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The Transcription factor USF2: A Master regulator of DNA damage response

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지도교수 이 정 희

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의과학과

서 관 우

서관우의 석사학위논문을 인준함

위원장 조선대학교 교 수 장인엽 (인)

위 원 조선대학교 교 수 유호진 (인)

위 원 조선대학교 조교수 이정희 (인)

2017년 11월

조선대학교 대학원

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국문초록

DNA 손상반응의 새로운 조절자로서의 USF2 기전연구

서 관 우

지도교수: 이 정 희

조선대학교 일반대학원

의과학과

DNA 손상반응에 주요 단백질 중 하나인 BRCA1은 DNA가 손상되었을 때 손상인식, 복구 및 세포주기 조절에 관여하고, 특히 상동재조합 활성에 중요한 단백질로 알려져 있다. 하지만 DNA 손상 반응에서 BRCA1의 자세한 작용기전은 여전이 더 연구되어야 할 부분이 많다. 본 연구에는 BRCA1과 결합한다고 알려진 USF2를 동정하였다. 전사인자로 알려진 USF2 단백질은 USF1과 이형 중합체를 이루고 있다. 그리고 USF2는 나선고리나선(helix-loop-helix)구조를 가지고 있으며, E-box라고 불리는

DNA의 CACGTG 염기서열을 인식하여 해당 유전자의 전사를 시작한다. 이과 같은 방법으로 USF2는 많은 유전자의 전사에 관여한다고 보고되어있다. 또한 USF2는 BRCA1과 결합하여 전사활성을 조절한다고 알려져 있지만 BRCA1과 연관하여 DNA 손상반응에의 연관성 연구는 전무한 실정이다. 따라서 본 연구에서 우리는 BRCA1과 결합을 통한 DNA 손상반응에서 USF2의 역할을 규명하고자 한다. USF2는 BRCA1과 결합한 다는 것은 이미 보고되어 있으며, 세포에 방사선 조사로 DNA 손상을 유도하였을 때 USF2와 BRCA1의 결합이 증가함을 확인하였다. clonal survival, 상동재조합, 비 상동 말단 결합 활성 분석법 등을 통해 USF2가 결여된 세포에서 DNA 손상 복구 활성이 떨어짐을 확인하였다. USF2는 핵 내에서 손상된 DNA가 있는 곳으로 이동하여 세포주기에 관계없이 DNA 손상 foci를 형성하였다. 게다가, DNA 손상반응 기전에서 USF2는 ATM과 ATR kinase의 하위조절자로써 작용하여, RBCA1, BARD1, Rad51에 의한 상동재결합 활성을 조절함을 밝혔다. 또한 USF2는 BRCA1의 RING domain 부위에서 결합함을 보였다. 이상의 연구결과를 토대로 BRCA1에 의한 새로운 DNA 손상 복구 기전을 규명함과 동시에 DNA 손상반응의 새로운 조절자로써 USF2의 가능성을 제시한다.

INTRODUCTION

The genome is always under the genotoxic risks by both endogenous and exogenous factor. These directly or indirectly cause DNA lesion, then can induce several DNA structural changes such as oxidation, depurination, depyrimidination, single strand breaks (SSB) or double strand breaks (DSB). SSBs or UV-induced DNA damage is repaired by base-excision repair (BER), and nucleotide repair (NER), mismatch repair (MMR) pathway, whereas DSBs are repaired by homologous recombination (HR), and non-homologous end joining (NHEJ). The most dangerous DNA damage is the DSBs because of the possibility to cause mutations, cancer, extensive structural rearrangement of the genome, acceleration of aging and immune deficiency. Because of these reasons, DNA damage response and repair are very important [1-4].

The DNA damage response is signaling cascades involving the orchestration of a variety of cellular events and these cascades involve a number of factors that facilitate DNA repair and damage checkpoint. The DDR at DSBs begins with the activation of two specific kinases, the ataxia telangiectasia mutated (ATM) kinase and the ataxia telangiectasia and rad3-related protein (ATR) kinase.

They promote DNA repair process and arbitrate in either apoptosis or checkpoint activation through p53-mediated mechanisms [5]. ATM and ATR kinases are on top of the DNA damage response pathway. These phosphorylate Ser/Thr-Glu (S/TQ) site of many proteins [6]. ATM and ATR phosphorylate H2AX at Ser 139 [7]. The mediator of the DNA damage checkpoint protein 1 (MDC1) binds phosphorylated H2AX(γ -H2AX), which is crucial to recruitment DNA damage or repair proteins [4]. The phosphorylated MDC1 triggers to recruit the ring finger protein 8 (RNF8) and the ring finger protein 168 (RNF168), the E3 ubiquitin ligase. RNF8, linked with the E2 conjugating enzyme UBC13, adds its lysine (K) 63-linked ubiquitin chain to histone H1 [8, 9]. Then, ubiquitinated H1 by RNF8 recruits RNF 168 to ubiquitinated H1 at DSB and subsequently, RNF168 ubiquitinates histone H2A on K15. Next, 53BP1 and BRCA1 are recruited to DNA damage sites, at the G1 or S/G2 phase cells, respectively. Finally, 53BP1 and BRCA1 function as an assembly platform to assist localize repair factors at DSB sites [10].

The choice between HR and NHEJ is dependent on cell cycle. NHEJ repairs DSBs throughout the cell cycle, whereas HR functions in S and G2 phase cells. When there are homolog sequences on the sister chromatid, HR is performed in cells to repair DSBs in a cyclin-dependent kinase (CDK)-

dependent manner. However, when the sister chromatid is absent, NHEJ mainly occurs [11, 12]. The regulation of every repair pathways is required to minimize genomic instability. The first regulation for DNA repair pathway choice is the processing of the breaks. DNA end resection inhibits NHEJ and permits the other pathways. Thus, DNA end resection is primarily considered DSB repair pathway choice. DNA end resection occurs in two phases that are a slow initial phase, catalyzed by MRN complex, and fast phase, catalyzed by either the exonuclease Exo1 or the helicase Bloom Syndrome Protein (BLM). To initiate the step of DNA resection, the form of the phosphorylation of CtIP is essential. Then it stimulates MRE11 endonuclease activity [13]. In addition, p-53-binding protein 1 (53BP1) and its effector proteins block DNA resection. So, repositioning of 53BP1 is also important for promoting DNA resection [14]. Breast and ovarian cancer susceptibility protein 1 (BRCA1) catalyzes de-phosphorylation and reposition of 53BP1, then reposition 53BP1 [12] fertilizes HR. Consequently, 53BP1 and BRCA1 play antagonizing roles in DSB repairs.

BRCA1 is already reported to the key regulator in HR and interact with BARD1 at its RING domain [15]. BRCA1 also interacts with CtIP and BRCA2. The interaction with CtIP involved in DNA end resection and interaction with BRCA2 through PALB2 are associated with recruitment of

RAD51 to DSBs [16]. First, BRCA1, through interaction with CtIP, promote end resection [12]. BRCA1-CtIP complex replaces 53BP1, and initiates DNA end resection [17]. End resection is progressed in three step, as initiation, elongation, and extension step. In initiation step, Meiotic recombination 11 homolog A (MRE11) endonuclease initiates through interaction with CtIP. Then DNA lesion is elongated by an MRE11 exonuclease. Finally, exonuclease 1 (EXO1) or Bloom Syndrome RecQ helicase (BLM) and DNA replication helicase/nuclease 2 (DNA2) extend the length of 3' overhang [18-20]. During end resection, replication protein A (RPA) bind to single strand DNA (ssDNA) [17]. Next, RPA, bound to ssDNA, is replaced by RAD51 [21]. For recruitment of RAD51, recruitment of BRCA2-PALB2 complex has to be recruited to DSBs [22], because RAD51 interacts with BRCA2 [23]. BRCA2-PARB2 complex contributes to form RAD51 filament and BRCA1 recruits BRCA2-PALB2 complex, interacted with RAD51, to DSBs site [22]. Then, damaged DNA is repaired. In summary, through interaction with BARD1, BRCA2, and CtIP, BRCA1 contributes to reposition of 53BP1, promoting end resection, and recruitment of RAD51 [12, 14].

