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Regulatory mechanism of RIF1 through interaction with RBPMS

조선대학교 대학원

의과학과

최 하 연

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의과학과

최 하 연

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지도교수 이 정 희

이 논문을 이학석사학위신청 논문으로 제출함

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조선대학교 대학원

의과학과

최 하 연

최하연의 석사학위논문을 인준함

위원장 조선대학교 교 수 유호진(인)

위 원 조선대학교 교 수 장인엽(인)

위 원 조선대학교 부교수 이정희(인)

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조선대학교 대학원

CONTENTS

KOREAN ABSTRACT ····· v

INTRODUCTION ····· 1

MATERIALS AND METHODS

1. Cell culture and treatment of ionizing radiation ····· 6

2. siRNA transfection ····· 7

3. co-Immunoprecipitation assay ····· 7

4. Western blot analysis ····· 9

5. Antibodies ····· 10

6. Proximity Ligation Assay (PLA) ····· 11

7. Colony survival assay ····· 13

8. Immunofluorescence microscopy ····· 13

9. Non-homologous end joining assay ····· 14

10. Homologous recombination assay ····· 15

11. Statistical analysis ····· 16

RESULTS

1. RBPMS interacts with RIF1 ····· 17

2. Defect of RBPMS is generated by RBPMS siRNA ····· 21

3. Depletion of RBPMS is hypersensitive to IR and increases DNA damage ····· 24

4. RBPMS is required for IR-induced RIF1 foci formation ····· 28

5. RBPMS enhances Non-homologous end joining ····· 33

6. RBPMS-depleted cells reduce in homologous recombination activity and RAD51 foci formation ····· 37

DISCUSSION

Discussion ····· 43

ABSTRACT

Abstract ····· 49

REFERENCES

References ····· 51

CONTENTS OF FIGURES

Figure 1. RBPMS binds to RIF1 according to IR treatment ····· 20

Figure 2. RBPMS is knockdowned by RBPMS siRNA transfection ····· 23

Figure 3. Depletion of RBPMS is sensitive to IR and is defective in DSB repair ···

····· 27

Figure 4. RBPMS-depleted cells were declined RIF1 foci formation after DNA

damage ····· 32

Figure 5. Absence of RBPMS reduces the activity of NHEJ ····· 36

Figure 6. Depletion of RBPMS impairs HR ····· 41

Figure 7. A schematic representation of the role of RBPMS in regulating the DSB

repair is shown ····· 42

국문초록

RBPMS에 의한 RIF1의 활성 조절 기전 연구

최 하 연

지도교수: 이 정 희

조선대학교 일반대학원

의과학과

DNA의 이중나선 절단 시 DNA는 상동결합재결합 또는 비 상동 말단결합으로 손상 복구가 이루어 진다. RIF1은 53BP1, Rev7, PTIP와 더불어 비 상동 말단 결합에 중요한 단백질로 알려져 있다. 본 연구에서는 Yeast two-hybrid screening을 통해 DNA 손상 복구에 필수적인 역할을 하는 RIF1과 결합하는 RBPMS 단백질을 동정하였다. RNA 결합 단백질인 RBPMS는 RRM(RNA Rec-

ognition Motif) family의 구성원으로써 세포의 전사와 mRNA processing에 관여한다. RBPMS는 세포 발달 및 성장을 조절하는데 매우 중요한 SMAD2/3 그리고 SMAD4를 통해 전사 활동을 촉진한다고 알려져 있다. 또한, RBPMS는 포유동물의 망막에서 신경세포의 선택적인 마커로 알려져 있지만 DNA 손상 반응에서의 역할은 전무한 실정이다. 따라서 본 논문에서는 RBPMS가 RIF1과의 결합을 통해 RIF1 활성을 조절하는 기전을 밝힘으로써 RBPMS의 DNA 손상반응에서의 새로운 역할을 규명하고자 한다. 먼저, 방사선 조사 후 세포 내에서 RBPMS와 RIF1의 결합이 증가하는 것을 확인하였다. 이는 방사선 조사에 따른 DNA 이중나선 절단 복구에서 RIF1이 매개된 DNA손상 반응에 RBPMS가 기능적으로 연관됨을 시사한다. RBPMS의 결핍은 세포의 DNA손상 복구 활성을 떨어뜨림을 colony survival assay와 late γ-H2AX염색법을 통해 확인하였다. 다음으로, RBPMS가 RIF1의 활성 조절에 미치는 영향을 알아보기 위해 먼저 RBPMS가 결핍된 세포에서 방사선 조사 후 RIF1의 기능을 조사한 결과 RIF1의 DNA손상 foci가 감소되는 것을 각 단백질에 대한 면역염색을 통해 확인하였다.

그러나, 53BP1과 BRCA1 손상 foci는 RBPMS가 있고 없음에 큰 영향을 받지 않았다. 더 나아가, RBPMS의 결손에 의해 상동재결합, 비 상동 말단 결합 활성이 저하됨을 DR-GFP와 EJ5-GFP 벡터 시스템을 통해 확인하였다. 뿐만 아니라 RBPMS가 결핍된 세포에서 RAD51 foci가 감소됨을 확인하였다. 따라서, 본 연구는 RNA 대사 단백질로 알려진 RBPMS가 RIF1과의 결합을 통해 DNA 이중나선 절단 시 손상복구에 관여됨을 밝힌 최초의 보고이다.

INTRODUCTION

Maintaining of genomic integrity is very important from constantly exposed to various toxic agents, such as ionizing radiation or DNA-damaging chemicals [1]. DNA double-strand breaks (DSBs) are generated by many of these agents, and must be repaired correctly to protect genomic stability. If DSBs are not accurately repaired, they may result in cell death, mutations and inappropriate chromosomal translocations, finally which can lead to cancer [2]. Thus, the proper choice of DSB repair pathway has a critical impact in DNA damage response (DDR). Mammalian cells have two major pathways for DSBs repair. It is called non-homologous end joining (NHEJ) and homologous recombination (HR).

NHEJ accounts for nearly all DSBs repair outside of the S and G2 phases. This pathway utilizes proteins that recognize, resect, polymerize and directly ligate together, with the resulting in loss of genetic information [3, 4]. As a re-

sult, NHEJ has another name called error-prone repair. RIF1 is the downstream of 53BP1, is recruited to DSBs via the N-terminal phospho-SQ/TQ domain of 53BP1 [5]. p53-binding protein 1 (53BP1) represents key DSBs repair protein, is an influential factor in anti-resection function. 53BP1/RIF1 with its down-stream proteins PTIP, REV7-shieldin complex inhibits the 5′- 3′ end resection of broken DNA ends, and promote non-homologous end joining depending on the ATM-mediated 53BP1 phosphorylation [6]. REV7 forms a complex with three proteins, RINN1 (CTC-534A2.2), RINN2 (FAM35A) and RINN3 (C20ORF196), and are called shieldin complex [7]. Studying of NHEJ by 53BP1-RIF1-REV7-shieldin remains still elusive.

DSBs are primarily repaired by error-free homologous recombination in S/G2 phase [8]. HR requires the use of homologous sequences to align DSB ends prior to ligation, and needs the presence of single-strand DNA at the DSB end [1]. Resection of DNA ends, which converts broken DNA into 5′ - recessed

ends suitable for HR [9]. Resection is initiated by CtIP-dependent stimulation of MRE11 endonuclease activity. MRN complex (MRE11/RAD50/NBS1) binds to DSB ends, promotes end resection [10]. Next, exonucleases such as Exo1/DNA2 expand resection, ssDNA overhangs generated by DNA resection are initially coated by replication protein A (RPA), which is replaced by RAD51 to facilitate homology searching and the subsequent steps of HR [11]. Therefore, DNA end resection is the key to determining HR and NHEJ, which occurs S/G2 than G1. HR utilizes an undamaged DNA template to DSBs repair, it has more faithfully than NHEJ. The breast cancer 1 (BRCA1) is a tumor suppressor involved in basic cellular functions necessary for cell replication and DNA synthesis, promotes HR by activating DNA end resection [12]. Antagonistic relationship between 53BP1 and BRCA1 has been very important in DNA repair. If NHEJ does not follow, repair switches to HR.

mRNA processing factor RBPMS is member of the RRM (RNA Recognition

Motif) family, and acts as a co-activator of transcription [13]. RBPMS physically interact with Smads, and is required to increase TGFB1/SMAD-mediated transactivation. RBPMS plays through SMAD2, SMAD3 and SMAD4 to enhance transcriptional activity, which is expressed as multiple alternatively spliced transcripts encoding different protein isoforms [14, 15]. TGF-β superfamily includes BMPs, GDFs, GDNFs, multifunctionally regulates a wide range of biological processes, such as morphogenesis, embryonic development, adult stem cell differentiation, immune regulation, wound healing, inflammation, and cancer [16]. In addition, RBPMS was reported on relation of selective marker of ganglion cells in the mammalian retina [17], but its function remains unknown in DNA damage response.

