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BANF1 regulates MDC1−mediated DNA damage response

조선대학교 대학원

의과학과

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BANF1에 의한 MDC1 활성 조절연구

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DNA 손상반응의 핵심 단백질 중 하나인 MDC1은 DNA가 손상되었을 때 손상인식, 복구 및 세포주기 조절에 관여하는 것으로 알려져 있다. 이런 MDC1의 조절기전을 밝히고자 MDC1을 bait로 yeast two-hybrid를 실시하였고 그 결과 MDC1과 결합하는 새로운 단백질 BANF1를 동정하였다. BANF1는 고도로 보존된 DNA 결합 단백질로써 유사분열, nuclear assembly, 바이러스 감염, 염색질 및 유전자

조절, DNA 손상반응을 비롯한 여러 경로에 관여하는 것으로 보고되어져 있다. 따라서 본 연구에서는 MDC1과 결합하는 새로운 단백질인 BANF1이 MDC1과 관련된 DNA 손상반응에 있어서 미치는 영향을 알아보고자 한다. 이를 위해 먼저 세포 내에서 과발현된 BANF1과 MDC1이 결합함을 확인하였다. 또한, 특정부위가 결핍된 돌연변이 MDC1 과발현을 통해 MDC1의 PST부위에서 BANF1가 결합함을 확인하였다. 다음으로, BANF1가 결핍된 세포에 여러 DNA 손상자극 (IR, HU, CPT)을 가한 후 세포손상 민감도 및 MDC1 활성을 조사하였다. 먼저, BANF1-MDC1 결합은 방사선 조사에 따른 MDC1 활성에는 아무런 영향이 없음을 clonal survival assay, HR/NHEJ activity, MDC1 손상 foci 관찰을 통해 확인하였다. 두번째로 BANF1-MDC1 결합이 HU처리에 따른 MDC1 활성에 미치는 영향을 clonal survival, MDC1과 RPA 염색을 통해 확인한 결과 BANF1-MDC1 결합은 기존에 알려진 HU에 대한 MDC1의 DNA 손상반응과는 무관함을 알 수 있었다. 마지막으로 BANF1가 결핍된 세포에 CPT를 처리하면, 정상세포에 비해 세포민감도가 증가하며, MDC1 손상 foci 및 세포주기 체크 단백질 Chk1의 활성이

감소됨을 확인하였다. 또한, CPT처리에 의해 BANF1가 DNA 손상부위로 이동함을 관찰하였다. 따라서 본 연구는 BANF1가 CPT손상에 의한 MDC1 활성을 조절하는 새로운 DNA 손상조절 물질임을 밝힌 최초의 보고이다.

INTRODUCTION

A large number of chemicals and γ-radiation and UV light have been associated with the etiology of cancer. Generation of DNA damage (also known as DNA lesions) induced by these agents is an important in the production of cancer. Gene mutations may generate by damaged DNA replication, which in turn may give rise to altered proteins. For this reason, a system is needed to protect DNA from DNA damage agents. Evolutionary processes gave rise to DNA repair systems that are efficient in repairing damaged DNA [1]. DNA damage response (DDR) an neutralize the negative effects of DNA damage as an important pathway to protect DNA molecules from DNA destruction [2].

DDR proteins can be divided into multiple groups according to their roles, but they can be divided into sensors, transducers, mediators, and effectors. The DDR cascade starts by the sensors that detect the DNA damage and transmit the initial

signal to the transducers. After, with the help of mediators, the transducer amplifies the signal and transmits it to the effectors. Effectors play a variety of roles, such as DNA repair, checkpoint activation, and apoptosis. One of the characteristics of DDR is that DDR protein is locally accumulated in the double strand breaks (DSB) site. In a manner of minutes, multiple DDR proteins assemble at double strand break sites. The accumulation of these proteins is not restricted to the break site itself, but rather spreads across large megabase domains flanking the DSB, forming microscopically visible foci. MDC1, also known as NFBD1, is considered an important element of DDR. MDC1 is known to be involved in homologous recombination (HR) /non-homologous end-joining (NHEJ) DSB repair pathways, G2/M checkpoint, intra-S-phase checkpoint and some DDR regulators' phosphorylation[3].

When DSB occurs, the cellular response are regulated by protein kinases, ataxia telangiectasia mutated (ATM), ATM and Rad3-related (ATR), and DNA-

dependent protein kinase (DNA-PK). Upon DNA damage, histone H2AX is phosphorylated by these kinases at the DSB site of Ser139 and becomes γH2AX. After, γH2AX is recognized by MDC1, which acts as a platform for recruitment of DDR factor mediate for DNA repair. The NBS1 subunit of the MRE11-RAD50-NBS1 (MRN) complex binds to MDC1 via direct interaction to multiple acid sequence motifs near the N-terminal of MDC1. Another is RNF8, an E3 ubiquitin ligase with an FHA domain that binds to a cluster of conserved threonine residues in MDC1 that are phosphorylated by ATM in response to DSBs to promote chromatin ubiquitylation events required for recruitment of DNA damage response mediator proteins such as 53BP1 and BRCA1[4].

In order to find a partner that interacts with MDC1 as the core protein in DDR, we identified BANF1 through the yeast two hybrid screening assay. Barrier-to-autointegration factor (BANF1) binds directly to histone, lamins and LEM do-

main proteins containing emerin in the nuclear membrane and is known to be involved in chromatin structure, mitosis, and generation.[5]. Recently, it has been reported that BANF1 controls DNA damage response to oxidative stress through regulation of poly[ADP-ribose] polymerase1 (PARP1) activity[6].

In the present study, we identified a novel binding protein of MDC1, BANF1, through yeast two-hybrid screening assay. In addition, we showed that depletion of BANF1 decreased survival rate and impaired DNA damage checkpoint after CPT. Our results point to a critical role for BANF1 in increasing MDC1 activity to induce DNA damage checkpoint to CPT.

MATERIALS AND METHODS

1. Cell culture and treatment

This cell lines (Hela, HEK293T and U2OS) were obtained from ATCC. They were cultured in DMEM (Dulbecco's modified Eagle's medium) supplemented with 10% FBS and penicillin (100units/ml), streptomycin (0.1mg/ml) at 37℃ in a 5% CO_2 incubator. 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. Double strand breaks (DSB) were induced after irritation of 5 Gy IR in exponentially growing cells. After that, it was recov-ered at 37℃ incubator with various times. Hydroxyurea (HU) and Camptothecin (CPT) reagents were obtained from sigma. Hydroxyurea (HU) was treated at 2mM concentration in all experiments except clonal survival. Camptothecin (CPT) was treated at 1uM concentration in all experiments except clonal surgical.

2. siRNA transfection

Hela, HEK293T and U2OS cells were transfected with siRNA oligonucleotide duplexes against BANF1 using Turbofect (Thermo Fisher scientific) according to the manufacturer's instruction. The siRNA sequences targeting BANF1 (BANF1 siRNA #1: 5'-GCUAUUGUCGUACUCACCU-3', BANF1 siRNA #2: 5'-GGCCUAUGUUGUCCUUGGC-3') designed and synthesized for transient transfection.

3. Immunoprecipitation assay

The total cell lysates were extracted with NP40 lysis buffer (1% NP40 buffer, 50mM Tris (pH 8.0), 150mM NaCl, 5mM EDTA) with protease inhibitors (Roche Diagnostic Corp.). Sample were added to the anti-MDC1(R2), GFP (Santa Cruz) and HA (Santa Cruz) antibodies at 4℃ for 24 hours. And then, sepharose-G beads (GE Healthcare) were added to the lysates, and beads were incubated at

4℃ for 4 hours on shaker. The beads were washed three times in NP40 lysis buffer with protease inhibitors, resuspended in equal volume 2X SDS sample buffer. The samples were extracted from the bead by boiling at 99℃ for 5min in heat block. The samples were then analyzed by western blotting using the appropriated antibodies.