Upstream stimulatory factor 2 (USF2) is a transcription factor, ubiquitously expressed, and

mainly forms a heterodimer with USF1 [24]. USF2, has a basic helix-loop-helix leucine zipper domain, recognizes CACGTG sequence called enhancer-box (E-box), and regulates expression through binding E-boxes of target genes [25]. All USF bind to specific DNA and recognize E boxes that resemble binding site of c-Myc. However, the function between USF and c-Myc is very different in cellular proliferation. The ability of USF2 in many cancer cell lines is impaired or lost, but c-Myc, known proto-oncogene, is not. They suggest that USF2, antagonized c-Myc, is involved in tumor suppression [26, 27]. Furthermore, USF2 has reported it is very important for the embryonic development, brain function, metabolism, iron homeostasis, fertility, and growth, but USF1 has metabolic function [28]. Though it is known that USF2 interacts with BRCA1 [29], its function in DNA-damage response (DDR) have not been identified.

MATERIALS AND METHODS

1. Cell culture and treatment

HeLa and HEK293T cells were purchased from ATCC. They were cultured in Dulbecco's modified Eagles' medium (DMEM) supplemented with 10% fetal bovine serum and streptomycin (0.1 mg/ml), penicillin (100units/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 0.5 % trypsin-EDTA before being passaged. Cells in exponential growth were harvested for subsequent experiments. To induce DNA double-strand breaks, exponentially growing cells were irradiated at 10 Gy from ^{137}Cs source (Gamma cell 3000 Elan irradiator, Best Theratronics) and allowed to recover at 37 °C incubator for various times.

2. siRNA transfection

HeLa and HEK293T cells were transfected with siRNA oligonucleotide duplexes against USF2,

ATM, ATR, and BRCA1 using Turbofect (Thermo Fisher scientific) according to the manufacturer's instruction. The siRNA sequences targeting USF2 (USF2 siRNA #1: 5'-TCCAGACTGTAAC-GCAGACAA-3', USF2 siRNA #2: 5'-GACACACCCTTACTCTCCAAA-3', USF2 siRNA #3: 5'-TCCTCCACTTGGAAACGGTAT-3'), ATM (ATM siRNA : 5'-UUCUCUUGCAAUCUCAU-CAGGACGC-3'), ATR (ATR siRNA : 5'-AAGACGGTGTGCTCATGCGGC-3'), and BRCA1 (BRCA1 siRNA : 5'-UCACAGUGUCCUUUAUGUA-3') designed and synthesized for transient transfection.

3. Immunoprecipitation assay

The whole cell lysates prepared by extracting with MILD lysis buffer (20mM HEPES (PH 7.4), 2mM EGTA, 50mM β-Glycerol phosphate, 1% Triton X-100, 10% Glycerol, and 1mM DTT) with protease inhibitors (Roche Diagnostic Corp.) The lysates were added to the anti-USF2 (Abcam), BRCA1 (Santa Cruz), HA (Santa Cruz), or GFP(Santa Cruz) antibodies at 4℃ for 24 hours. And then, protein A/G plus-agarose beads(Santa Cruz Biotechnology), G-sepharose and A-sepharose(GE Healthcare) were added to the lysates, and beads mixtures were incubated at 4℃ for 4hours with

shaking. The beads were washed five times in MILD lysis buffer without protease inhibitors, resuspended in equal volume 2X SDS sample buffer. The samples were extracted from the bead by boiling at 95℃ for 5 min. The samples were then analyzed by western blotting using the appropriate antibodies.

4. Western blot analysis

Cells were lysed in NP-40 lysis buffer (50mM Tris–HCl (pH 8.0), 150mM NaCl, 1% NP-40, 10mM NaF, 1mM Na_3VO_4, 5mM EDTA, and 1mM EGTA) with protease inhibitors (Roche Diagnostic Corp.). Cell lysates were collected by centrifugation at 12,000rpm for 30min. Protein concentrations were measured using the Bradford assay (Bio-Rad). Equal amounts of protein were separated by 6–15% SDS–PAGE followed by electrotransfer onto a polyvinylidene difluoride membrane (Millipore, Bedford, MA, USA). The membranes were blocked for 1 hours with TBS-T (10mM Tris-HCl (pH 7.4), 150mM NaCl and 0.1% Tween-20) containing 5% skim milk and then incubated at 4℃ with primary antibodies(1:1000). The blots were washed six times for 10min 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 with 10min and developed using an enhanced chemiluminescence detection system (ECL; GE Healthcare, Buckinghamshire, UK).

5. Antibodies

We used the following primary antibodies: Rabbit polyclonal anti-USF2 (sc-862, Santa Cruz Biotechnology), rabbit polyclonal anti-USF2 (ab125184, Abcam), mouse monoclonal anti-BRCA1 (sc-6954, Santa Cruz Biotechnology), rabbit polyclonal anti-BRCA1 (sc642, Santa Cruz Biotechnology), rabbit polyclonal anti-BARD1 (sc-11438, Santa Cruz Biotechnology), Goat polyclonal anti-ATR (sc-1887, Santa Cruz Biotechnology), mouse monoclonal anti-ATM (LF-MA0246, Abfrontier), monoclonal anti-Cyclin A (sc-271645, Santa Cruz Biotechnology), rabbit polyclonal anti-RAD51 (sc-83495, Santa Cruz Biotechnology), mouse monoclonal anti-γ-H2AX (05-636, Millipore), mouse monoclonal anti-β-actin (sc-47778, Santa Cruz Biotechnology), monoclonal anti-GFP (sc-9996, Santa Cruz Biotechnology), rabbit polyclonal anti-GFP (sc-8334, Santa Cruz Biotechnology), mouse monoclonal anti-HA(sc-7392, Santa Cruz Biotechnology), and rabbit polyclonal anti-HA (600-401-384, Rockland Immunochemicals) antibodies.

6. Clonal survival assay

After treatment with IR, $1x10^3$ cells were immediately seeded onto a 60mm dish in duplicate and grown for 2-3weeks at 37℃ to allow colony formation. Colonies were fixed with 95% methanol for 10min and stained with 1% methylene blue in 20% ethanol and counted. The fraction of surviving cells was calculated as the ratio of the plating efficiencies of treated cells to untreated cells.

7. Immunofluorescence microscopy

To visualize nuclear foci, cells were grown on glass coverslips and were irradiated with 10 Gy of ionizing radiation (IR). Cells were then washed twice with 0.01M PBS, fixed with 4% paraform-aldehyde for 10 min and ice-cold 98% methanol for 5 min, followed by permeabilization with 0.5% Triton X-100 for 15 min at room temperature. Next, the coverslips were washed three times with 0.01M PBS and then blocked with 2% BSA in 0.01M PBS for 1hrs. The cells were single or double immunostained with primary antibodies against various proteins overnight at 4°C. Next, the cells were washed with 0.01M 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.

8. Non-homologous end joining assay

To measure the NHEJ repair, stable cells lines expressing HeLa EJ5-GFP reports 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. After 48hours, the percentage of GFP-positive cells which had repaired the DSBs generated by I-SecI was determined by flow cytometry. For each analysis, 10,000 cells were processed and each experiment was repeated three times.

9. Homologous recombination assay

DR-GFP assay: To measure the HR repair, stable cell lines expressing DR-GFP reports 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. HeLa DR-GFP cells were transfected with Control and USF2 siRNA, after 4 hours transfected with 1 μ g of I-SceI–expressing vector. After 48hrs, the percentage of GFP-positive cells which had repaired the DSBs generated by I-SecI was determined by flow cytometry. For each analysis, 10,000 cells were processed and each experiment was repeated three times.

10. 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).