In this study, we identified RBPMS as a novel binding partner of RIF1 by yeast two-hybrid assay. We confirmed interaction between RBPMS and RIF1 by proximity ligation assay (PLA) and endogenous co-immunoprecipitation (IP).

Next, RBPMS-depleted cells were more sensitive to IR than treatment of HU, MMC as detected by colony survival and resulted in DNA damage repair defect, shown by late γ-H2AX staining. Moreover, we showed that RBPMS depletion is not significantly affected the recruitment of 53BP1 and BRCA1 foci but decreased the recruitment of RIF1 foci after ionizing radiation (IR). Also, RBPMS-depleted cells impaired non-homologous end joining and homologous-recombination repair activities. In addition, knockdown of RBPMS decreased Rad51 foci after DNA damage (IR). Based on these results, we propose a significant function of RBPMS in DNA double strand break repair through interaction with RIF1.

MATERIALS AND METHODS

1. Cell culture and treatment of ionizing radiation

HeLa and U2OS cells were purchased from ATCC. They were cultured in Dulbecco's modified Eagles's medium (DMEM) supplemented with 10% fetal bovine serum and streptomycin (0.1 mg/ml), penicillin (100 units/ml) at 37℃ in a 5% CO_2 incubator. Cell growth was monitored under an inverted microscope. Upon reaching 70-80% confluency, cells were digested with 1x trypsin-EDTA before being passaged. Cells in exponential growth were harvested for subse-quent experiments. To induce DNA double strand breaks, exponentially growing cells were irradiated at 5 Gy from ^{137}Cs source (Gamma cell 3000 Elan irradiator, Best Theratronics) and allowed to recover at 37 ℃ incubator for various times.

2. siRNA transfection

HeLa and U2OS cells were transfected with siRNA oligonucleotide duplexes against RBPMS using RNA IMAX (Invitrogen) according to the manufacture's instruction. The siRNA sequences targeting RBPMS (RBPMS siRNA: 5′-GUUUGCUAAGGCAAACACGAA-3′), siRIF1 (RIF1 siRNA: 5′-GACTCACATTTCCAGTCAA-3′) designed and synthesized for transient transfection.

3. co-Immunoprecipitation assay

The whole cell lysates prepared by extracting with NP-40 buffer (50 mM Tris (pH 8.0), 150 mM Nacl, 1% NP-40, and 5 mM EDTA) with protease inhibitors (Roche Diagnostic Corp.). The lysates were added to anti-RBPMS antibody (Atlas) at 4 ℃ overnight. And then, G-sepharose (GE Healthcare) was added to the lysates, and beads mixtures were incubated at RT for 1 hour with shaking.

The beads were washed three times in NP-40 buffer without protease inhibitors, resuspended in equal volume 2 x SDS sample buffer. The samples were extracted from the bead by boiling at 99 ℃ for 5 min. The samples were then analyzed by western blotting using the appropriate antibodies.

4. Western blot analysis

Cells were lysised in RIPA buffer (50 mM Tris (pH 7.5), 150 mM NaCl, 1% NP-40, 0.5% sodium deoxycholate (NADOC), 0.1% sodium dodecyl sulfate(SDS)) with protease inhibitors (Roche Diagnostic Corp.). Cell lysates were collected by centrifugation at 15,000 rpm for 30 min. Protein concentrations were measured using the Bradford assay (Bio-Rad). Equal amounts of protein were separated by 6-15% SDS-PAGE followed by electro transfer onto a polyvinylidene difluoride membrane (Millipore, Bedford, MA, USA). The membranes were blocked for 1 hour with TBS-t (10 mM Tris (pH 7.4), 150 mM NaCl and 0.1% Tween-20) containing 5 % skim milk and then incubated at 4 ℃ with primary antibodies (1:1000) overnight. The blots were washed six times for 10 min with 0.1% Tween-20 containing TBS-t and then incubated for 2 hours with peroxidase-conjugated secondary antibodies (1:4000) at RT. The membranes were washed six times for 10 min, and developed using an enhanced chemilumi-

nescence detection system (ECL; GE Healthcare, Buckinghamshire, UK).

5. Antibodies

We used the following primary antibodies: Rabbit polyclonal anti-RBPMS (HPA056999, Atlas), Mouse monoclonal anti-RBPMS (sc-293285, Santa Cruz) Rabbit polyclonal anti-RIF1 (A300-567A, Bethyl), Mouse monoclonal anti-ß-actin (sc-47778, Santa Cruz), Mouse monoclonal anti-53BP1 (612523, BD), Mouse monoclonal anti-BRCA1 (sc-6954, Santa Cruz), Rabbit ploYclonal anti-RAD51 (ab63801, Abcam), Rabbit monoclonal anti-Mad2L2/REV7 (ab180579, Abcam) and Mouse monoclonal anti-γ-H2AX (05-636, Millpore).

6. In situ proximity ligation assay (PLA)

The primary antibodies need to be optimized through concentration titration to make sure that they work properly before the star of proximity ligation assay, and IgG from the same isotype and species was usually used as negative control. The cells were transferred on the coverslip in plates and grown to 50-70% confluency. The cells were washed with PBS, fixed with 4% formaldehyde for 15 min, permeabilized with 0.3% Triton X-100 for 10 min, washed, and then blocked using Duolink blocking buffer for 1 hour. The coverslips were transferred into a humidified chamber and then a small volume of primary antibodies targeting the proteins under investigation were pipetted onto each coverslip. Atigen-antibody interaction will mostly accomplish overnight at 4 ℃ with gentle agitation. The two corresponding PLA probes were mixed and diluted in the antibody diluent. Allow the mixture to sit for 20 min at RT. The samples were washed with 0.05% Tween-20 containing PBS twice, and then incubated with

PLA probes MINUS and PLUS (DUO92005, Sigma) for 1 hour at 37 ℃. The samples were washed in 1x wash buffer A (0.01 M Tris, 0.15 M NaCl, 0.05% Tween-20, pH 7.4) for 5 min twice under gentle agitation, and the probes were ligated with two other circle-forming DNA oligonucleotides by ligation-ligase solution for 30 min at 37 ℃. The samples were washed in 1x wash buffer A for 2 min twice under gentle agitation and two added oligonucleotides by enzymatic ligation were amplified via rolling circle amplification by the incubation with am-plification-polymerase solution over 90 min at 37 ℃. The samples were washed in 1 x wash buffer B (0.2 M Tris, 0.1 M NaCl, pH7.5) for 10 min twice followed by 0.01 x wash buffer B for 1 min by diluting 1 x buffer B (1:1000) in high purity water. The samples were dried in hood for approximately 10 min at RT in the dark, the cells were mounted using Vectashield mounting medium with 4, 6-diamidino-2-phenylindole (Vector Laboratories, Burlingame, CA, USA). Fluo-rescence images were taken using a confocal microscopy (Zeiss LSM510 Meta:

Carl Zeiss) analyzed with ZEN software

7. Colony survival assay

After treatment with IR, $1x10^3$ cellswere immediately seeded onto a 60 mm dish in duplicate and grown for 2-3 weeks at 37 ℃ to allow colony formation. Colonies were fixed with 100% methanol for 10 min and stained with 1% Methy-leneblue in 20% Ethanol and counted. The fraction of surviving cells was calcu-lated as the ratio of the plating efficiencies of treated cells to untreated cells.