4. Western blot analysis

Briefly, cells were washed with 1X PBS, and then lysed in RIPA lysis buffer (1% NP40 buffer, 0.5% sodium deoxycholate, 50mM Tris (pH7.5), 150mM NaCl, 5mM EDTA, 0.1% SDS) with protease inhibitors (Roche Diagnostic Corp.). The cell lysates were centrifuged at 15,000rpm for 20 min, and the supernatant cel-lected. In order to amount of protein 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 (pH7.4), 150mM NaCl and 0.1% Tween-20) containing 5% skim milk and then incubated at 4℃ with primary antibodies (1:1000) for over-night. The membranes were washed six times for 10min with washing buffer (0.1% Tween 20 containing TBS-T) and then incubated for 2 hours with pe-roxidase-conjugated secondary antibodies (1:4000) at RT. The membranes were washed six times for 10min with washing buffer (0.1% Tween 20 contain-ing TBS-T) and developed using an enhanced chemiluminescence detection system (ECL; GE Healthcare, Buckinghamshire, UK).

5. Antibodies

We used the following primary antibodies: Mouse monoclonal anti-BANF1 (sc-166324, Santa Cruz Biotechnology), Mouse monoclonal anti-BANF1 (H00008815-M07), mouse monoclonal anti-MDC1 (M2444, Sigma-aldrich), rabbit polyclonal MDC1(R1, R2), mouse monoclonal anti-BRCA1 (sc-6954, Santa

Cruz Biotechnology), mouse monoclonal anti-53BP1 (612523, BD biosciences), mouse monoclonal anti-γ-H2AX (05-636, Millipore), mouse monoclonal anti-RPA (NA18-100UG, MilliporeSigma), mouse monoclonal anti-Chk1 (2360S, Cell Signaling Technology), rabbit polyclonal anti-Chk1 p317 (AF2054, R&D systems), rabbit monoclonal anti-Chk1 p345 (2348S, Cell signaling Technology), mouse monoclonal anti-β-Actin (sc-47778, Santa Cruz Biotechnology), mouse monoclonal anti-HA (sc-7392, Santa Cruz Biotechnology), rabbit polyclonal anti-GFP (NB600-308, Novus Biologicals) antibodies.

6. Clonal survival assay

After Treatment with IR, HU and CPT, $1x10^3$ 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 then transfected. After cells treated HU, CPT and were irradiated with 5Gy of ionizing radiation (IR). Cells were then washed twice with 0.01M PBS, fixed with 4% paraformaldehyde for 10min and ice-cold 98% methanol for 5min, followed by permeabilization with 0.5% Triton X-100 for 15min at room temperature. Next, the coverslips were washed three times with 0.01M PBS and then blocked with 5% BSA in 0.01M PBS for 1hrs. The cells were single or double immunostained with primary antibodies against various proteins overnight at 4℃. Next, the cells were washed six times with 0.01M PBS and then stained with Alexa Fluor 594 (red, Molecular Probes) or Alexa Fluor 488 (green, Molecular probes) conju-gated secondary antibodies, as appropriate. After washing, the cells were

mounted using Vectashield mounting medium with 4, 6-diamidino-2-phenyl-indole (Vector Laboratories, Buflingame, 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-SceI-induced DSBs are repaired by NHEJ in HeLa EJ5-GFP cells, the purogene 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 recombiation 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 used iGFP as the template for nascent DNA synthesis, which results in retoration of a GFP expression cassette. HeLa DR-GFP cells were transfected with Control and BANF1 siRNA, after 4 hours transfected with 1 μg of I-SceI-expressing vector. After 48 hours, 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 preented as the mean ± standard deviation (SD). Analysis were performed using software (Image J) and Excel (Microsoft).

RESULT

1. BANF1 interacts with MDC1.

MDC1 is one of the core proteins in DDR[3]. To gain insight into the regulation of MDC1 function in DNA damage response, we searched for novel interacting partner of MDC1 by yeast two hybrid screenings using C-terminal BRCT domain of MDC1 as a bait and identified new MDC1 interacting protein, BANF1. The BANF1, which is a DNA-binding protein that links DNA to structural proteins in nuclear envelope, was identified as involved in DDR [7]. However, it is not known what the interaction between MDC1 and BANF1 will do in DDR. Therefore, we investigated how BANF1 and MDC1 interaction play a role in DDR based on IR, HU, and CPT, which are DNA damage drugs. First, to confirm results of yeast two hybrid assay, HEK293T cells were transiently transfected with an expression construct encoding full length BANF1 tagged with GFP and a second construct that

expressed full length MDC1 with HA and co-immunoprecipitation assay was performed. Immunoprecipitation assay revealed that full length MDC1 tagged with HA was associated with full length BANF1 tagged with GFP in both control and CPT treated cells and that the level of binding increased after CPT treatment (Figure1. A). Next we tried to determine which region of MDC1 is required for its interaction with BANF1. In order to do so, we generated several MDC1 construct tagged with HA (Figure1. B) and carried out co-immunoprecipitation assay in indicated transfected cells. We found that deletion of MDC1 PST domain abolished binding of BANF1 with MDC1, whereas normal binding was observed when constructs containing full-length HA- tagged MDC1 and other internal MDC1 deletion mutant containing PST domain of MDC1 was used (Figure1. C). These results indicated that PST domain of MDC1 is required for binding to BANF1 .