RESULTS

1. USF2 interacts with BRCA1

BRCA1 is the most important key regulator in DDR. However, the role of USF2 in DDR is unknown though it is already known that USF2 interacts with BRCA1 by Cable, P. L. et al [29]. To verify that USF2 interacts with BRCA1 in HeLa cells, and investigate changing interaction between BRCA1 and USF2 after treatment of ionizing radiation (IR), we performed the co-immunoprecipitation assay. HeLa cells were induced DNA damage by IR, then recovered for 3 hours. The two protein interacted each other in undamaged cells, but the interaction between two proteins increased in IR-induced damaged cells (figure 1A, and B). Then, our endogenous interaction data between USF2 and BRCA1 were further confirmed by exogenous co-immunoprecipitation with GFP-USF2 and HA-BRCA1. HEK 293T was transfected with full-sequence GFP-USF2 and HA-BRCA1 vector. Exogenous co-immunoprecipitation assay was performed by using anti-HA antibody and immunoblotting was carried out with an anti-GFP antibody, and reversely. These results were similar to endogenous data (figure 1C). In our data, USF2 typically interacts with BRCA1, and USF2-BRCA1 interactions

were enhanced after treatment of IR. Because USF2 typically interacts with BRCA1, a key regulator of DNA damage response and interaction of two protein increased under IR-induced damage, we inferred that USF2 has an important role in DDR.

Figure 1

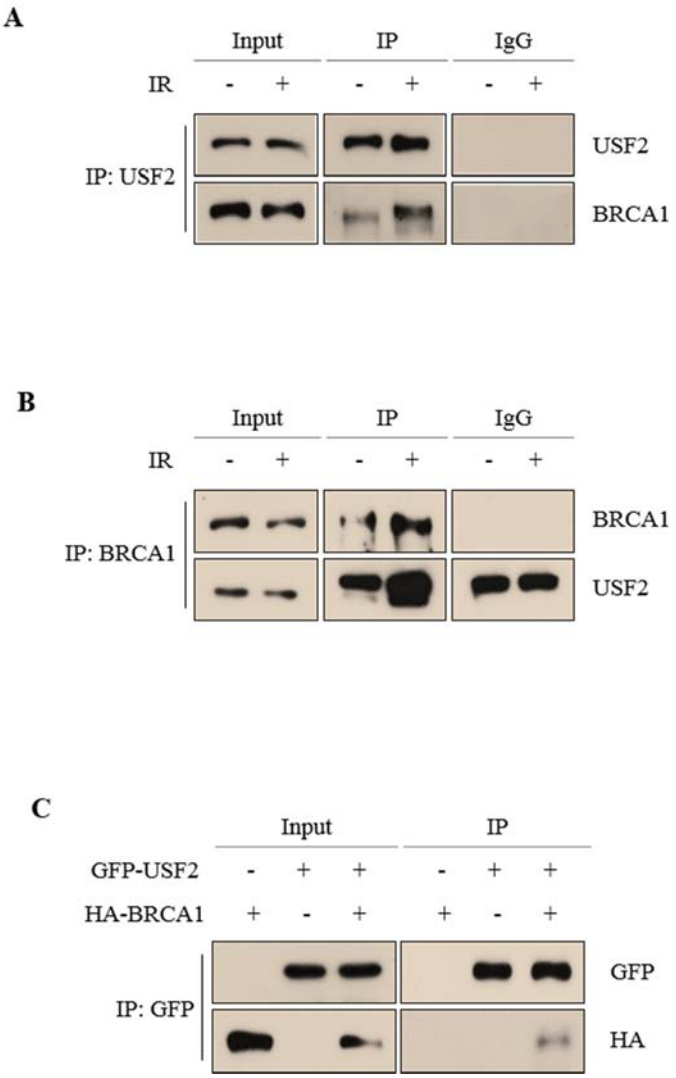

Figure 1. USF2 binds to BRCA1.

(A, and B) Endogenous immunoprecipitation assay (IP) used HeLa cells after 3 hours past treatment of IR-induced DNA damage. IR-induced damaged cells were lysed in the MILD lysis buffer, and total lysates were performed immunoprecipitation using anti-USF2 and BRCA1 antibodies. Normal rabbit and mouse IgG were used for negative control. (C) HEK293T cells were transfected with full sequence GFP-USF2 and HA-BRCA1 expression vectors. The total proteins were subjected to immunoprecipitation using an anti-GFP antibody followed by western blotting using anti-HA or GFP antibodies.

2. Depleted USF2 cells show increased DNA damage sensitivity.

We found that interaction of USF2-BRCA1 increase after IR-induced damage. So, we wondered what will happen to depleted-USF2 cells in DNA damage response. First, we generated three type of siRNA against USF2, recognizes CDS and 3`UTR sequence of USF2-mRNA. We named these siRNA to siUSF2 #1, siUSF2 #2, and siUSF2 #3, respectively (figure 2A). To check how efficient this siRNA interfered expression of USF2 gene, we performed western blot assay using USF2 anti-body. The result showed that the expression of USF2 was reduced by more than 7-80% in USF2 siRNA-treated cells, compared with that of control siRNA-transfected cells. All of USF2 siRNA work efficiently, we used siUSF2 #3 to next experiment. To investigate the potential role of in DDR, we carried out clonal survival assay after IR treatment. The cells, transfected control or USF2 siRNA, were treated with an indicated dose of IR, then monitored for 10 to 14 days. We found that depleted-USF2 cells exhibited decline of colony numbers after treatment with IR, compared to control cells. These data indicated that USF2-knockdown cells are hypersensitive to IR-induced DNA damage (figure 2B, D). Depleted-USF2 cells are exhibited less colony compare to control cells. Therefore, we could suppose that USF2 regulate DNA damage response.

Figure 2

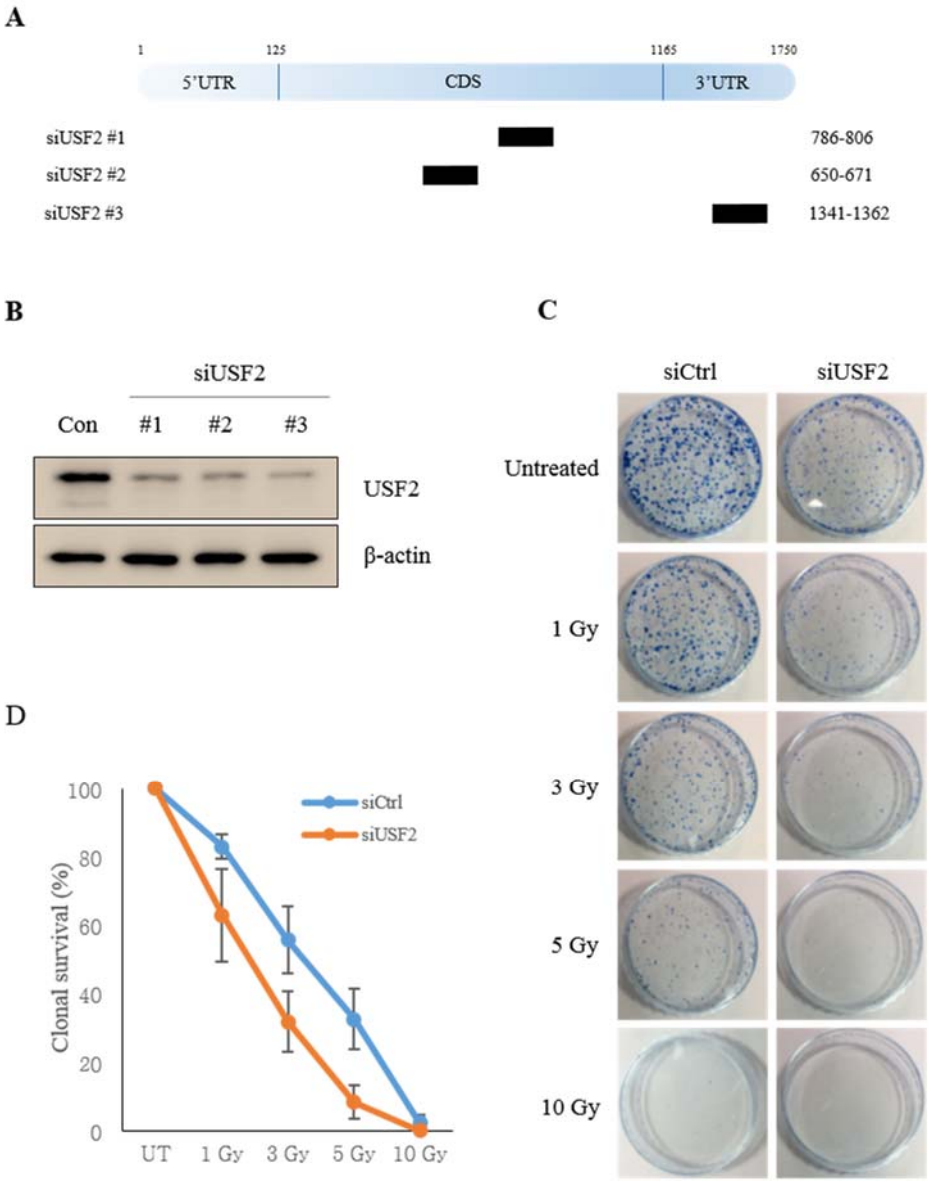

Figure 2 Depletion of USF2 is sensitive to IR.