8. Immunofluorescence microscopy

To visualize nuclear foci, cells were grown on glass coverslips and were ir-radiated with 5 Gy of ionizing radiation (IR). Cells were then washed twice with 0.01 M PBS, fixed with 4% paraformaldehyde for 15 min and 100% methanol for 5 min, followed by permeabilization with 0.3% Triton X-100 for 15 min at RT. Next, the cover slips were washed three times with 0.01 M PBS and then

blocked with 5% BSA in 0.01 M PBS for 1 hour. The cells were immunostained with primary antibodies against various proteins overnight at 4 ℃. Next, the cells were washed with 0.01 M PBS and then stained with Alexa Fluor 488 (green, Molecular Probes) or Alexa Fluor 594 (red, Molecular Probes) conjugated secondary antibodies, as appropriate. After washing, the cells were mounted using Vectashield mounting medium with 4, 6-diamidino-2-phenylindole (Vector Laboratories, Burlingame, CA, USA). Fluorescence images were taken using a confocal microscopy (Zeiss LSM510 Meta: Carl Zeiss) analyzed with ZEN software.

9. Non-homologous end joining assay

To measure the NHEJ repair, stable cells lines expressing HeLa EJ5-GFP report were generated by transfection using turbofectamine. EJ5-GFP contains a promoter that is separated from a GFP coding region by puromycin resistance gene, which is flanked by two I-SecI sites that are in the same orientation. When

the I-SecI-induced DSBs are repaired by NHEJ in HeLa EJ5-GFP cells, the puro gene is removed, and the promoter is rejoined to the rest of the GFP expression cassette, leading GFP expression. EJ5-GFP cells were transfected with control siRNA, RBPMS siRNA, RIF1 siRNA, and RBPMS, RIF1 double siRNA after 5 hours transfected with 0.5 ug of I-SceI. After 48 hours, the cells fixed 4% paraformaldehyde and stained 5 ug/ml Hoechst (Sigma) for 1 hour. The images were shown at x20 magnification using an IN Cell Analyzer 2500 HS (GE Healthcare)

10. Homologous recombination assay

To measure the HR repair, stable cell lines expressing U2OS DR-GFP report were generated by transfection using turbofectamine. DR-GFP is shown along with the HDR product that uses *iGFP* as the template for nascent DNA synthesis, which results in restoration of a GFP expression cassette. DR-GFP

cells were transfected with control siRNA, RBPMS siRNA, RIF1 siRNA after 5 hours transfected with 0.5 ug of I-SceI. After 48 hours, the cells fixed 4% paraformaldehyde and stained 5 ug/ml Hoechst (Sigma) for 1 hour. The images were shown at x20 magnification using an IN Cell Analyzer 2500 HS (GE Healthcare).

11. Statistical analysis

Data in all of the experiments are presented as the mean ± standard deviation (SD). Analyses were performed using software (Image J) and Excel (Microsoft).

RESULT

1. RBPMS interacts with RIF1.

Together with 53BP1, RIF1 is known to be an important key regulator of DNA damage response. However, its regulation and mechanism of action are only partially understood. In order to better characterize the DDR regulatory connection relevant to RIF1 and RBPMS, a yeast two-hybrid screen was performed.

To confirm the interaction between RBPMS and RIF1, we performed Proximity Ligation Assay (PLA) staining that detects protein-protein binding. HeLa cells were induced with 5 Gy ionizing radiation (IR) for 3 hours and were then fixed. The cells were each other stained with an anti-RIF1 antibody and an anti-RBPMS antibody. RBPMS was ligated to PLA probes MINUS and PLUS. Normal rabbit IgG was used as negative control of PLA staining. RIF1 and RBPMS binding was indicated by red dots (Figure 1A and 1B).

To investigate the interaction between RIF1 and RBPMS in vivo, endogenous

co-immunoprecipitation (IP) assay and western blotting was performed using the anti-RBPMS, anti-RIF1 antibodies. The rabbit IgG was used as negative control of co-IP assay. As shown in Figure 1C and 1D, RIF1 increased binding to RBPMS and the same result was obtained when reversed. These results suggest that RBPMS interacts with RIF1 and would be play a role in DNA damage re-sponse.

Figure 1

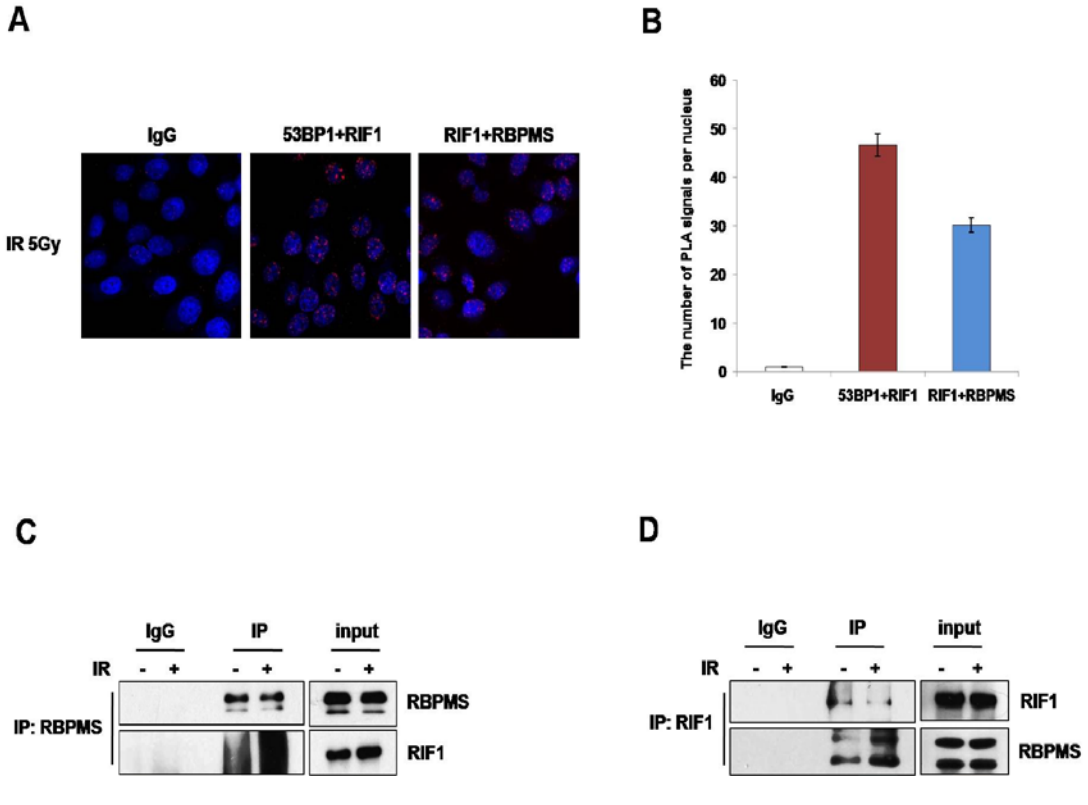


Figure 1. RBPMS binds to RIF1 according to IR treatment.

(A) HeLa cells were untreated or treated with 5 Gy IR for 3 hours and were then fixed. The cells were stained with the anti-RIF1 antibody and anti-RBPMS antibodies. RBPMS was ligated through PLA probes MINUS and PLUS. **(B)** Quantification of PLA signal. **(C)** HeLa cells were untreated or treated with 5 Gy IR for 3 hours. Whole cell lysates were subjected to immunoprecipitation (IP) using an anti-RBPMS antibody followed by Western blotting using the anti-RBPMS, RIF1antibodies. **(D)** The same experiment Fig 1C, were performed IP using an anti-RIF1antibody.