Figure 1

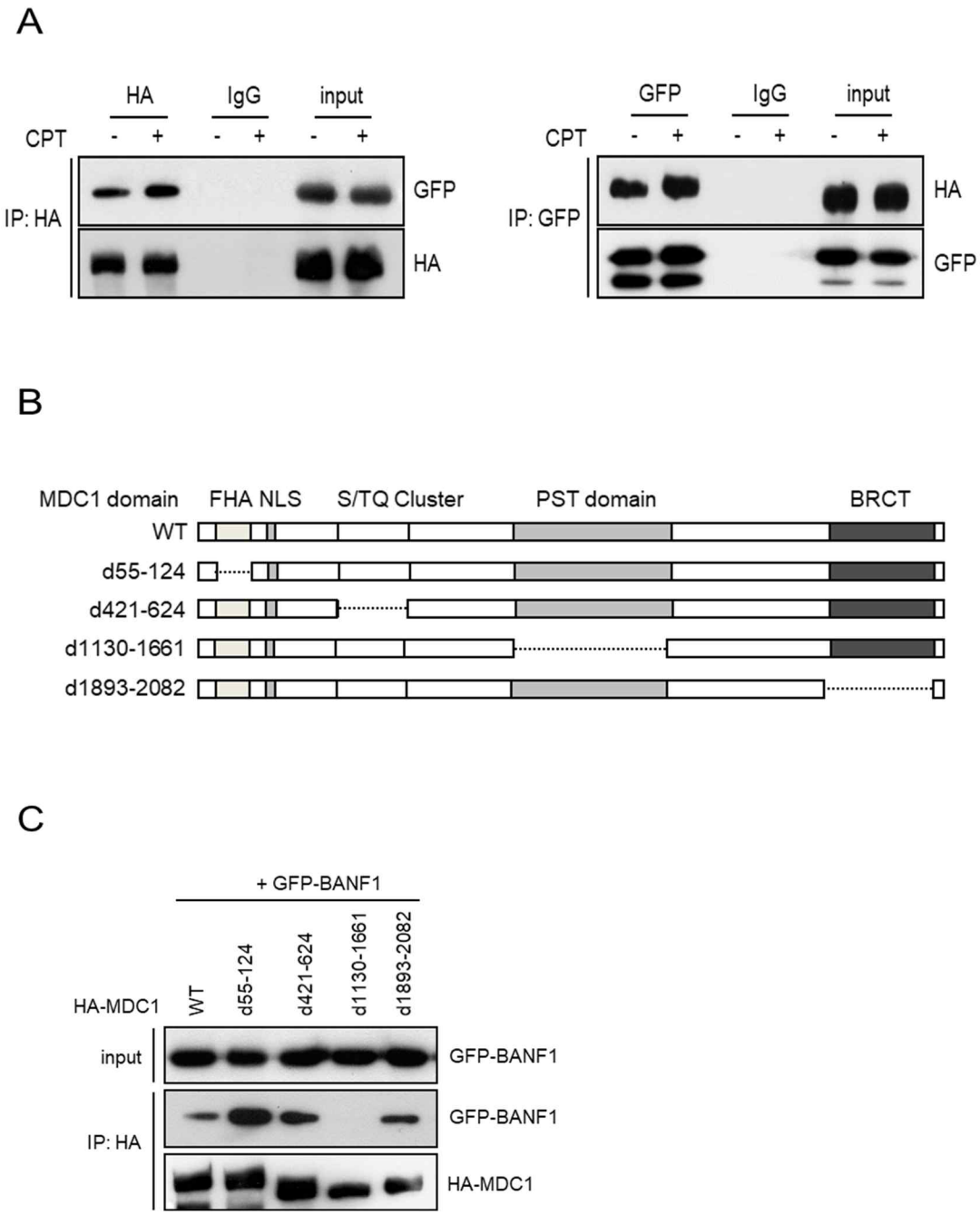

Figure 1. BANF1 interacts with MDC1.

(A) exogenous immunoprecipitation assay (IP) used HEK293T cells were transfected with full sequence GFP-BANF1 and HA-MDC1 expression vectors. After 48hours, the cells untreated or treated with 1 μM CPT during 3 hours. Cells were lysed in the NP40 lysis buffer. The total proteins were subjected to immunoprecipitation using an anti-GFP or HA antibodies followed by western blotting using anti-HA or GFP antibodies. (B) Schematic diagram of MDC1 domain. (C) HEK293T cells were transfected with indicated expression vectors. The total proteins were subjected to immunoprecipitation using an anti-HA antibodies followed by Western blotting using anti-GFP or HA antibodies.

2. BANF1 interacts with MDC1 does not affect MDC1-mediated DNA damage response in IR.

IR is said to cause DSB. When DSB occurs, DNA damage response works. ATM kinase first activates after IR treatment, and then H2AX was phosphory-lated, which called to γ-H2AX. MDC1 is then known to be recruited to γ-H2AX .[8]. It is questionable whether BANF1 can regulates MDC1's recruitment to IR-induced DSB site through interaction with MDC1. Control and BANF1 siRNA were transfected in Hela cells, after 48 hours, treated with IR (5 Gy), and then stained with anti-MDC1 antibody. We found that depletion of BANF1 not effect to foci formation of MDC1 to IR (Figure2. A and B).

There are two type repair pathways for DSBs: Homologous Recombination (HR) and Non Homologous End Joining (NHEJ). Homologous recombination (HR) occurs when BRCA1 becomes recruitment in downstream mode after MDC1 is re-cruitment in γ-H2AX. On the other hand, when 53BP1 becomes recruitment in

downstream, nonhomologous end joining (NHEJ) occurs[9]. Even though BANF1 can not regulates the recruitment of MDC1 to DSB sites, we investigated that regulates the DDR protein recruitment of MDC1 downstream. We investigated whether BANF1 regulates BRCA1 and 53BP1 foci after IR. Control and BANF1-depleted Hela cells were treated with IR for 6 hrs, and performed immunostaining using anti-BRCA1, anti-53BP1 antibodies. As a result, BRCA1 and 53BP1 foci were difference between control and BANF1-depleted cells. (Figure3. A ~ D).

BRCA1 is known to be related in homologous recombination (HR) and 53BP1 is known to be related in non-homologous end-joining (NHEJ). [9]. (Figure3. A ~ D) Since BRCA1 foci and 53BP1 foci were formed less in the absence of BANF1, it was measured whether HR and NHEJ were affected by existence or absence of BANF1. First, siRNA-Control, BANF1#1, and BANF1#2 were transfected, HR was measured using DR-GFP cells, and NHEJ was measured using EJ5-GFP cells. As a result, HR did not differ significantly with or without BANF1

(Figure4. A and B). Similarly, NHEJ was no difference between control and BANF1-depleted cells. (Figure 4. C and D).

Finally, in order to confirm whether BANF1 helps cells survive in IR induced DNA damage, control and BANF1#1, BANF1#2 siRNA were transfected with Hela cells, incubated for 2 days, 5 Gy IR treatment, after 2 weeks stained Methylene blue . As a result, it was confirmed that the clonal survival was constant regardless of whether BANF1 was present or not in DNA damage (Figure5. A ~ C).

We measured the rate-γ-H2AX foci using the DNA data marker γ-H2AX to determine whether BANF1 affects the repair of DSB by IR through interaction with MDC1. First, control and BANF1 siRNA were transfected in Hela cells, and then treated with 5 Gy IR, fixed after 1 hour and 24 hours and stained with rH2AX antibody. As a result, γ-H2AX foci was formed more when BANF1 was absent than when BANF1 was present (Figure6. A and B). This indicates that

DSB repair occurred less in the absence of BANF1. We wanted to know if BANF1 would respond when we actually gave IR. Therefore, we untreated or treated with 5Gy IR in Hela cells, fixed after 0.5hr, 1hr, 3hr, 4hr, 5hr, and stained using BANF1 antibody, then fluorescently stained and then counted BANF1 foci. As a result, BANF1 foci treated IR, found the most after 4hr, and then decreased, thus confirming what role BANF1 plays in IR (Figure7. A and B).