(A) Schematic diagram of human USF2 siRNA sequences. siUSF2 #1 and #2 recognize CDS sequence, and siUSF2 #3 are recognize 3` UTR sequence of USF2 mRNA. (B) HeLa cells were transfected with siRNA-control and siRNA-USF2 #1, 2, and 3. After 48hrs, the expression level of USF2 was confirmed by western blotting using anti-USF2 antibody. ß-actin was used as loading control. (C and D) USF2 were hypersensitive to IR damage. Control and USF2 depleted Hela cells were untreated or treated with 1, 2, 5 and 10Gy of IR. After 2 weeks, cells were fixed with Methyl alcohol and stained with methylene blue, then the number of surviving colonies were counted. Representative figure(C) and quantification of cell viability (D). The cell viability of untreated cells is defined as 100%.

3. USF2-depleted cells are defective in HR and NHEJ repair

We found that USF2 are hypersensitivity to IR, which makes DSBs of DNA. DSBs of DNA are repaired in two pathway, HR or NHEJ. To estimate the involvement of USF2 in HR and NHEJ repair, we used DR-GFP cells and EJ5-GFP cells, respectively. EJ5-GFP cells contain 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 flanked 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. In this system repair via NHEJ is monitored using flow cytometry to measure the percentage of cells expressing GFP (Figure 3A). We found that depleted-USF2 was lowered NHEJ by 60% in HeLa EJ5-GFP cells (Figure 3B and C). Next, DR-GFP was constructed using the homology-directed repair (DRD) product that used intense GFP (iGFP) as the template for nascent DNA synthesis, which results in the restoration of a GFP expression cassette (figure 3D). Notably, we found that in depleted-USF2 cells, the level of HR repair was also decreased (Figure 3E and F). Taken together, USF2 regulates HR and NHEJ repair.

Figure 3

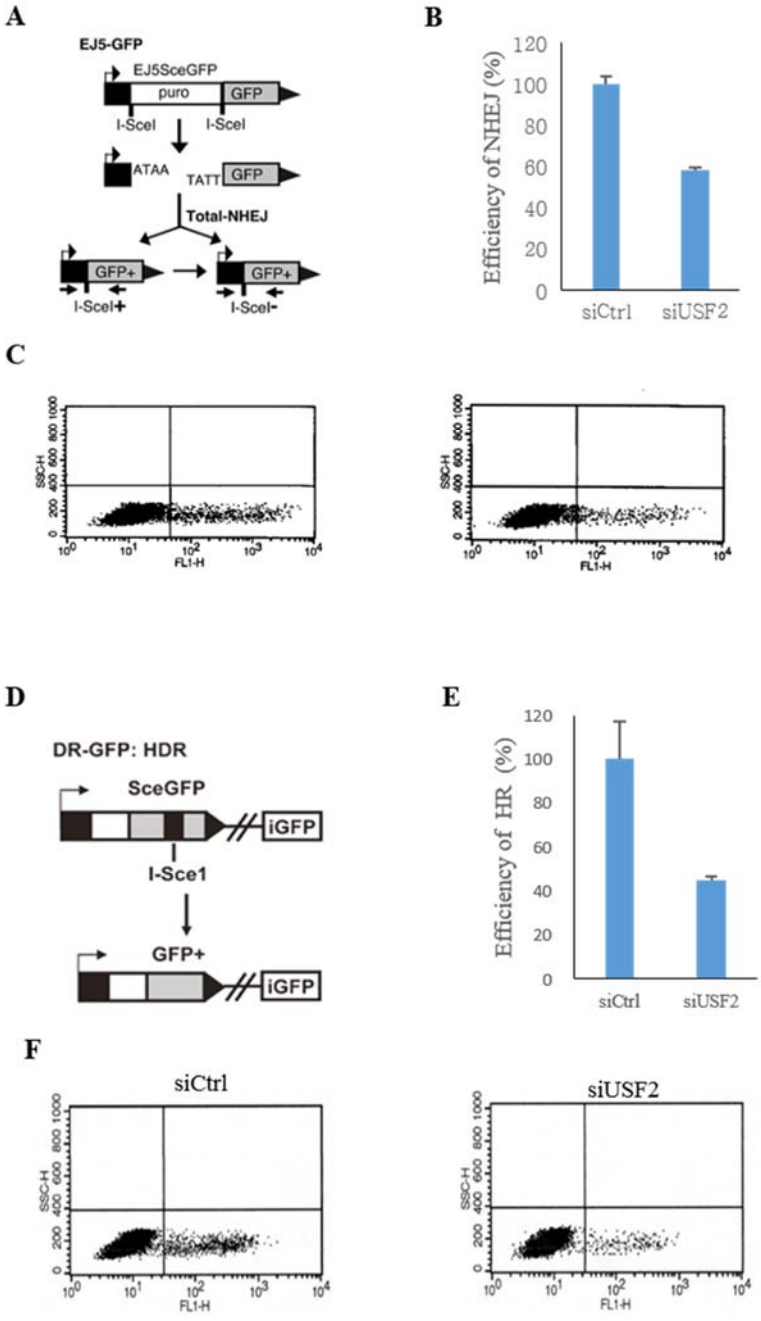

Figure 3. Depletion of USF2 impairs HR and NHEJ repair

(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 and C) EJ5-GFP HeLa cells were transfected with Control, USF2 siRNA for 4hours and then transfected with an I-SceI expression vector. After 48hours, the population of the GFP-positive cells was measured by flow cytometry (B). Quantification of cells with GFP expression in Control, USF2 siRNA cells. The percentage of GFP expressing cells determined (C). (D) DR-GFP is shown along with the HDR product that uses iGFP as the template for nascent DNA synthesis, which results in the restoration of a GFP expression cassette. (E and F) The efficiency of HR was measured in HeLa cells that contained DR-GFP and had been transfected with either control, USF2 siRNA. When the DSB is repaired, the reporter construct will then express GFP that can be measured by flow cytometry. Quantification of

cells with GFP expression in Control, USF2 siRNA-transfected cells. The % of GFP expressing cells determined. Results are shown as means ±SD (n=3).

4. USF2 forms DNA damage foci at G1 and S/G2 phase cells.

We found that potential role of USF2 should be associated with DSBs repair through clonal survival assay, interaction with BRCA1, and decreasing HR, and NHEJ. We wondered that USF2 are recruited to damage site. To investigate that USF2 can form foci at DNA lesion, we carried out IF assay. The cells were used for IF at the indicated time after IR damage. USF2 foci primarily appeared at 30 minutes after IR treatment and the cells, including USF2 foci in Nuclei, increased until 3 hours after IR treatment. Then, USF2 foci-included cells decreased (figure 4A and B). USF2 foci appeared at 30 minutes after IR treatment was a quite interesting event. Namely, USF2 played a role at the early DNA damage response. In contrast, the level of USF2 protein expression showed constant at the time points of IR damage (figure 4C). This supposed that USF2 may regulate many functions of other protein in DDR. We found that USF2 formed foci after IR treatment, but we needed to confirm that USF2 foci really formed as DNA-damage response protein at DNA lesion. To confirm that USF2 are co-localized with γ-H2AX, as DNA damage marker, at DNA lesion, we stained with anti-USF2 and γ-H2AX antibodies. This resulted in USF2 are co-localized with γ-H2AX. Therefore, we confirmed that USF2 foci are real foci by IR-induced DNA double-strand breaks (figure 4D, E).