2. Defect of RBPMS is generated by RBPMS siRNA.

To determine the functional consequence of RIF1-RBPMS interaction, we investigated the effect of RBPMS on IR-induce RIF1 activation. First, we designed the RBPMS siRNA targeting of CDS sequence of RBPMS-mRNA by the targeting prediction methods of the several algorithms. Figure 2A showed that schematic diagram of RBPMS siRNA sequence. To observe the effect of the designed siRNA, we performed western blot analysis using an anti-RBPMS antibody after siRNA transfection. Also, we checked the expression levels of 53BP1, RIF1 and REV7 as binding partners of RBPMS in RBPMS knockdown cells. The β-actin was used as loading control (Figure 2B). Western blot analysis showed that RBPMS siRNA worked efficiently for the next experiments.

Figure 2

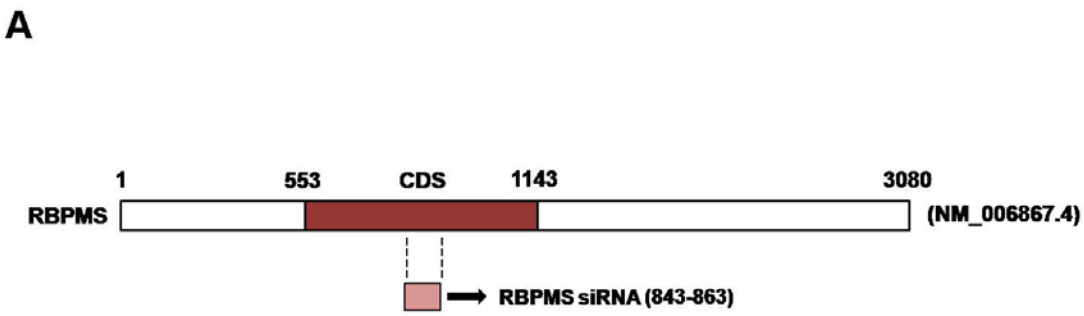


Figure 2. RBPMS is knockdowned by RBPMS siRNA transfection.

(A) Schematic diagram of human RBPMS siRNA sequence. The position of CDS (dark red), we designed RBPMS siRNA (light red). **(B)** HeLa cells were transfected with control siRNA and RBPMS siRNA. After 48 hours, cells lysates were analyzed by western blotting using anti-RBPMS, anti-53BP1, anti-RIF1 and anti-REV7, anti-β-actin antibodies.

3. Depletion of RBPMS is hypersensitive to IR and increases DNA damage.

To determine whether RBPMS is involved in the DNA damage response, we exposed control and RBPMS knockdown HeLa cells to IR, HU or MMC, and then performed cell survival assay. After 14 days, we found that depletion of RBPMS exhibited decrease of colony numbers after treatment with IR compared to control cells. However, RBPMS-depleted cells did not result in increased sensitivity to HU or MMC. These results suggested that RBPMS-depleted cells were the most sensitive to IR out of three DNA-damage agents (Figure 3A, 3B and 3C). We next investigated whether RBPMS affects DSBs repair by measuring γ-H2AX staining. γ-H2AX is indicator of DNA damage repair as well as sensing marker of DNA damage. We found that RBPMS-depleted cells showed increase in signal intensity of γ-H2AX staining compared control cells. Control cells quickly formed γ-H2AX foci following exposure to IR, and these foci were almost completely disappeared 18 hours after the exposure, indicating efficient DNA repair. RBPMS-depleted cells also quickly formed γ-H2AX foci after IR

exposure, but 18 hours later, large amount of foci still remained, indicating lacked DNA repair (Figure 3D and 3E). These results suggest that RBPMS promotes DSB repair.

Figure 3

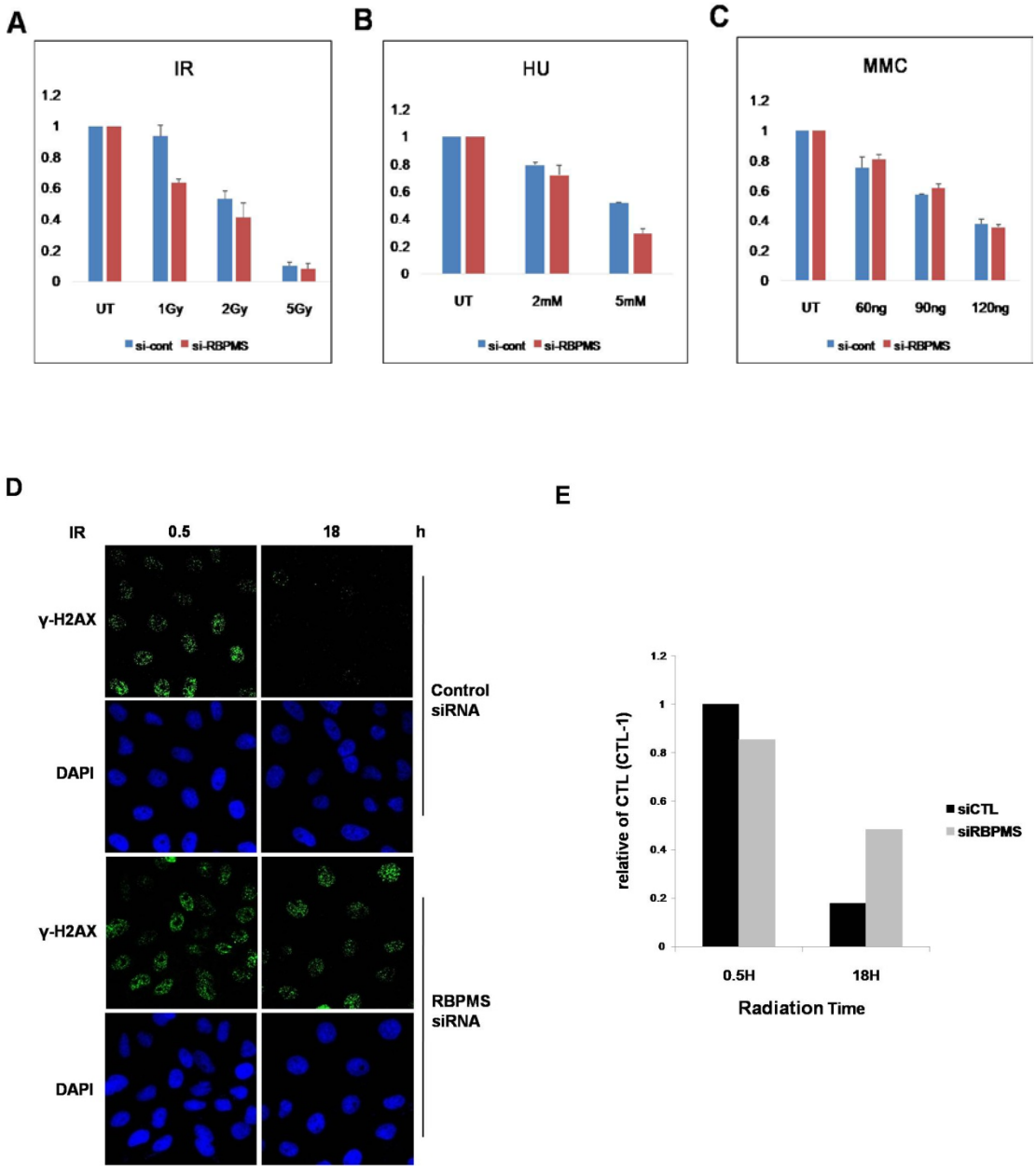


Figure 3. Depletion of RBPMS is sensitive to IR and is defective in DSB repair.

(A, B, C) RBPMS-depleted cells are hypersensitive to IR in HeLa cells. HeLa cells were transfected with control or RBPMS-siRNA, and exposed to the indicated doses of IR **(A)**, HU **(B)** and MMC **(C)**. After 2 weeks, cells were stained with methylene blue, and the number of surviving colonies was counted. The cell viability of untreated cells is defined as 1. **(D)** Control- and RBPMS-depleted HeLa cells were untreated or treated with 5Gy γ-irradiation and were then fixed after 0.5, 18 hours. Cells were stained with an anti-r-H2AX antibody and the nuclei were counterstained using DAPI. **(E)** Quantification of Fig 3D. Data are presented as means ±SD (n=3).

