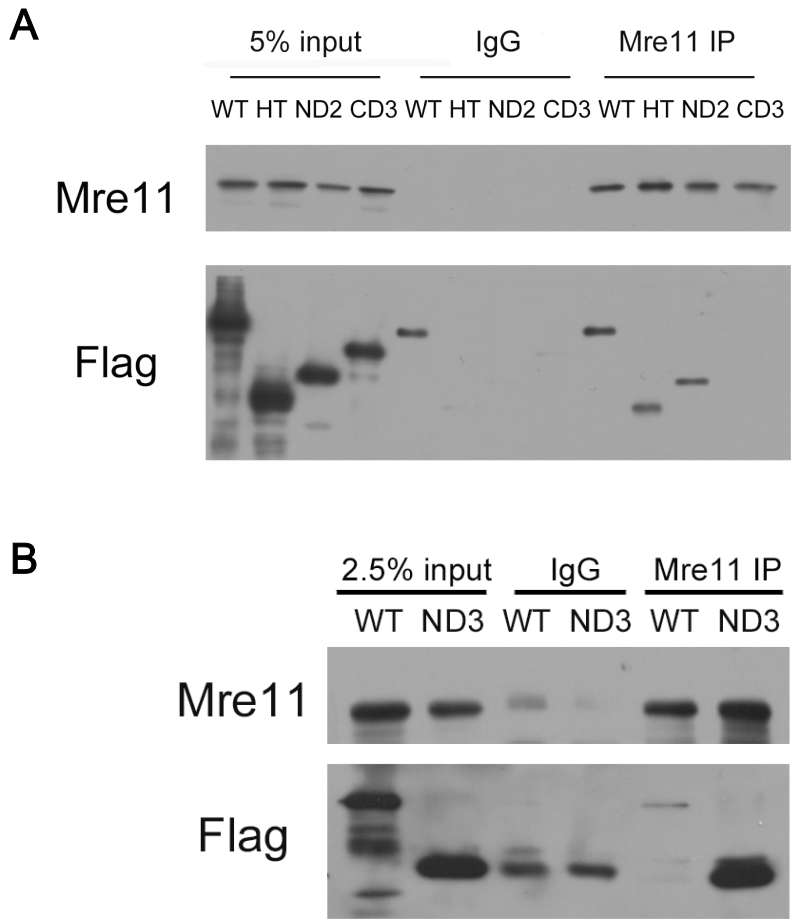


Figure 7. C-terminus of RecQL4 interacts with Mre11 directly, without DNA mediation

Immunoprecipitation is repeated with as before, with benzonase (0.25U/㎕) treated for 6 hours at 4°C additionally. (A) N-terminus, C-terminus, and helicase truncated mutants are subjected to immunoprecipitation. (B) N-terminus deletion 3 mutant (C-terminus domain) is subjected to immunoprecipitation. % input, % of the whole input; IgG, negative control; Mre11 IP, immunoprecipitated sample with Mre11 antibody.

3.3 RecQL4 recruits Rad51 to DSB sites

Having confirmed the sites of interaction between RecQL4 and Mre11 in the presence of DSBs, the next question was whether RecQL4 plays any role in the DNA repair pathway. As mentioned previously, Rad51 binds to single-stranded DNA during the initial phase of homologous recombination. After suppressing expression of RecQL4, and subsequently overexpressing wildtype or Walker 'A' mutant form of RecQL4 respectively, the presence and/or co-localization of Rad51 and γH2AX foci were checked by immunostaining. γH2AX is used as a double strand break marker.

Cells expressing wildtype RecQL4, and treated to NCS, show increased numbers of Rad51 and γH2AX foci, compared to non-treated cells (Fig. 8A).. This confirms that NCS treatment has worked sufficiently to cause DSBs in the cells, and also that Rad51 is recruited to these damage sites for DNA repair effectively. The co-localization of foci are checked by the presence of yellow signals in the merged pictures. However, when cells are induced to express Walker 'A' mutant instead, the number of Rad51 foci significnalty decreases, and the ones that show do not co-localize with γH2AX foci. This result suggests that effectively working RecQL4 is necessary for the recruitment of Rad51 to the damage sites, and possibly needed for effective DNA repair pathway to begin.