Figure 2

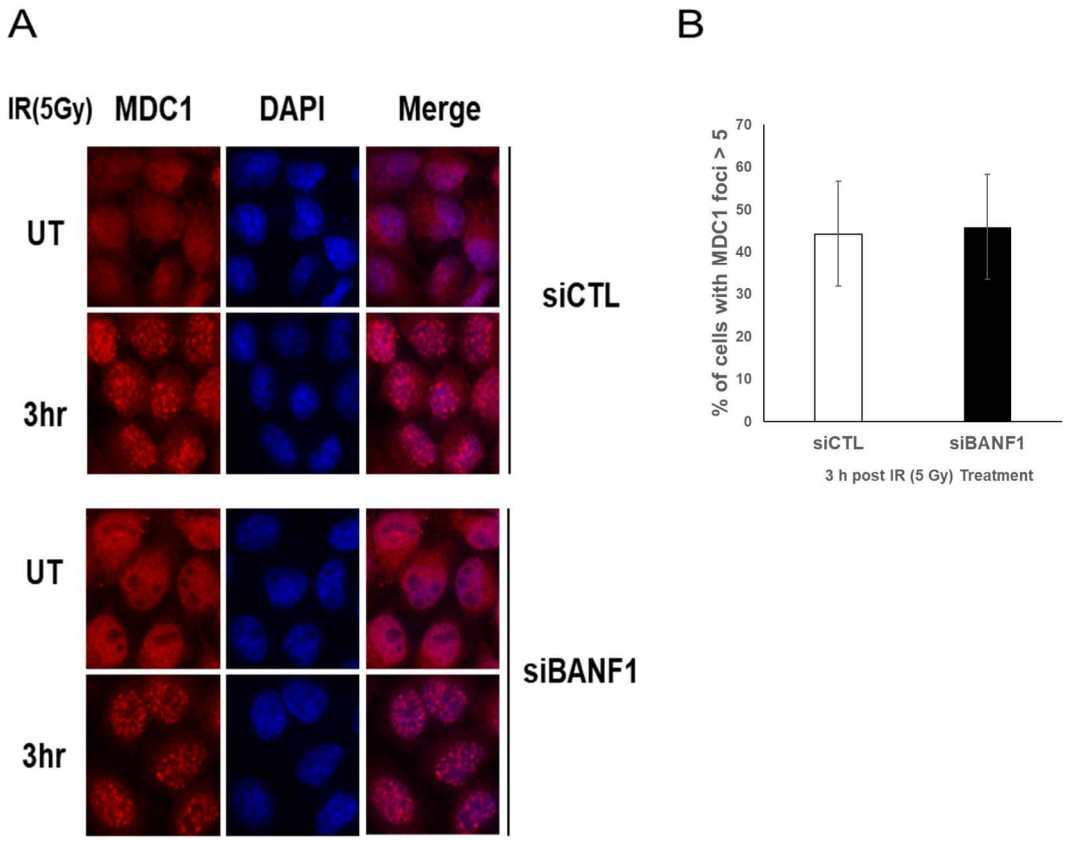

Figure 2. BANF1 does not affect MDC1 foci recruitment to IR.

(A) Control and BANF1 siRNA were transfected in HeLa cells, and then incubated for 2days. Next, the cells were untreated or treated with 5Gy γ-irradiation, and then were fixed at the indicated time. Cells were stained with an anti-MDC1 antibody and DAPI solution. (B) Quantification of Fig2A.

Figure 3

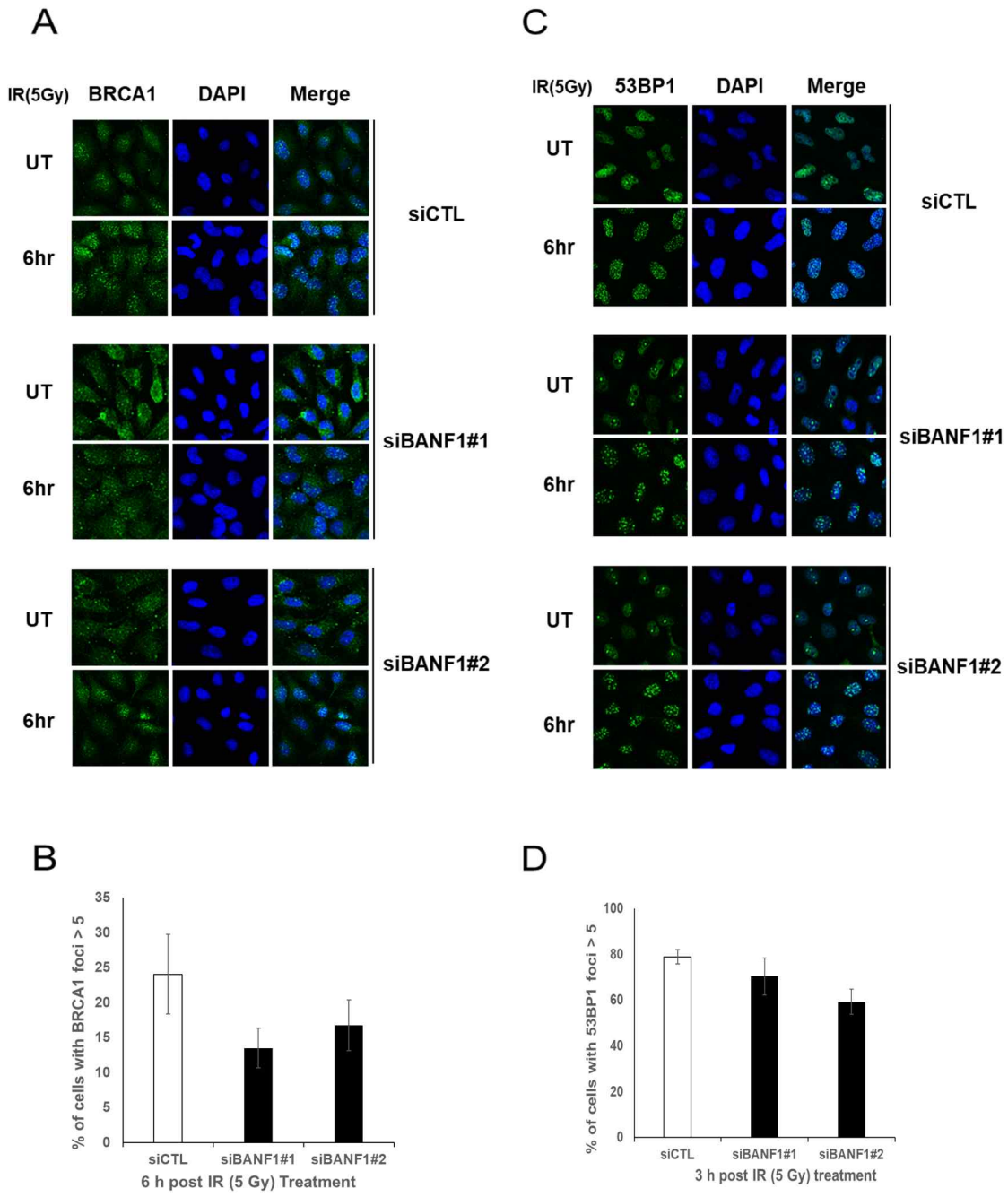

Figure 3. BANF1 affects BRCA1, 53BP1 foci formation after IR.

(A) Control and BANF1#1, BANF1#2 siRNA were transfected in Hela cells, and then incubated for 2 days. Next, the cells were untreated or treated with 5Gy γ-irradiation, and then were fixed at the indicated times after IR. Cells were stained with an anti-BRCA1 antibody and the DNA was counterstained using DAPI solution. (B) Quantification of Fig3A. (C) Control and BANF1#1, BANF1#2 siRNA were transfected in Hela cells, and then incubated for 2 days. Next, the cells were untreated or treated with 5Gy γ-irradiation and then were fixed at the indicated times after IR. Cells were stained with an anti-53BP1 antibody and the DNA was counterstained using DAPI solution. (D) Quantification of Fig3C.