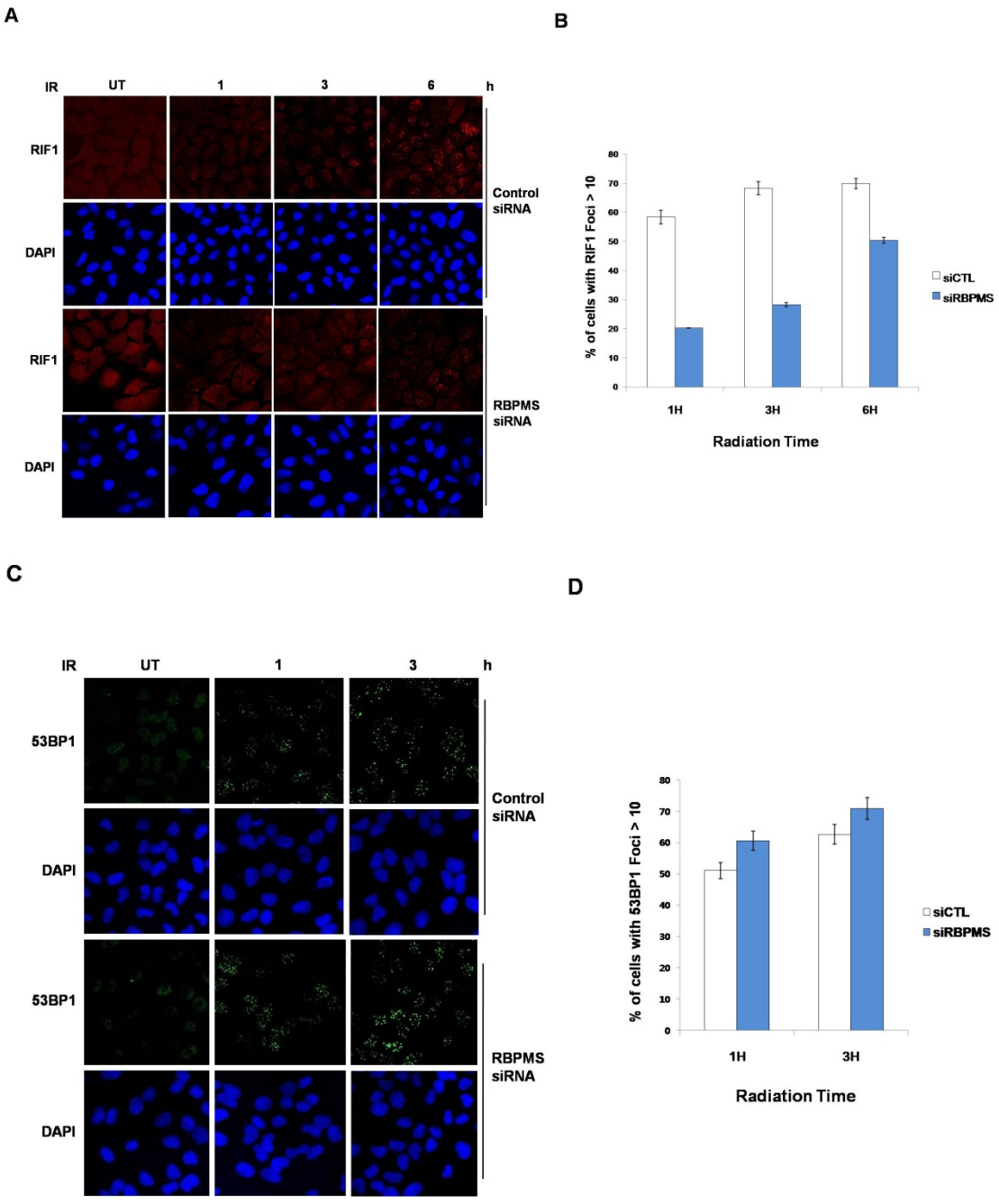
4. RBPMS is required for IR-induced RIF1 foci formation.

To above results, the interaction between RBPMS and RIF1 suggested that RBPMS was involved in IR-induced damage repair. To investigate this further, we looked for foci-formation of RIF1 and RIF1-related damage protein. Control- and RBPMS-depleted cells were treated with 5 Gy IR to induce DSBs and harvested in different time intervals. Cells were fixed by using 4% PFA and immunofluorescence assay performed. Our data showed that knockdown of RBPMS cells decreased a great number of RIF1 foci following IR exposure than control cells (Figure 4A and 4B).

53BP1 is upstream of RIF1, has been reported to be involved in DNA damage repair. Next, we wondered that RBPMS also affects 53BP1 recruitment following IR treatment. To test this question, we performed immunostaining assay with an anti-53BP1 antibody after IR treatment. As a result, RBPMS-depleted cells still formed 53BP1 foci, because 53BP1 acts upstream of RIF1 protein (Figure 4C and 4D). Furthermore, we examined the BRCA1 foci formation, which represents

the opposite pathway of 53BP1 in DSBs repair. Like the previous experiment, we performed IF with an anti-BRCA1 antibody after ionizing radiation (IR). BRCA1 foci formation was not affected by the presence or absence RBPMS (Figure 4E and 4F). Together, our data suggested that RBPMS is needed for RIF1 foci formation at DSBs, but not 53BP1 and BRCA1 recruitments at DSBs.

Figure 4



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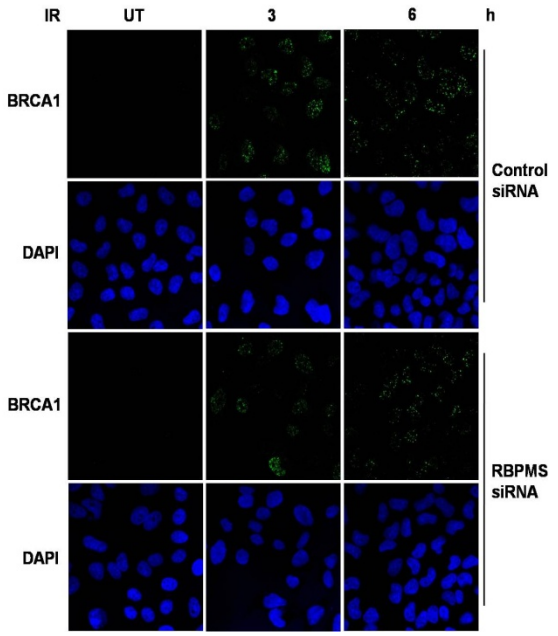

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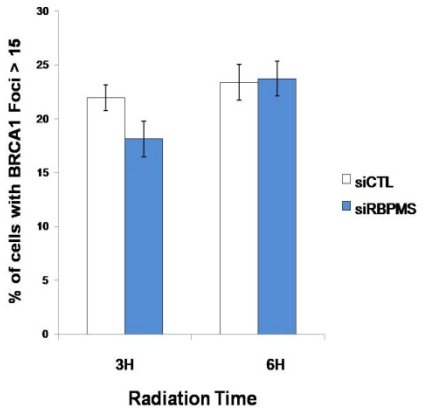


Figure 4. RBPMS-depleted cells were declined RIF1 foci formation after DNA damage.