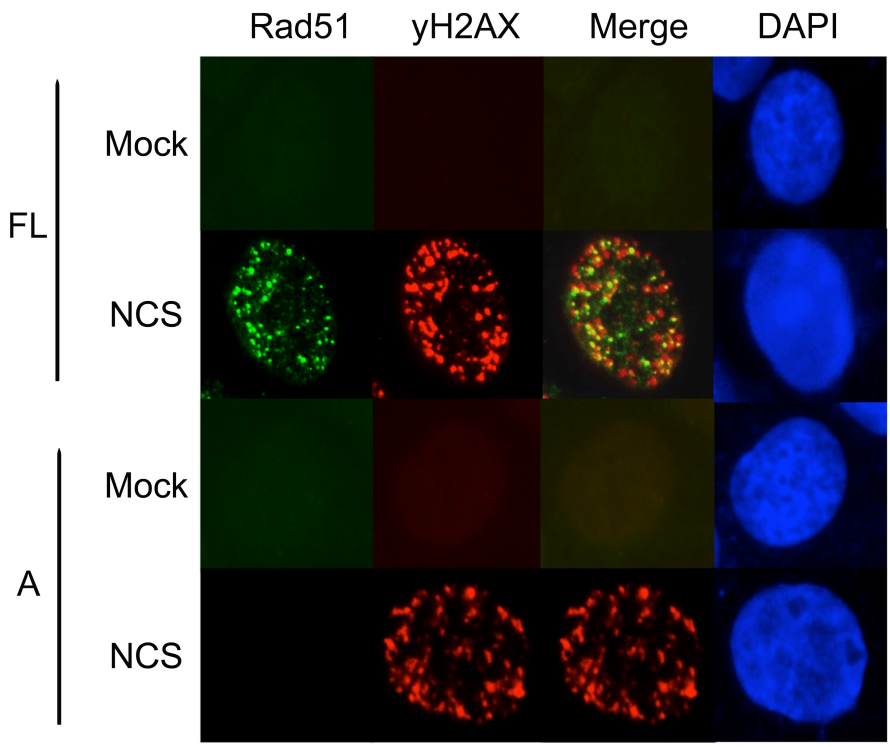


Figure 8. Co-localization of yH2AX and Rad51 in NCS-treated cells.
U2OS Tet-on RecQL4 expressing stable cells are used. Endogeneous RecQL4 is knocked down, and WT and Walker 'A' mutant of RecQL4 is induced, respectively. NCS treatment is the same as described before. Merged image of green and red signals shows colocalization between Rad51 and yH2AX. Anti-Rad51, green; anti-yH2AX, red; DAPI, blue.

4. Discussion

Protein domain work is essential in identifying the function of the protein, since the prediction of protein function is based on the combination of the protein domains. Each domain is a structural unit of the protein, thus knowing which domain is involved in a certain process is critical in answering the role of a protein in that process.

The RecQL4 protein plays multiple roles in the maintenance of genome stability. In vivo studies suggest that the N-terminus is essential for cell viability and embryogenesis (Abe et al., 2011), whereas the C-terminus (including the helicase domain) is responsible for genome integrity and preventing cancer predisposition (Mann et al., 2005). To add to this, the N-terminal Sld2 domain is known to play an important role in DNA replication initiation and the formation of pre-replicative complex, required for the proper assembly of CDC45-MCM2-7-GINS complex at the replication fork. The helicase domain is truncated in many RTS patients, but not much is known about the function of this domain. When DSBs are formed, Mre11 of the MRN complex first recognizes and binds to the damage site for the consequent DNA repair pathway to begin. Here, RecQL4 is shown to be directly interacting with Mre11. This suggests a possibility that RecQL4 has a function to play in DNA repair mechanism. By showing further the direct interaction between Mre11 and RecQL4 via its C-terminus domain, it is evident that RecQL4 may also be crucial in DNA repair and thus in the maintenance of genome integrity, The findings of this study that the C-terminus may play a crucial role in DNA repair pathway, will hopefully contribute to further the understanding of the mechanisms of RTS syndrome and genomic instability.

In addition, the fact that properly working RecQL4 is needed to recruit Rad51 to the damage sites is important in proving that RecQL4 could play a critical role specifically in homologous recombination. This could be confirmed further by observing for the foci of Replication protein A (RPA), which is another important protein required for homologous recombination to occur. Furthermore, HR assays using the Isce-I system, where the level of successful homologous recombination repair are measured, could be performed for acquisition of more quantitative data.

Having identified the physical region that is involved in the DNA repair mechanism, it can be expected that follow-up studies will be able to explain the details of the sophisticated DNA repair mechanism. It is hoped that the findings of this study will ultimately contribute to the understanding of the genetic disease RTS, and also tumorigenesis and agenig as well.

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요약(국문초록)

DNA 이중 나선 손상은 세포의 생존에 가장 심각한 위협이기 때문에 정교한 DNA 손상 복구 경로를 통해 신속하고 정확하게 수선되어야 한다. RecQL4는 RecQ helicase family의 일원으로서, DNA 복제 개시 과정에서 중요한 역할을 한다고 알려져있다. 특히 N-termincal Sld2 domain이 DNA 복제 개시와 pre-replicative complex 형성에 관여한다고 알려져있다. 그러나 추가적으로 RecQL4가 DNA 이중나선 손상이 일어났을 때에도 중요한 역할을 한다는 것이 밝혀졌다. DNA 손상이 있을시 가장 초기에 손상부위를 인식하여 수선기작이 일어날 수 있게하는 Mre11-Rad50-Nbs1 (MRN) complex와 RecQL4가 DNA 이중 나선이 손상되면 상호작용하는 것이 확인되었다. 본 연구에서는 RecQL4가 MRN complex 중에서도 Mre11과 직접적인 상호작용을 한다는 것을 확인하였다. 또한, RecQL4가 손상부위에 Rad51을 활성화 시키는 역할을 수행한다는 것도 확인할 수 있었다. RecQL4 유전자의 helicase domain에 돌연변이가 생길 경우 Rothmund-Thomson Syndrome (RTS)라는 유전질환의 원인이 되는데, 이 질환은 골육종과 소아 백내장 등을 유발하는 희귀질환이다. 따라서 본 RecQL4의 추가적인 역할과 기능에 대한 연구가 관련 유전 질환, 게놈 보전과 DNA 수선 기작에 대해 보다 더 자세하게 이해하는데 기여하리라 생각한다.

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주요어 : RecQL4, Mre11, MRN complex, DNA수선기작, DNA손상, RTS

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