Figure 4

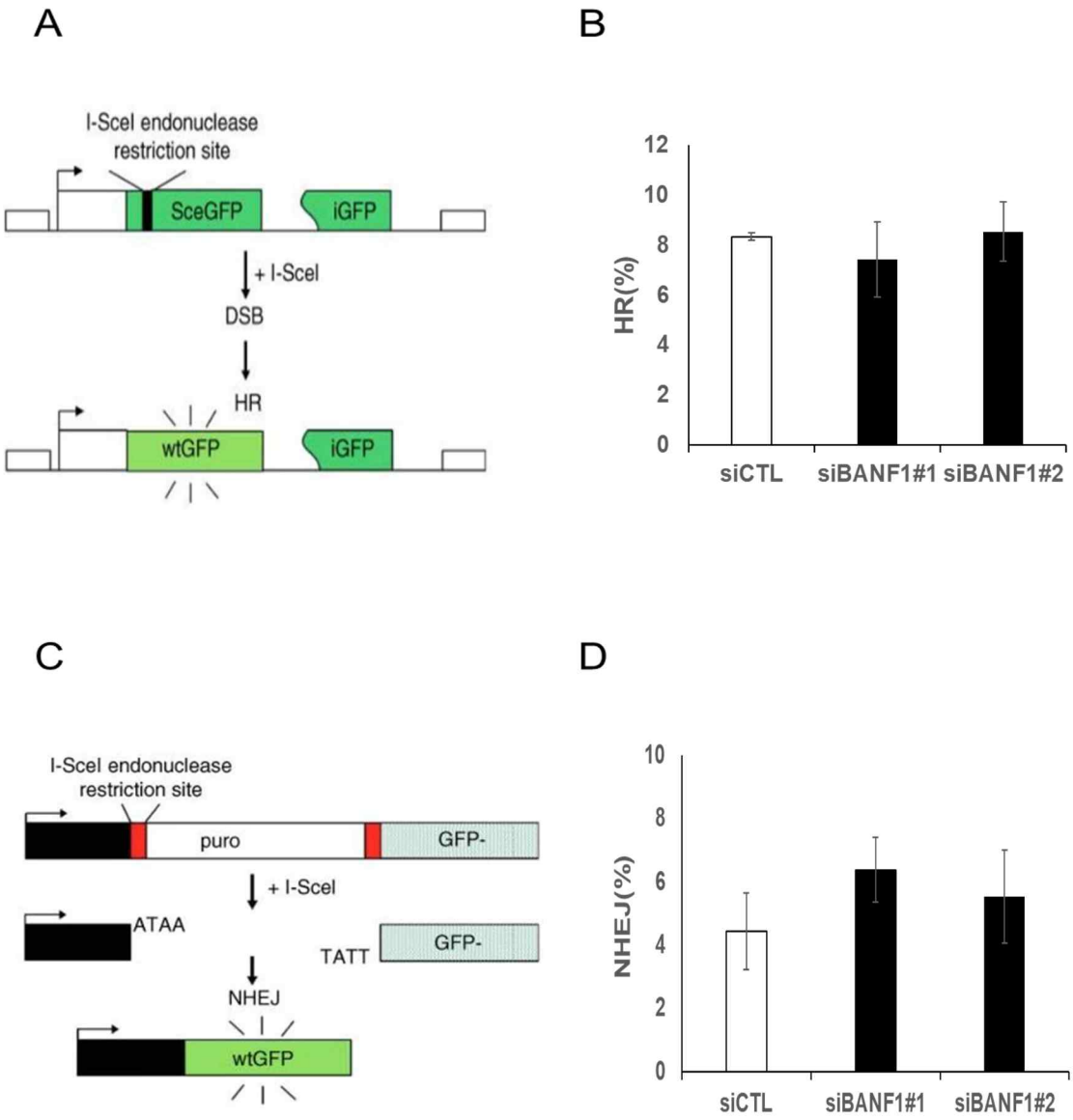

Figure 4. BANF1 does not affect HR/NHEJ.

(A) 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. (B) Control and BANF1#1, BANF1#2 siRNA were transfected in Hela cells that contained DR-GFP and then were transfected with with an I-SceI. Next, the cells were incubated for 3 days. The cells were fixed, and then were stained with heocast solution. (C) Schematic diagram of the NHEJ assay based on the EJ5-GFP reporter, which contains two tandem endonuclease cut site for the I-SceI. (D) Control and BANF1#1, BANF1#2 siRNA were transfected in HeLa EJ5-GFP cells, and then were transfected with an I-SceI. Next, the cells were incu-bated for 2 days. The cells were fixed, and then were stained with heocast solution.

Figure 5

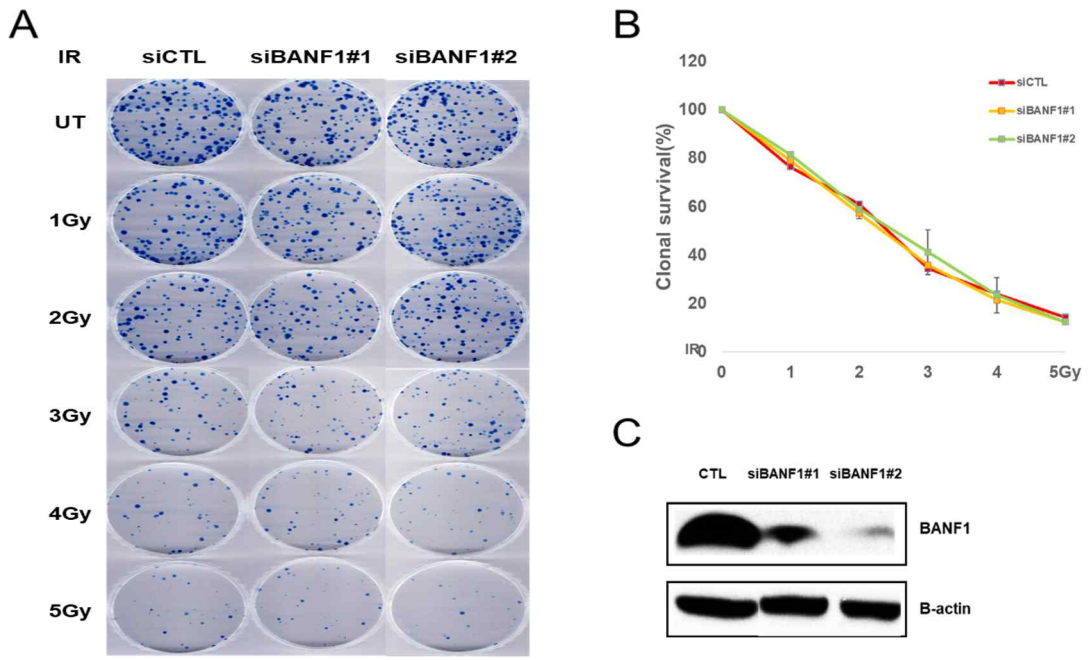

Figure 5. BANF1 does not affect clonal surviving to IR.

(A) Control and BANF1#1, #2 siRNA were transfected in Hela cells, then incu-bated for 2 days and then untreated or treated with 1, 2, 3, 4 and 5Gy IR. After 2weeks, cells were stained with methylene blue. (B) Quantification of cell viability fig5A. (C) Cells were transfected with control and BANF1#1, #2 siRNA. After 48hrs, the expression level of BANF1 was confirmed by western blot analysis using BANF1 antibody. β-actin was used as loading control.