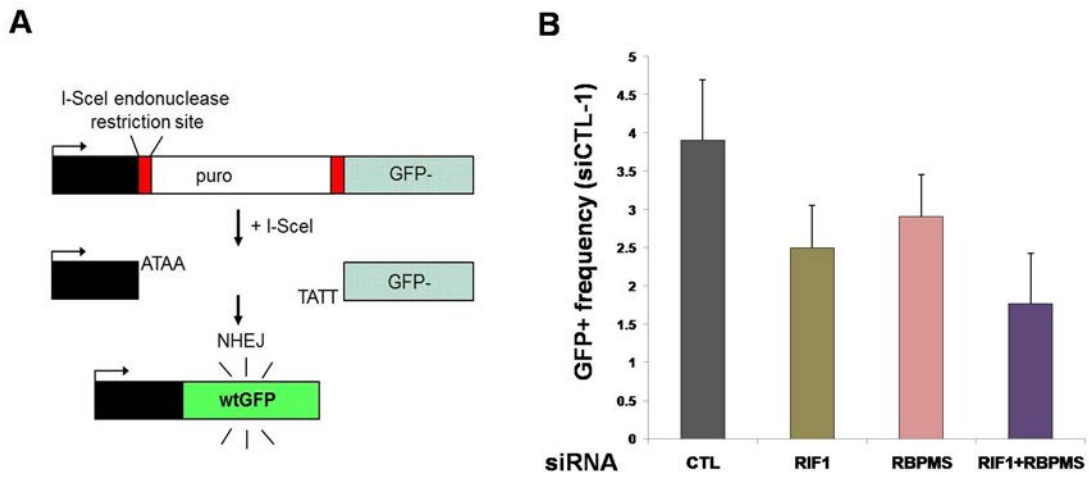
Control- and RBPMS-depleted HeLa cells were untreated or treated with 5 Gy irradiation and were then fixed at the indicated times. **(A)** The cells were stained with an anti-RIF1 antibody and nuclei were stained with DAPI. **(B)** Quantification of RIF1 foci > 10 in control- and RBPMS- depleted cells. **(C)** The cells were stained with an anti-53BP1 antibody and nuclei were stained with DAPI. **(D)** Quantification of cells with 53BP1 foci in nuclei. **(E)** The cells were stained with an anti-BRCA1 antibody and nuclei were stained with DAPI. **(F)** Quantification of cells with BRCA1 foci in nuclei. The histogram shows the number of cells with foci. Results are shown as mean ±SD (n=3).

5. RBPMS enhances Non-homologous end joining.

DNA double strand breaks (DSBs) must be repaired accurately to maintain genomic stability. Mammalian cells have usually two DNA repair pathway, named by NHEJ or HR. Based on previous studies, we confirmed that RBPMS promoted DSB repair. Now, we will explain the two routes in detail with RBPMS. To determine the involvement of RBPMS in Non-homologous end joining (NHEJ) mediated repair, we used EJ5-GFP cells, which contain an promoter that is separated from a GFP coding cassette by a puro gene that is flanked by two I-SceI sites that are in the same orientation (Figure 5A). The DSBs are caused by two I-SceI sites, resulting in DNA damage is repaired by NHEJ, the puro gene is excised. And then, the promoter is joined to the rest of the expression cassette, leading to restoration of the GFP+ gene. Because the two I-SceI possess complementary 3′ overhangs, I-SceI site could be restored by NHEJ. Also, a reconstructed I-SceI sites were also able to be re-cutted and restored to result in an

I-SceI resistant site [18]. We discovered that an RBPMS knockdown resulted to decreased NHEJ, and these effects were more declined with double- knockdown of RIF1 and RBPMS. As positive control, the absence of RIF1 reduced NHEJ (Figure 5B and 5C). Thus, our findings suggest that RBPMS promotes NHEJ through interaction of RIF1.

Figure 5



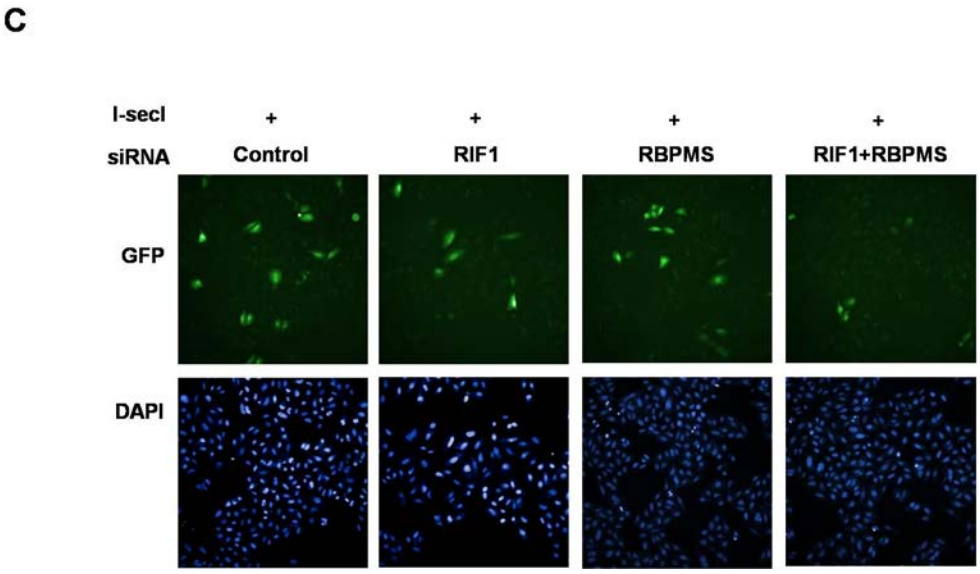


Figure 5. Absence of RBPMS reduces the activity of NHEJ.

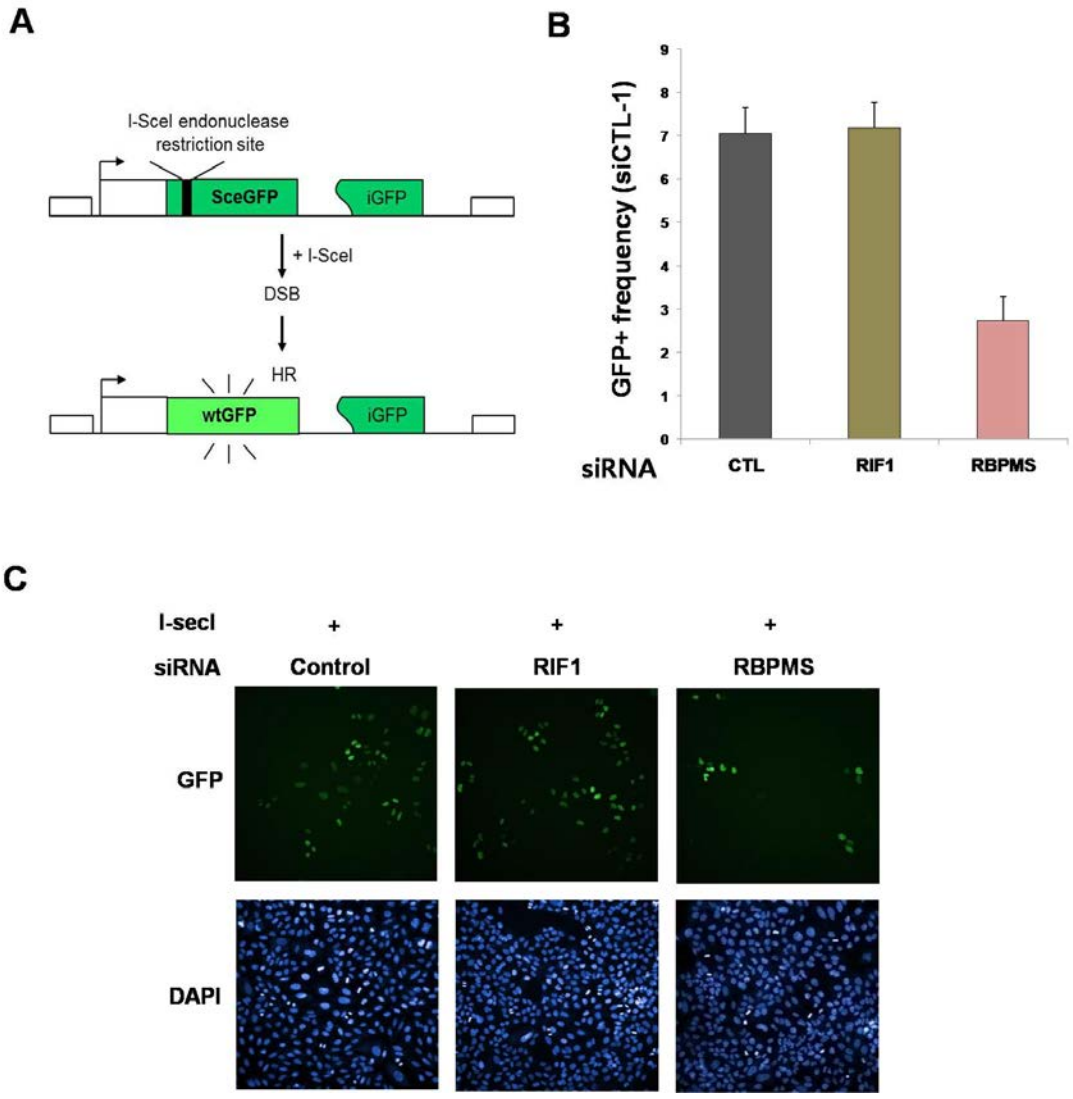
(A) A diagram for the NHEJ assay based on the EJ5-GFP reporter, which contains two tandem endonuclease cut sites for the I-SceI. EJ5-GFP contains a promoter that is separated from a GFP coding cassette by a puro gene that is flanked by two I-SceI sites in the same orientation. Once the puromycin gene is excised by the two I-SceI induced DSBs, the promoter is joined to the rest of the expression cassette by NHEJ repair, leading to restoration of the GFP+ gene. (B), (C) HeLa EJ5-GFP cells were transfected with control, RIF1, RBPMS , both RIF1 and RBPMS siRNA for 5 hours and then transfected with an I-SceI. After 43 hours, the population of the GFP-positive cells was measured by IN Cell Analyzer 2500 HS. The percentage of GFP expressing cells determined. Results are shown as means ±SD (n=3).