NHEJ-related protein foci, such as RIF1 and 53BP1, was found throughout the cell cycle, and HR-related protein foci, such as BRCA1, CtIP, and RAD51, was found in the G2/S phase of cell cycle. We observed that USF2 foci was detected in most cells. We supposed that USF2 are associated with G1 and S/G2 phase cell. To more detailed address IR-induced USF2 foci at the stage of the cell cycle, we co-stained the IR-treated cells with USF2 antibody and markers for S/G2 phase, cyclin A antibody. This signified that USF2 forms foci at G1 and S/G2 phase cells (figure 4F). Base on this data, we suggest that USF2 are accumulated to DNA lesion from early time after IR treatment and USF2 play a role throughout the cell cycle.

Figure 4

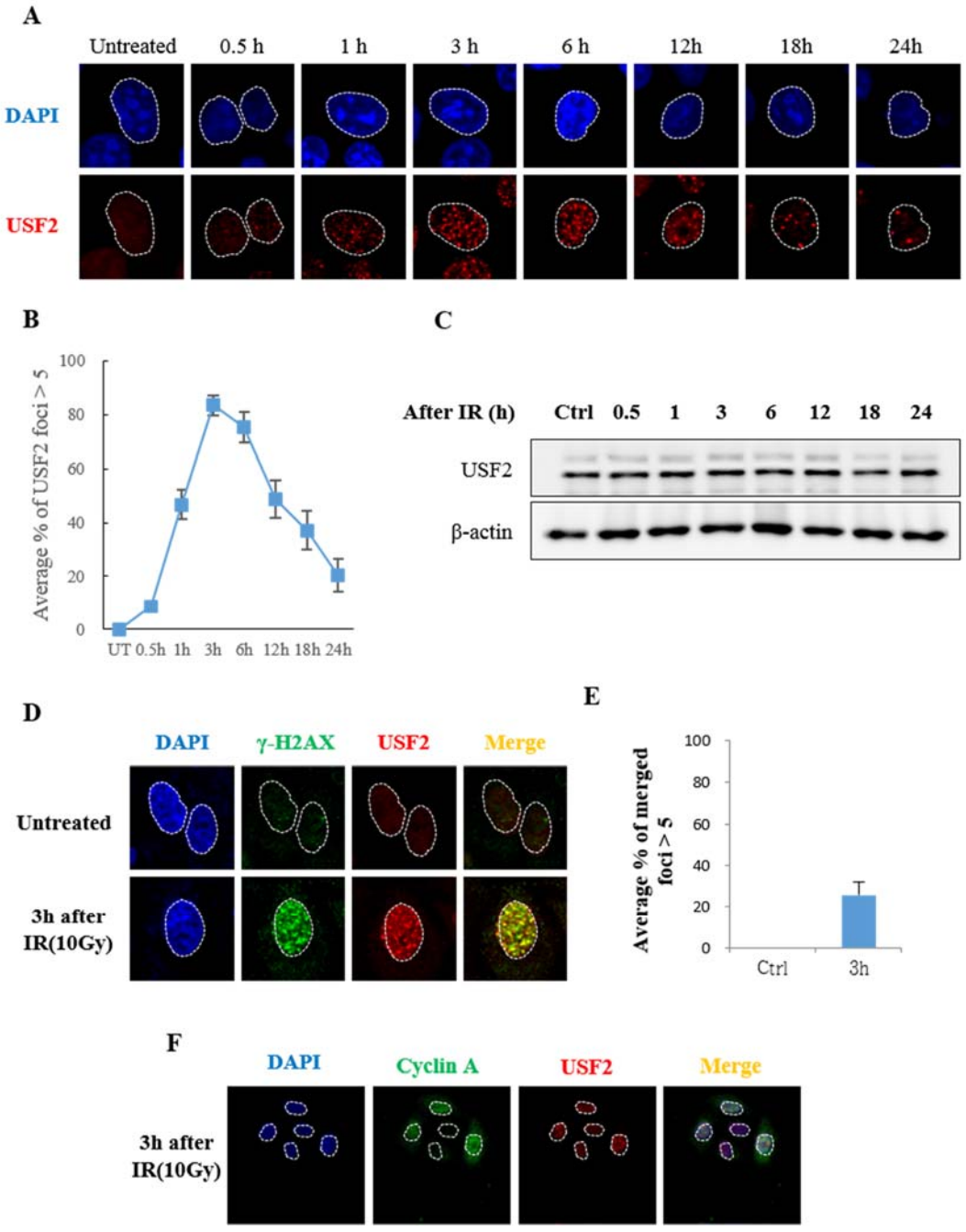

Figure 4. USF2 is recruited to DNA damage sites.

(A) HeLa cells were incubated for 48 hours and fixed following indicated time after IR treatment. Then the cells were stained with anti-USF2 antibody. Nuclei were stained with DAPI. (B) Quantification of cells with USF2 foci in nuclei. The percentage of USF2 foci, above five, were determined. (C) HeLa cells were lysed at the indicated time after IR treatment using lysis buffer. The expression level of USF2 was confirmed by western blotting using anti-USF2 antibody. β-actin was used as loading control. (D) HeLa cells were incubated for 48 hours and fixed at 3 hours after IR treatment. Then, the cells were stained with anti-USF2 and γ-H2AX antibodies. The nuclei were visualized by DAPI staining. (E) Quantification of cells with merged USF2 and γ-H2AX in nuclei. The percentage of merged USF2-γ-H2AX foci. The percentage of merged USF2, γ-H2AX foci, above five, were determined. (F) The cells were co-stained with anti-USF2 and Cyclin A antibodies to IF. The % of cells, included USF2 foci above five, determined. Results are shown as means ±SD (n=3).

5. Recruitment of USF2 is decreased by ATM and ATR kinase.

As shown figure 3 and 4, USF2 regulates HR and NHEJ and forms USF2-damage foci through-out the cell cycle. So, we wondered what USF2 regulate, and what regulates USF2. To investigate which step USF2 play a role in, we generated siRNA against ATM, ATR, and BRCA1. ATM and ATR are generally known as a sensor of DNA damage. As DNA is damaged, ATM or ATR is activated through phosphorylation itself, then phosphorylate downstream proteins of them. BRCA1, as one of the ATM downstream proteins, is a very important mediator to regulate HR. It is known that BRCA1 is usually phosphorylated by ATM or ATR to repair. In this result, when ATM and ATR, as DNA damage sensor, were knocked down, USF2 foci was reduced by more than 50%, when BRCA1 was knocked down, USF2 was not changed (figure 5A, B), although USF2 protein level was constant (figure C). Thus, USF2 is likely to function downstream of ATM/ATR kinase and contributes to the recruitment of BRCA1 into the DSB sites.