Figure 6

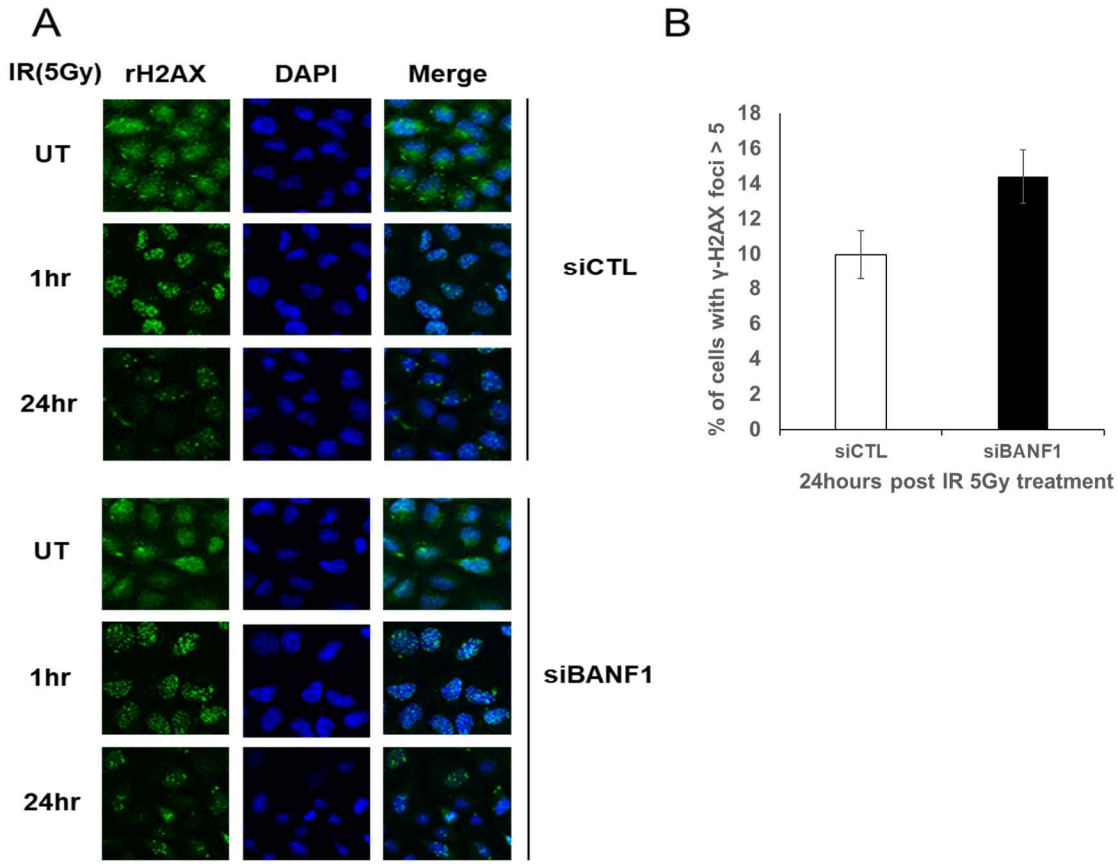

Figure 6. Depletion of BANF1 enhanced γ-H2AX foci formation to IR.

(A) Control and BANF1 siRNA were transfected in Hela cells, and then incubated for 2 days, and then untreated or treated with 5Gy γ-irradiation. Next, the cells were then fixed following indicated times after IR. Hela cells were stained using an anti-γ-H2AX antibody and DAPI solution. (B) Quantification of Fig6A.

Figure 7

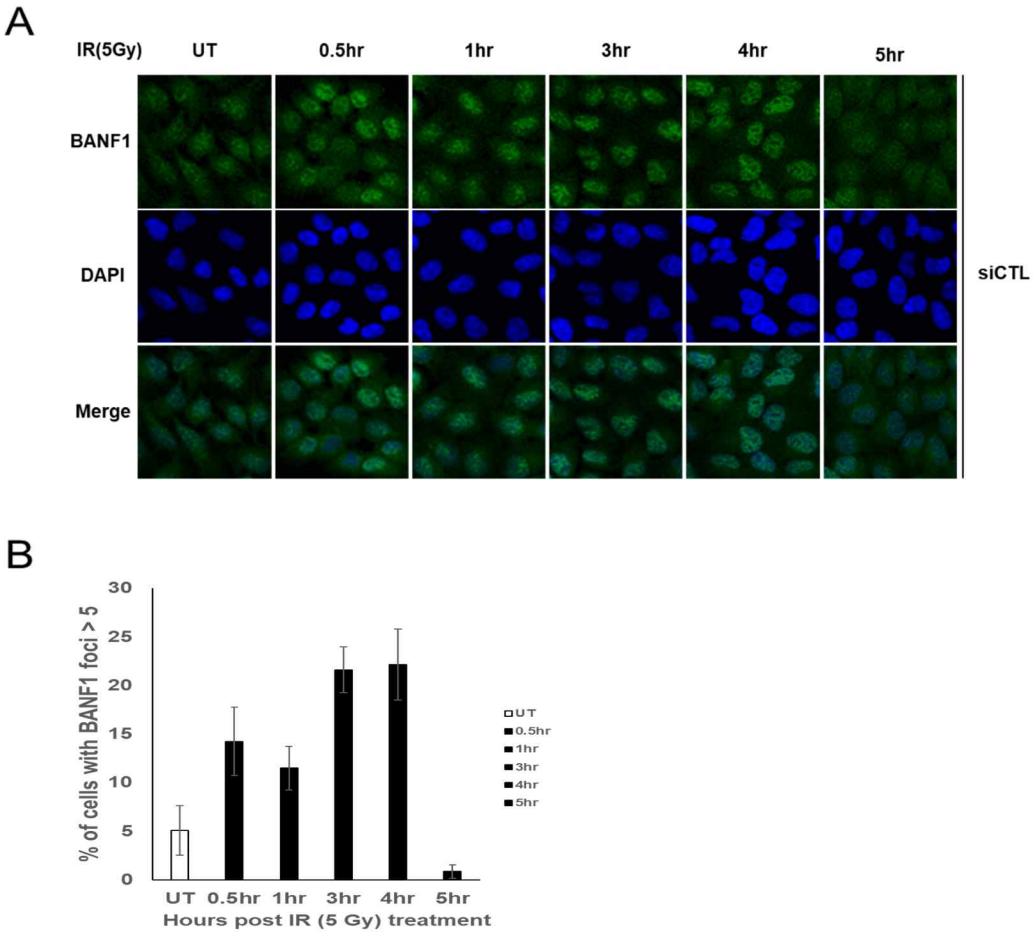

Figure 7. BANF1 responds to ionizing radiation (IR).

(A) HeLa cells were grown in 60mm dish for 2 days, then induced DNA damage by 5Gy γ -irradiation. Next, the cells were fixed indicated time after IR. Then the cells were stained using anti-BANF1 antibody and DAPI solution. (B) Quantification of Fig7A.

3. BANF1 interacts with MDC1 does not affect MDC1-mediated DNA damage response in HU.

Hydroxyurea (HU) induces replication stress by preventing ribonucleotides (NTPs) from converting to deoxyribonucleotides (dNTPs) as inhibitors of ribounucleotide products. [10, 11]. Continuous treatment of such HU leads to DSB. [12]. In general, ATMs are activated mainly in DSBs, whereas ATR is known to be mainly responsive to replication stress or blocking replication. [13]. When such a replication stress occurs, TopBP1 and RPA are recruited and stored in the replication fork part. RPA is known to stabilize the single strand DNA, and TopBP1 is known to play an important role in DNA replication and replication checkpoint control. The ATR activation domain in TopBP1 interacts directly with ATR-ATRIP to activate ATR kinase. TopBP1 also interacts with Rad9 phosphorylated by the N-terminal tendon BRCT1-2, which is known to be necessary for Chk1 activation. [14, 15]. It has been reported that H2AX/MDC1 is required as an upstream of

these TopBP1. [15]. Therefore, we know that MDC1 is recruitment in DNA damage site during HU treatment, so we confirmed whether BANF1 affects MDC1 recruitment during HU treatment. First, Control and BANF1 siRNA were transfected to Hela cells, then untreated or treated with 2mM HU, fixed after 6 hours and then stained with anti-MDC1 antibody. The results confirmed that BANF1 could not regulate MDC1 recruitment in HU-induced DNA damage (Figure8. A and B).

As mentioned earlier, when replication stress occurs and replication forks are formed, RPA is coated on ssDNA to stabilize ssDNA[15], We identified whether BANF1 affects RPA foci recruitment. First, the control and BANF1 siRNA were transfected in Hela cells, then untreated or treated with 2mM HU, fixed after 3 hours, and then stained using anti-RPA antibody. As a result, there was no difference in RPA foci with or without BANF1 (Figure9. A and B)

We actually performed clonal survival to confirm the survival of DNA

damage from HU based on the presence or absence of BANF1. First, control and BANF1#1, #2 siRNA ere transfected in Hela cells, and then untreated or treated with 1, 2, 3, 4, 5mM HU during 3 hours, then media change was carried out, incu-bated for 2~3weeks, and then cells were stained using methylene blue. As a result, it was confirmed that there was no significant difference in clonal survival at HU regardless of whether there was a BANF1 or not (Figure10. A and B).

We wanted to know if BANF1 responded to HU-induced DNA damage. so we fixed it according to the time zone shown after Hela cells with 2mM HU treat-ment and then stained it with anti-BANF1 antibody. As a result, BANF1 foci was formed the most in the 18 hours and then decreased, so we could estimate what BANF1 played in the HU (Figure11. A and B).