6. RBPMS-depleted cells reduce in homologous recombination activity and RAD 51 foci formation.

To explain the role of RBPMS in the Homologous recombination(HR), we performed HDR assay. DR-GFP was made using the homology-directed repair product that used iGFP (intense GFP) as DNA synthesis template, which re-stores of the GFP expression cassette. In the DR-GFP reporter strain, DSBs produced by I-SceI, and cuts specific recognition sites located in the GFP gene (Figure 6A). In this experiment, analysis of repair efficiency through HR is measured by using the % of cells expressing GFP. Notably, we discovered that RBPMS-deficient cells were also decreased in HR repair (Figure 6B and 6C). For confirm HR, we observed to RAD51 foci formation. RAD51 is DNA-binding proteins that help maintain genome stability during DNA replication. Also, RAD51 binds both ssDNA and dsDNA, and is best known for its actions in catalyzing strand invasion during double strand break (DSB) repair by homologous recom-

bination (HR). In addition, RAD51 has essential functions at replication forks, including in regulating fork reversal, that are genetically separable from DSB repair [19]. Depletion of RBPMS impaired the recruitment of RAD51 at DSB site (Figure 6D and 6E). In conclusion, RBPMS-depleted cells were reduced HR as well as NHEJ. This suggests the possibility that RBPMS can regulate HR by unknown mechanism.

Figure 6



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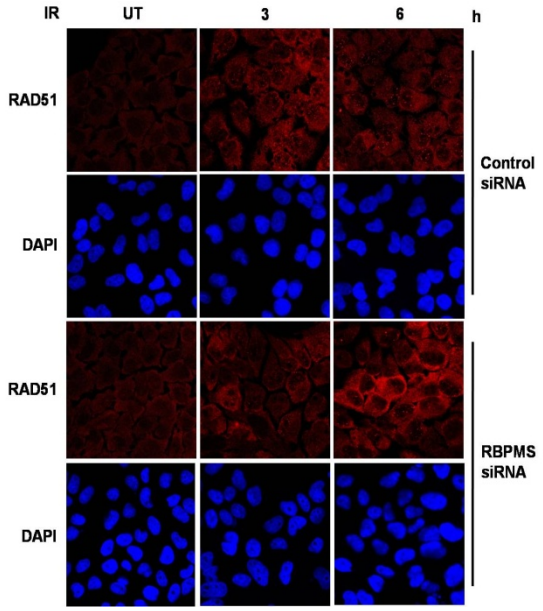

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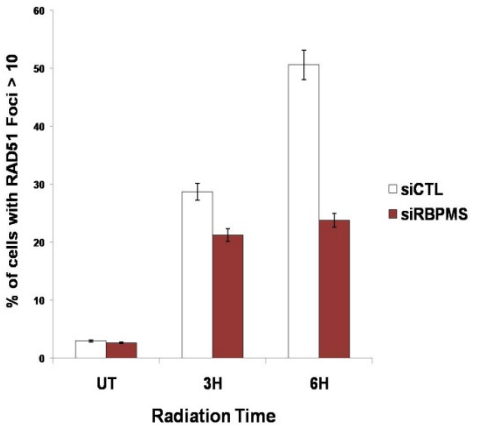

Figure 6. Depletion of RBPMS impairs HR.

(A) DR-GFP is shown along with the HDR product that uses iGFP as the tem-plate for nascent DNA synthesis, which results in the restoration of a GFP ex-pression cassette. (B), (C) The efficiency of HR was measured in U2OS cells that contained DR-GFP and had been transfected with control, RIF1 and RBPMS siRNA for 5 hours and then transfected with an I-SceI. After 48 hours, the population of the GFP-positive cells was measured by IN Cell Analyzer 2500 HS. Quantification of cells with GFP expression in control, RIF1, RBPMS siRNA transfected cells. The percentage of GFP expressing cells determined. Results are shown as means ±SD (n=3). (D) Control- and RBPMS-depleted HeLa cells were untreated or treated with 5 Gy IR to make DSBs and were then fixed at the indicated times. The cells were stained with an anti-RAD51 antibody and nuclei were stained with DAPI. (E) Quantification of cells with foci in nuclei.