Figure 5

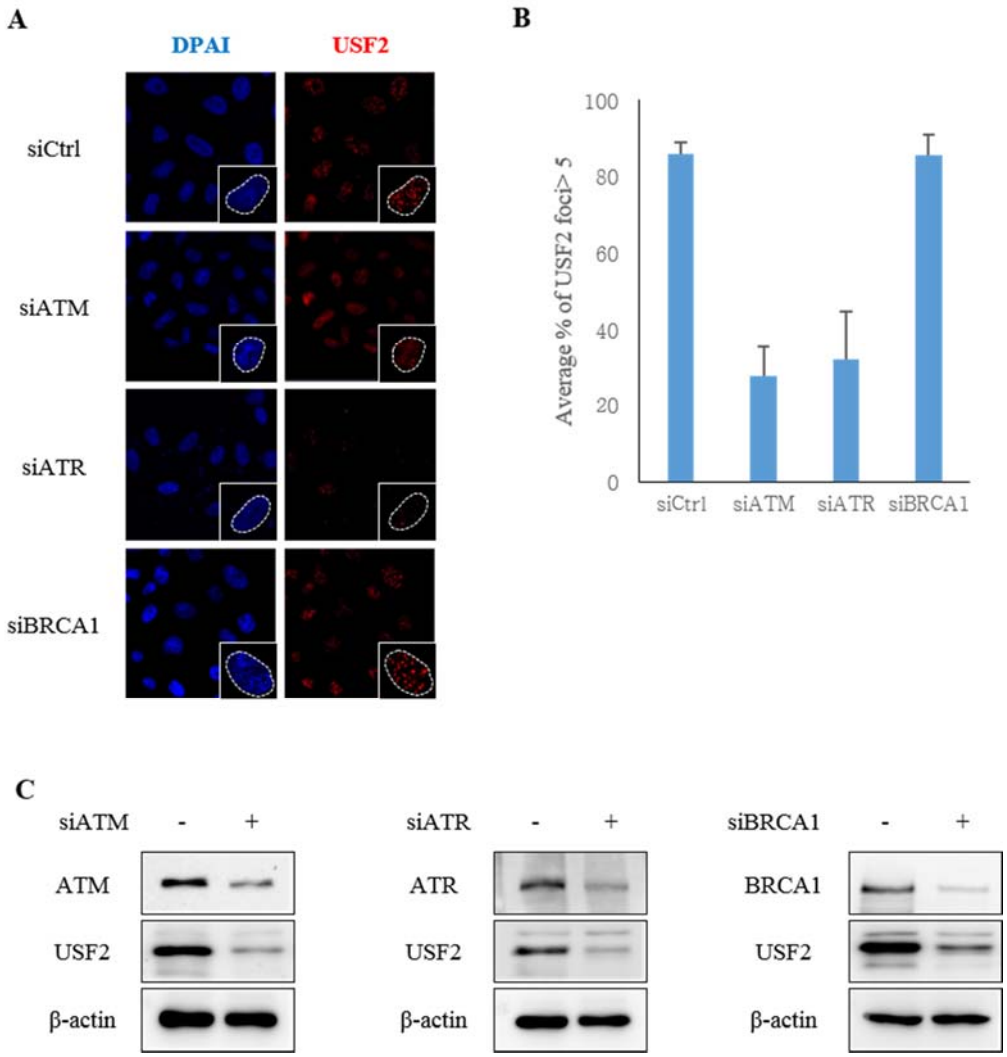

Figure 5. ATM and ATR kinase regulated USF2 foci formation into the DSB sites.

(A) Endogenous ATM, ATR, and BRCA1 was depleted by siRNA, recognized a specific sequence of ATM, ATR, and BRCA1 mRNA. HeLa cells were transfected siRNA against ATM, ATR, and BRCA1, respectively. Then, the cells were treated with IR (10 Gy) and stained with anti-USF2 antibody. DAPI staining was performed to incubate the positions of nuclei. (B) The numbers of USF2 foci in each nuclei, above five of foci, were quantified. (C) HeLa cells were transfected with each siRNA. After 48 hours, the expression level of ATM, ATR, BRCA, and USF2 were confirmed by western blotting using anti-ATM, ATR, BRCA1, and USF2 antibodies. β-actin was used to loading control.

6. USF2 regulates BRCA1-mediated HR through interaction with BRCA1 RING domain.

BRCA1 is a key regulator in HR. BRCA1, for having a function for HR processing, has to interact with BARD1. BARD1 binds to RING domain of BRCA1. The BRCA1-BARD1 complex is critical. As BRCA1 doesn't interact with BARD1, DSBs aren't repaired [18]. We wanted to know how BRCA1 and BARD1 recruitment change in USF2-depleted cells. To investigate this, the cells strained with anti-BRCA1 and BARD1 at 6 hours after IR treatment, respectively. The cells above five of BRCA1 foci and BARD1 foci were reduced by approximately 40 % (figure 6A, B). Because the cells, above five of USF2 foci, didn't reduce, we could expect that recruitment of BRCA1 and BARD1 were reduced. Furthermore, since the cells, including BRCA1 and BRCA1 foci, were reduced, we also thought that RAD51 foci are reduced in USF2-depleted cells. To confirm that, we stained cells with the anti-RAD51 antibody. As we guess, the cells, including RAD51 foci were reduced by approximately 50% (figure 6C). We found that USF2 regulated BRCA1, BARD1, and RAD51. To further identify which regions of BRCA1 interact with USF2, we generated RING domain deletion vector of HA-tagged BRCA1, named HA-BRCA1 mutant type (HA-BRCA1 mt). This vector was deleted initial 109 residues of BRCA1 CDS sequence, and we already generated USF2

and BRCA1 full sequence vector, named GFP-USF2, HA-BRCA1 wild-type (HA-BRCA1 wt) (figure 7A). we performed a co-immunoprecipitation assay using anti-HA and GFP antibodies. HEK 293T was transfected with full-sequence GFP-USF2 and mock, GFP-USF2 and HA-BRCA1 wt, and GFP-USF2 and HA-BRCA1mt vector. Co-immunoprecipitation assay was performed by using anti-HA antibody and immunoblotting was carried out with an anti-GFP antibody. Reciprocally, GFP-USF2 immunoprecipitated together with HA-BRCA1 using anti-HA antibody. This resulted in that GFP-USF2 interacted with HA-BRCA1 wild-type but didn't interact with HA-BRCA1 mutant type, deleted RING domain (figure 7B and C). These data showed that the RING domain of BRCA1 is very important in the interaction between BRCA1 and USF2. Taken together, we suggest that USF2 regulates BRCA1-mediated HR through interaction with BRCA1 RING domain.

Figure 6

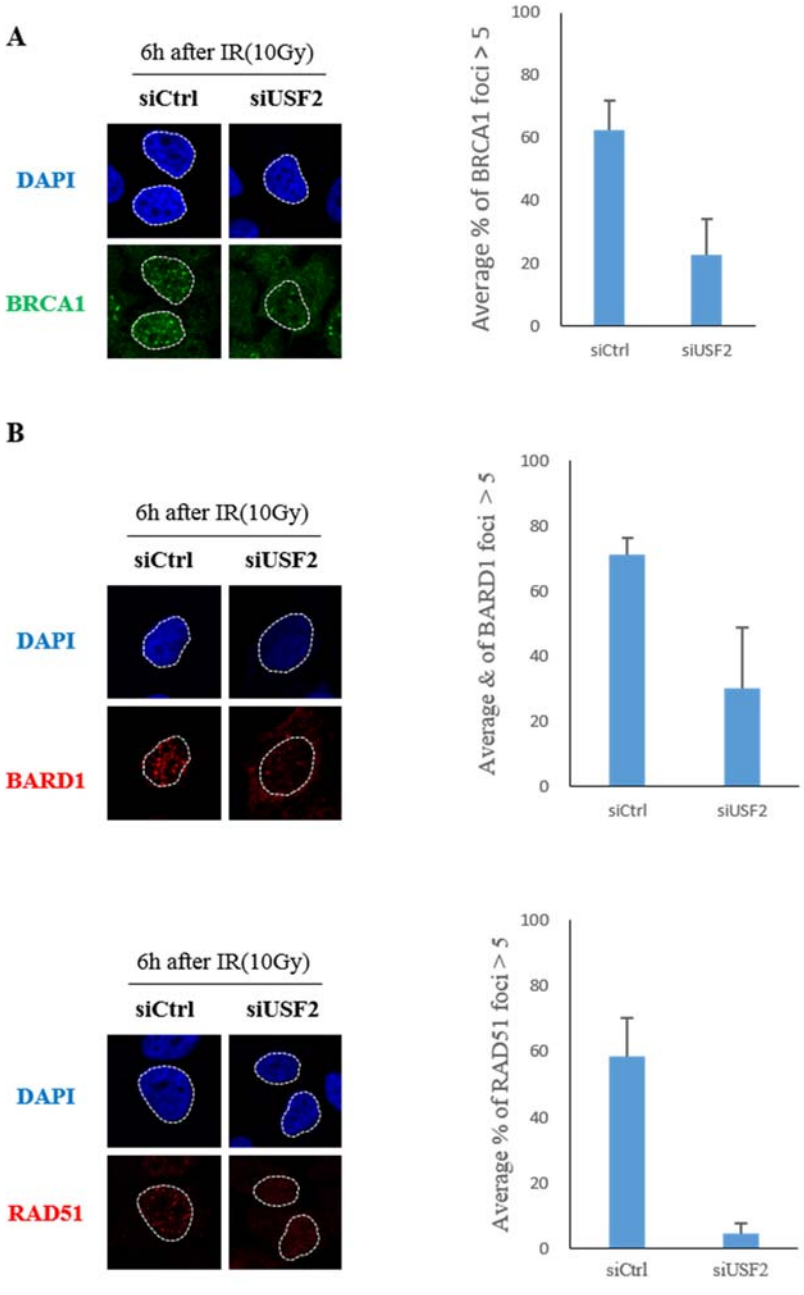

Figure 6. USF2 promotes BRCA1-related HR activity.