Figure 8

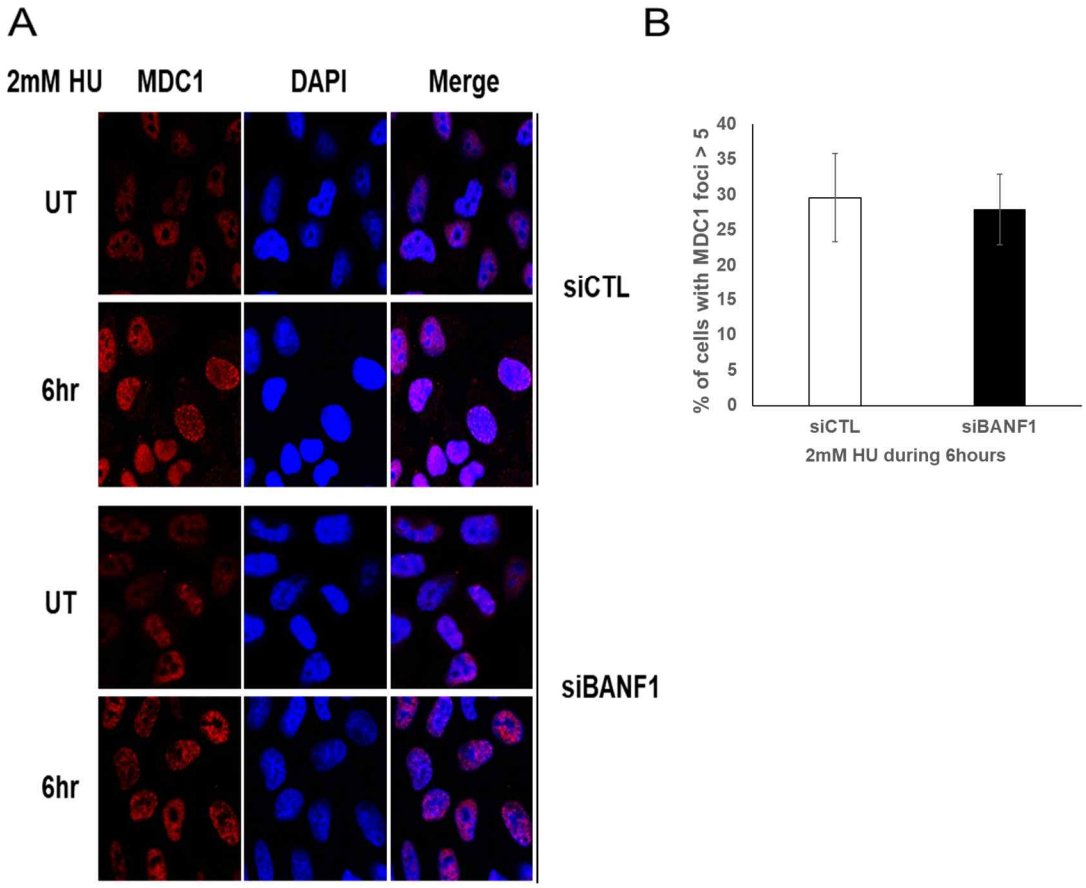

Figure 8. BANF1 does not affect MDC1 foci recruitment to HU.

(A) Control and BANF1-depleted cells were untreated or treated with 2mM HU during 6 hours in Hela cells, and then fixed. Hela cells were stained using anti-MDC1 antibody and DAPI solution. (B) Quantification of Fig8A.

Figure 9

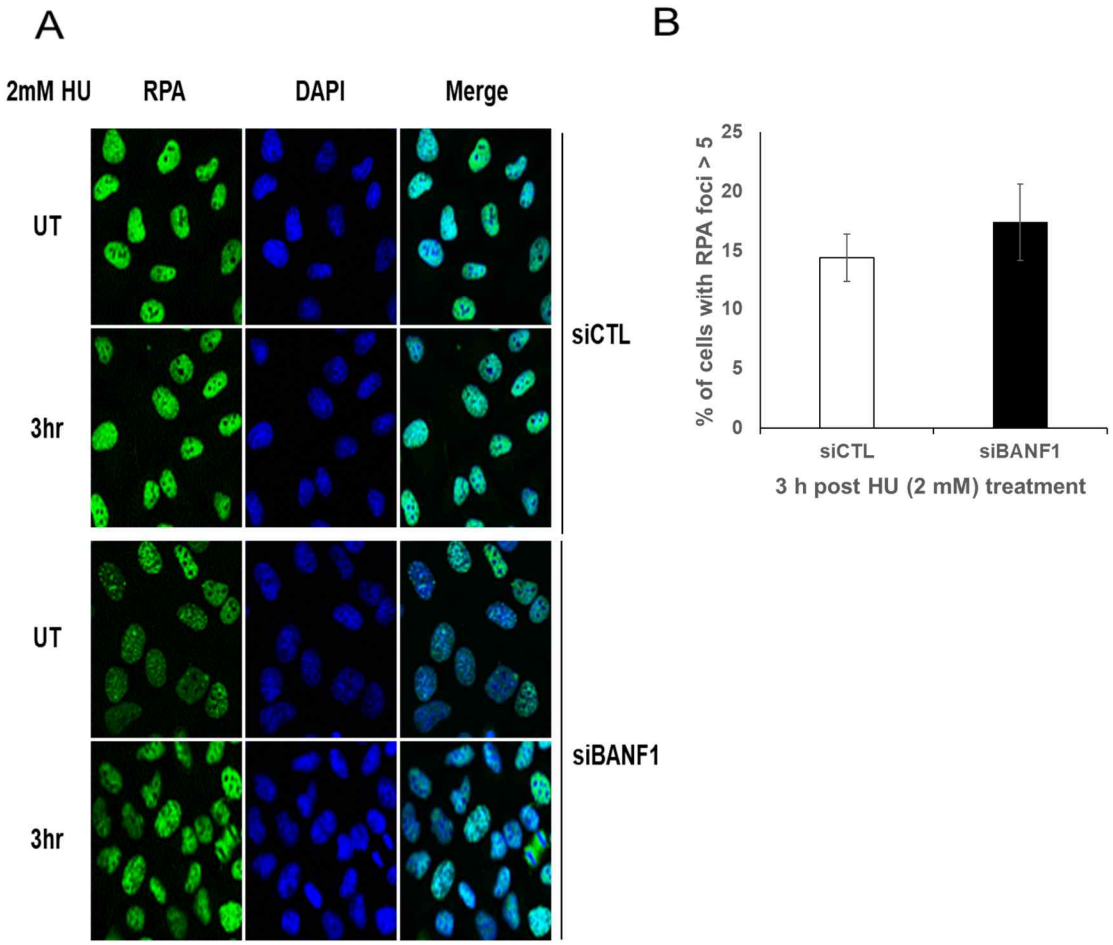

Figure 9. BANF1 does not affect RPA foci recruitment to HU.

(A) Control and BANF1-depleted HeLa cells were untreated or treated with 2mM HU during 3hrs, and then fixed. Hela cells were stained using anti-RPA antibody and DAPI solution. (B) Quantification of Fig9A.