Figure 7

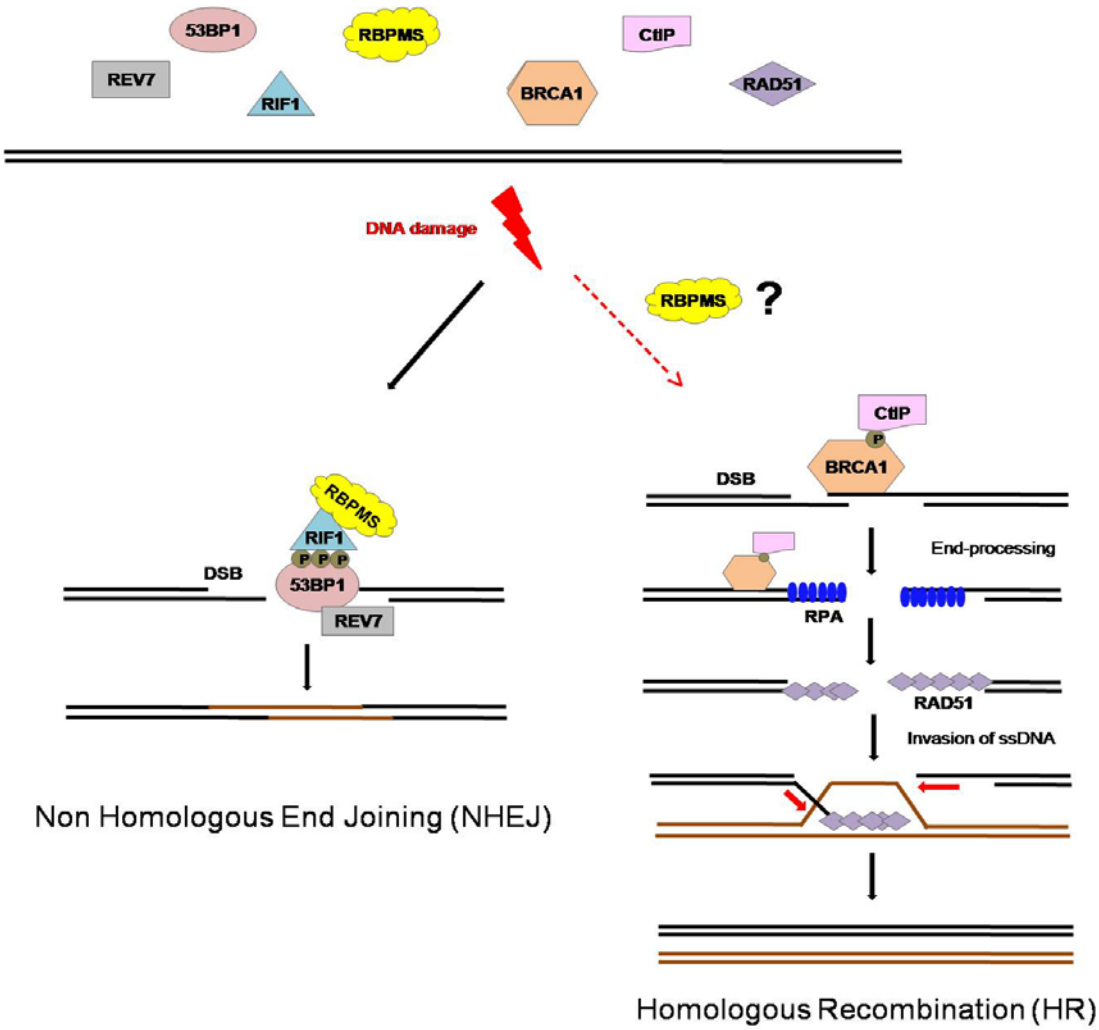

Figure 7. A schematic representation of the role of RBPMS in regulating the DSB repair is shown.

See Discussion for Details.

DISCUSSION

DNA double-strand breaks (DSBs) are the most dangerous type of DNA damage because they can result in the loss of large chromosomal regions [3]. Also, DSBs occur to the genomic instability, and induce cancer. So, DSBs must be repaired for tumor suppression. In mammalian cells, DSBs are repaired by the non-homologous end joining (NHEJ) and homologous recombination (HR). DSBs end structure is important factor affecting the two repair pathway. For example, one-ended DSBs in S phase are mainly repaired by HR because of the lack of a partner DSB end for NHEJ. On the other hand, two-ended DSBs, which are mainly induced by ionizing radiation, are repaired by either NHEJ or HR in G2 cells [20]. Because NHEJ uses proteins that recognize, resect, polymerize and directly ligates together with the resulting in loss of genetic information [3, 4]. The contrary, HR utilizes homolog sequences on the sister chromatid [21], has another name called error-free repair.

In this study, we discovered that RBPMS is a novel binding partner of RIF1

by yeast two-hybrid screening. We confirmed that RBPMS interacts with RIF1 through proximity ligation assay (PLA) and endogenous co-immunoprecipitation assay. Endogenous RIF1 and RBPMS interacted with each other and that this interaction increased upon exposure to IR irradiation. To determine whether RBPMS is involved in the DNA damage response, we exposed control and RBPMS knockdown HeLa cells to IR, HU, MMC. As a result, RBPMS-depleted cells were hypersensitive to IR, which affected reduction of colony formation as detected by colony survival assay. Also, RBPMS-depleted cells resulted in damage of DNA repair to IR, as detected by late γ-H2AX staining. Based on this research, we propose that RBPMS is involved in DSB repair. Next, we investigated that foci formation of RIF1 and RIF1-related damage protein in RBPMS-depleted cells compared to control cells by using IF assay. As a result, knockdown of RBPMS reduced IR-induced RIF1 foci, but 53BP1 foci were still remained in RBPMS-depleted cells. So, we confirmed BRCA1 foci formation,

which represents the opposite protein of 53BP1 in DSBs repair pathway. However, BRCA1 foci formation was not affected by the presence or absence RBPMS. Next, we discovered that absence of RBPMS impaired NHEJ repair activity through EJ5-GFP vector system. Therefore, we expected that HR would be promoted by depletion of RBPMS, and be increased RAD51 foci formation. To investigate this hypothesis, we measured cells with GFP expression by DR-GFP vector system. However, both HR and RAD51 foci formation did not enhance. Thus, we proposed that both repair activity of NHEJ and HR decrease in RBPMS-depleted cells. Maybe, RBPMS regulates HR through unknown mechanism which is independent of RIF1. The model of RBPMS function was suggested in Figure 7.

RNA-binding protein with multiple splicing (RBPMS) is mRNA processing factor, is implicated as the retinal ganglion cell, heart, oocyte and gastrointestinal smooth muscle development. These RNA-binding proteins contain a single RNA

recognition motif (RRM), and their targets and molecular function have not yet been identified [22]. Also, RBPMS is expressed as multiple alternatively spliced transcripts encoding different protein isoforms [14]. Another studies revealed that this factor has been shown to physically interact with Smads and enhance transforming growth factor-β (TGF-β)-mediated Smad2/3 transcriptional activity in mammalian cells. TGF-β superfamily includes BMPs, GDFs, GDNFs, multifunctionally regulates a wide range of biological processes, such as morphogenesis, embryonic development, adult stem cell differentiation, immune regulation, wound healing, inflammation, and cancer [15, 16]. However, little is known about DNA-damage response. Here, we have identified RBPMS as a novel binding partner of RIF1 in DNA damage response (DDR).

However, we still wondered that why both NHEJ and HR activities decreased in RBPMS-depleted cells. In general, these two pathways are known to play an antagonistic role and act like switches. But ironically, our data were not. In addi-

tion, deficiency of RBPMS reduced RAD51 recruitment after IR. It is unclear how RBPMS regulates HR repair pathway. However, we found that RBPMS regulated RIF1 foci formation but not 53BP1 and BRCA1 after IR. This is the first report that RBPMS increase NHEJ through binding of RIF1. As we said before, regulatory mechanism of RBPMS-related HR remains elusive. Therefore, we must have done to further demonstrate with detailed mechanism of RBPMS. First, we will perform to pull-down assay of RBPMS for finding of the RBPMS-binding partner which is related HR or DNA damage response. Moreover, we will research for RBPMS binding site of RIF1, and RIF1-binding site of RBPMS using deletion constructs. More further studies are needed to explain the mechanism of RBPMS in DNA damage response.

In summary, we have identified the novel mechanism of RBPMS-related DNA double strand breaks repair. We showed that defect of RBPMS resulted in cellular hypersensitivity and inhibited DSBs repair to IR. In addition, we showed

that knockdown of RBPMS affected several foci recruitments after irradiation. Finally, we found that RBPMS regulates DSB repairs. These results suggest a novel role of RBPMS in DSB repair through increase of NHEJ by interaction of RIF1.

ABSTRACT

Regulatory mechanism of RIF1 through interaction with RBPMS

Ha-Yeon Choi

Advisor: Prof. Jung-Hee Lee, Ph.D.

Department of Biomedical sciences,

Graduate school of Chosun University

RIF1 is downstream of 53BP1, promotes non-homologous end-joining (NHEJ) repair at DSBs (double strand breaks). In this study, we identify the RBPMS, is known RNA binding protein and mRNA processing factor, as a novel binding partner of RIF1 by yeast two-hybrid assay. RBPMS has been shown to selective marker of ganglion cells in the mammalian retina, but its function re-mains unknown in DNA damage response (DDR). We showed that RBPMS en-hanced its binding partner to RIF1 after ionizing radiation (IR). Depletion of

RBPMS was resulted in cellular hypersensitivity and impaired DNA damage re-pair to IR, as detected by colony survival assay and late γ-H2AX staining. RBPMS-depleted cells were decreased the RIF1 foci to IR. However, we showed that a 53BP1 and BRCA1 damage focus are not significantly affected in RBPMS absence or presence condition. Furthermore, depletion of RBPMS was shown the decrease of non-homologous end-joining (NHEJ) and homologous-recombination (HR) repair activities. In addition, RAD51 foci were declined in deficient RBPMS cells. Thus, our combined results suggest a novel role for RBPMS in DSB repair through increase of NHEJ by association with RIF1.

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