(A and B) HeLa cells were transfected with siRNA-control or siRNA-USF2. After 48 hours, the cells were induced DNA damage by IR treatment. For 6 hour, the cells were recovered, then fixed using the method of methyl alcohol and acetone buffer. Then, the cells were stained with anti-BRCA1 (A), BARD1 (B) antibodies. Nuclei were stained with DAPI. (C) HeLa cells were fixed by using 4% PFA protocol and stained with anti-RAD51 antibody. Nuclei were stained with DAPI. The average % of cells, included each foci above five, determined. Results are shown as means ±SD (n=3).

Figure 7

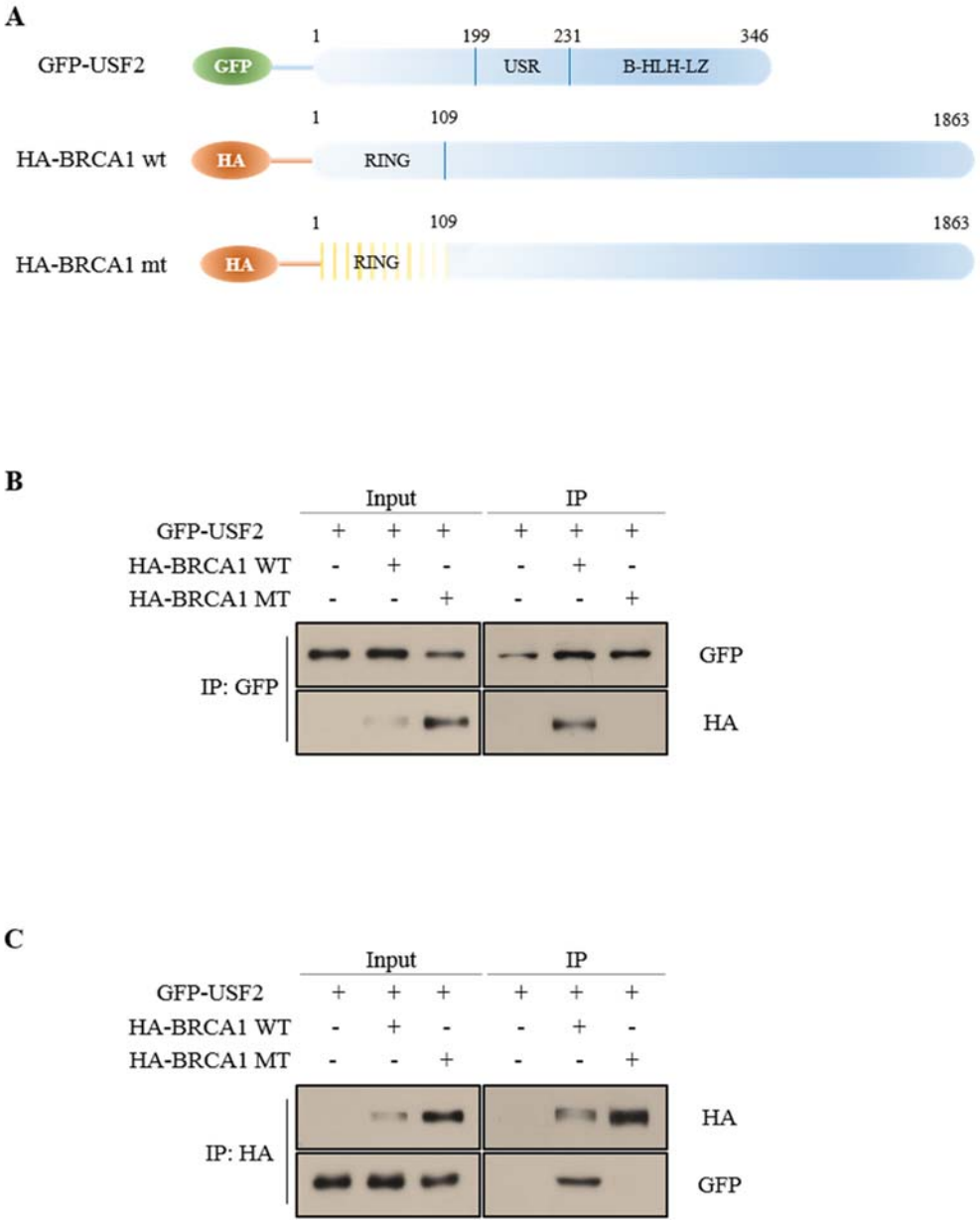

Figure 7. The RING domain of BRCA1 is required for interactions with USF2.

(A) Schematic diagram of USF2 and BRCA1 expression vectors. GFP-USF2 and HA-BRCA1 wild-type vectors express the full length of USF2 and BRCA. HA-BRCA1 mutant type vector expresses BRCA1 without RING domain. (B and C) HEK293T cells were transfected with full sequence GFP-USF2, HA-BRCA1 wild type, and HA-BRCA1 mutant type expression vectors. The total proteins were subjected to immunoprecipitation using an anti-GFP (B) or HA antibodies (C) followed by western blotting using anti-HA or GFP antibodies.

Figure 8

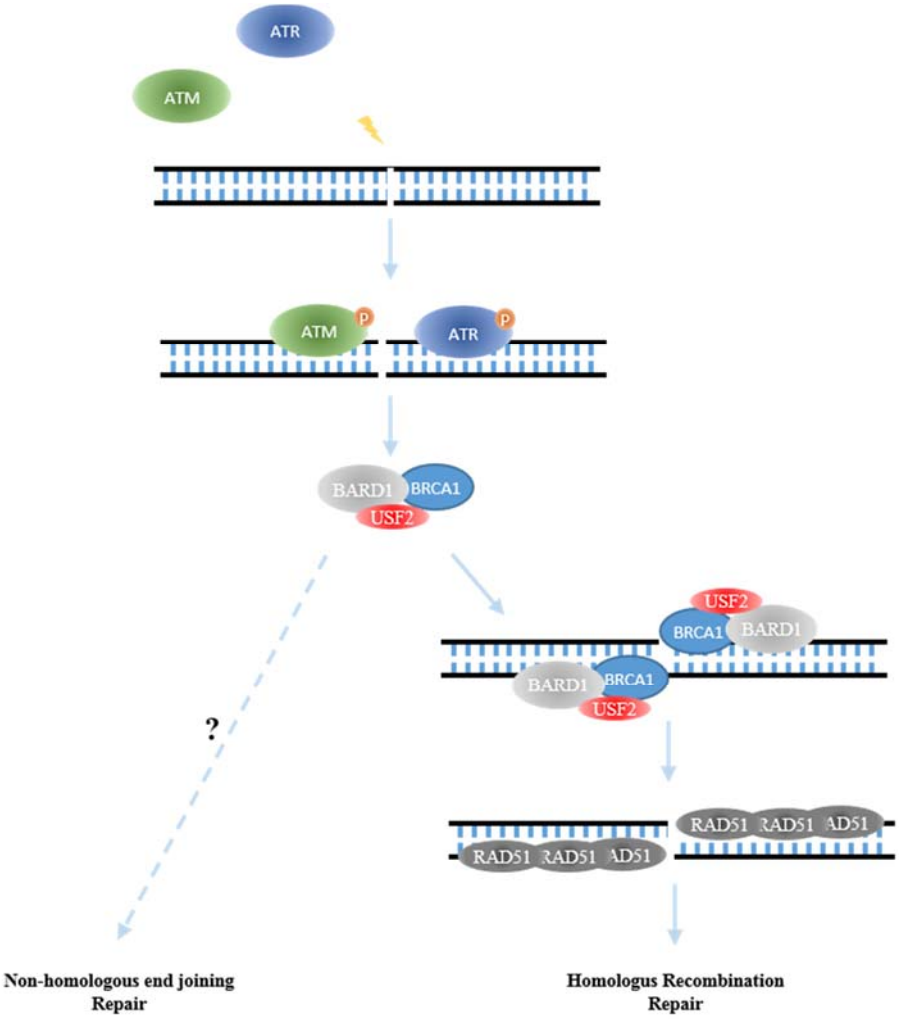

Figure 8. A schematic representation of the role of USF2 regulating BRCA1-mediated HR

DISCUSSION

In eukaryotic cells, it is a very serious event that DNA is damaged by endogenous or exogenous source. Because this event has a possibility to cause gene mutation, cancer, and apoptosis, repairing damage DNA is very important. Double strand break repair pathway are broadly classified HR and NHEJ. HR repairs damaged DNA base on homology, whereas NHEJ repairs the lesion without homology. But, because NHEJ has a higher risk to be mutated, HR is most important. That is the reason why BRCA1, a key regulator in HR, is very important.

Previous studies have shown that USF2 interacts with BRCA1, regulates the expression of BRCA1-targeted genes involved cellular response. Because, BRCA1 is a critical regulator of DDR. We primarily studied about USF2 in DDR. First, we confirmed that USF2 interacts with BRCA1 through co-immunoprecipitation, additionally, the interaction between USF2 and BRCA1 is enhanced by IR. Base on this result, we supposed that USF2 may involve in DDR (Figure 1). Next, to investigate what happens to the depleted-USF2 cell, we generated siRNAs against USF2. All of siRNA against USF2 efficiently knocked down USF2 expression. Through clonal survival assay, we

found that deficient-USF2 cells were hypersensitive to IR damage compared to normal cells. We guessed that USF2 have some role to repair IR-induced damaged DNA (figure 2). To investigate this assumption, we measured alteration of repair base on EJ5-GFP and DR-GFP system. Generally, HR and NHEJ antagonize each other, but all repair efficient of HR and NHEJ decreased in Lacking USF2 cells (figure 3). Through IF assay, we showed that USF2 foci increased after treatment of IR. Interestingly, USF2 was recruited to the lesion from early time to DNA damage, and it was confirmed by co-immunostaining assay with γ-H2AX and USF2 antibodies. This result suggests a possibility that USF2 play a role with comparative early step in DDR (figure 4). Then, to determine what regulates USF2, and what USF2 regulate, we generated several siRNA, against ATM, ATR, and BRCA1. This result in that ATM and ATR regulated USF2, and when BRCA1 is knocked down, USF2 foci didn't change (figure 5). In this result, we assumed that USF2 may regulate the BRCA1 recruitment, then we found that USF2 regulate BRCA1. Additionally, BARD1, known to constantly interact with BRCA1, and RAD51 also were regulated by USF2 (figure 6). As shown figure 6, when USF2 was defected, BRCA1 and BARD1 were not accumulated at DNA lesion. This result is reasonable be-

cause USF2 regulated BRCA1. But, we wondered how to regulate BRCA1 recruitment to DNA lesion. We reminded that BRCA1 interacts with BARD1 at RING domain of BRCA1, and USF2. USF2 had a possibility to be associated with the interaction between BRCA1 and BARD1. Through co-immunoprecipitation assay, USF2 bound to RING domain of BRCA1. It showed that USF2 can have the possibility to impair interaction between BRCA1 and BARD1 (figure 7).

In our study, we identified that DNA damage response is processed in order ATM/ATR, USF2, BRCA1, and RAD51, but the specific position of USF2 has been undemonstrated yet. For a demonstration of specific USF2 position in DDR, relation with downstream proteins of ATM/ATR, such as MDC1, RNF8, RNF168, NBS1, 53BP1, and BRCA1, need to be studied. Next, we were very curious why both repair activity of HR and NHEJ decreased in USF2-depleted cells. Normally, HR and NHEJ activity antagonize each other, but our data were not. Additionally, USF2 formed foci in G1 and S/G2 phase cells. Therefore, we supposed that USF2 could be also involved in NHEJ pathway. As the result, we can assume USF2 is an upstream regulator of 53BP1 and BRCA1 in DDR, and we also found that USF2 binds to BRCA RING domain. Its RING domain is very important for interaction with BARD. It is already known that BRCA1-BARD1 complex contributes repositioning of

53BP1. In order to prove this, we can confirm the binding of BRCA1 and BARD1 according to the presence of absence or USF2 through further studies.

USF2 is also known to interact with upstream stimulatory factor 1 (USF1), continuously [30]. We don't know how the interaction of USF1 and USF2 affects to DDR yet. As shown in figure 5, USF2 is a downstream protein of ATM/ATR kinase. ATM/ATR kinase phosphorylate substrate proteins on either a serine or threonine residue that is followed by glutamine residue (so, named SQ/TQ motif). In DNA damage response, most proteins have modification forms, such as phosphorylation and ubiquitination. USF1 has phosphorylation site by DNA-PK [31]. Thus, for USF2 specific study, the interaction of USF1 and USF2 has to be considered. Additionally, we have searched the possibility that USF2 has modification form. It is already known that USF2 is phosphorylated at serine 155 and threonine 230 by GSK3β[32], but they were not S/TQ site, phosphorylated by ATM/ATR. Through seeking whether there are amino acid sequences, S/TQ, we identified two putative TQ motifs in the USF2 amino acid sequence, threonine 115 and threonine 210 (data not shown). However, we don't know that USF2 can be phosphorylated at these site, the study about modification of USF2 are necessary.

Above this, USF2 antagonizes against Myc. Myc is commonly overexpressed in cancer progression, but USF2 can inhibit Myc-mediated cellular transformation [33], and it is also reported that USF2 inhibits cell proliferation [34]. Thus, we guess that USF2 may be important to inhibit cancer progression through antagonizing Myc.

In summary, when DNA is damaged by IR treatment, initially, ATM is autophosphorylated. Then, phosphorylated ATM/ATR are recruited to doubles strand breaks site. Phosphorylated ATM/ATR on DSBs recruit its downstream proteins. In homologous recombination, USF2, one of ATM/ATR downstream proteins, usually interacts with BRCA1, and interaction of USF2-BRCA1 is enhanced in damaged cells. Then, the USF2-BRCA1-BARD1 complex is recruited to DSBs. Finally, RAD51 is recruited, then DSBs are repaired. As shown figure 3, USF2 is also involved in NHEJ pathway and downregulated NHEJ activity in USF2-depleted cells, but we don't know yet how USF2 impairs NHEJ pathway (figure 8).

Here, we showed that USF2 is an important regulator of HR through interaction with BRCA1. As we already mentioned, relation with the proteins, interacted with USF2, should be determined. Taken together, we propose that USF2 are a critical regulator in HR.

ABSTRACT

The Transcription factor USF2: A master regulator of DNA damage regulator

Gwan-Woo Seo
Advisor: Prof. Jung-Hee Lee, Ph.D.
Department of Biomedical Sciences,
Graduate school of Chosun University

BRCA1, one of the major proteins in DNA damage response, is known to be involved in the recognition, repair and cell cycle regulation of damaged DNA, especially in homologous recombination activities. However, the precise mechanism of BRCA1 in these DNA damage response remains to be elucidated. This study identified USF2, which is known to interact with BRCA1. The USF2 protein, known as a transcription factor, forms a heterodimer with USF1. USF2 has a helix-loop-helix probe and recognizes the CACGTG base sequence of DNA called E-box and begins transcription of the gene. It has been reported that USF2 is involved in the transcription of many genes. However, it has not been studied how USF2 is involved in the mechanism of DNA damage

repair with BRCA1. In this study, we were investigating a novel mechanism of USF2 involvement in DNA damage response by binding to BRCA1. USF2 has been reported to interact with BRCA1 and it has been confirmed that the binding of USF2 to BRCA1 is increased by irradiation. In the lacking USF2 cells, the degradation of DNA repair activity was confirmed by clonal survival, homologous recombination, and non-homologous end joining activity assay. USF2 accumulates into the nucleus where the damaged DNA was present and formed damage foci throughout the cell cycle. In the DNA damage repair mechanism, moreover, USF2 is to function downstream of ATM/ATR kinase, and the depleted-USF2 cells inhibited the recruitment of BRCA1, BARD1, and RAD51 to DSB sites. Therefore, this study demonstrates USF2 is a novel regulator of the DNA damage response and suggests a molecular mechanism for BRCA1-related Homologous recombination.

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