Figure 10

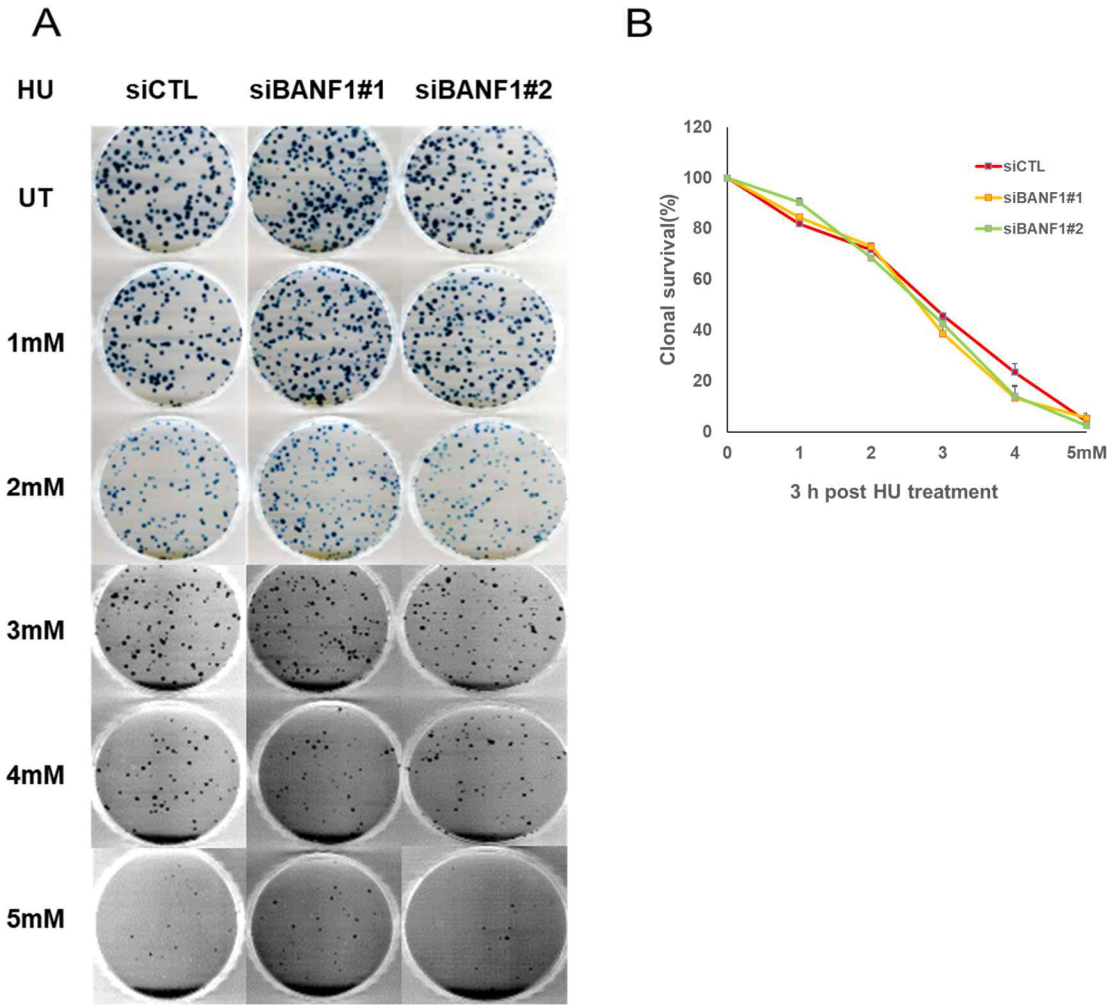

Figure 10. BANF1 does not affect clonal surviving to HU.

(A) Control and BANF1#1, #2 siRNA were transfected in HeLa cells, and then untreated or treated with 1, 2, 3, 4 and 5mM HU during 3 hours. After 2weeks, cells were stained with methylene blue. (B) Quantification of cell viability Fig10A.

Figure 11

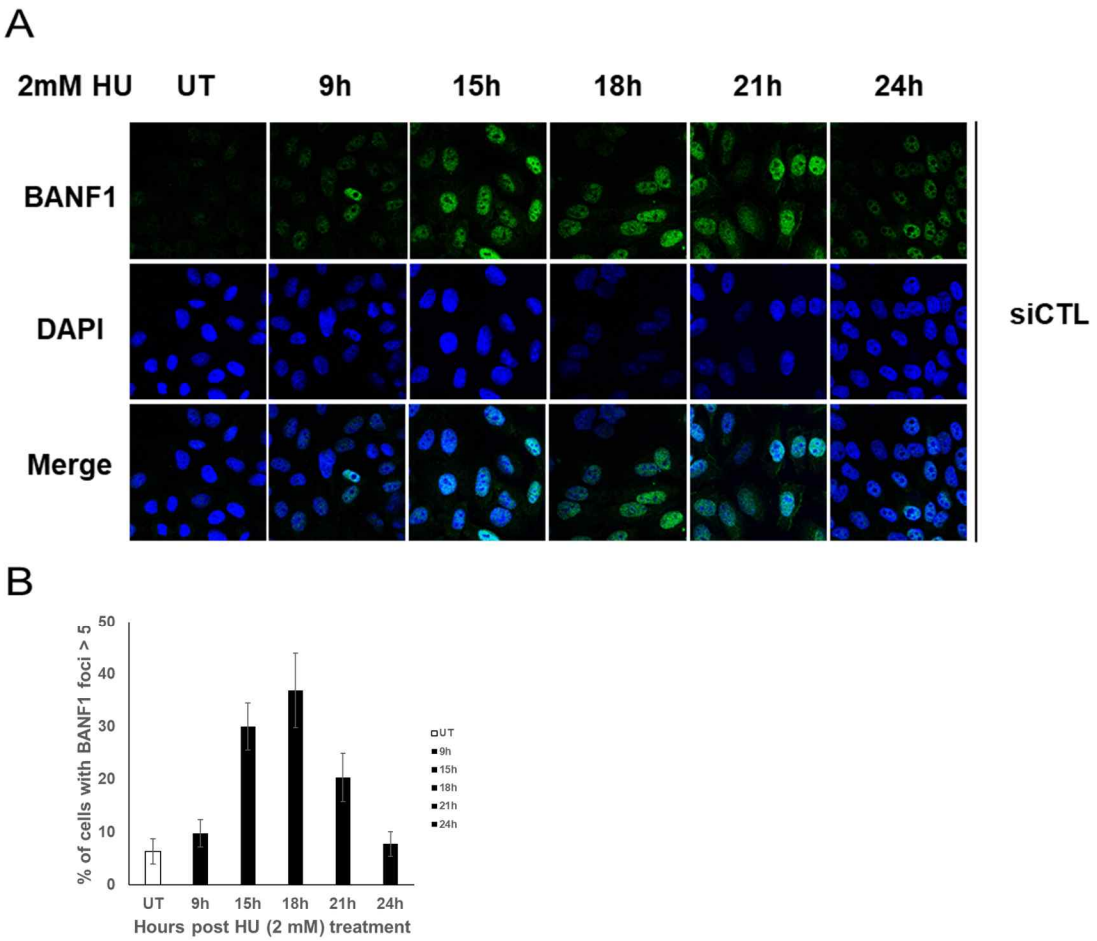

Figure 11. BANF1 responds to Hydroxyurea(HU)

(A) Hela cells were grown in 60mm dish for 2 days, and then fixed following in-dicated time 2mM HU. Then the cells were stained with anti-BANF1 antibody and DAPI solution. (B) Quantification of Fig11A.

4. BANF1 interacts with MDC1 regulates MDC1-mediated DNA damage response in CPT.

Camptothecin(CPT) is the replication stress drug such as the HU, but the mechanism works differently other than the HU. CPT inhibits the function of topoisomerase as a topoisomerase poison. Topoisomerase is an enzyme that eliminates the formation of supercoils by dissolving twisted double helix structures during replication. Therefore, when CPT is treated, the topoisomerase is inhibited, supercoil is formed during replication, and replication stress is generated[16, 17]. Similarly, during CPT treatment, MDC1 is recruitment with DNA damage site[17]. In order to confirm whether BANF1 regulates MDC1 recruitment during CPT treatment in Hela cells, we confirmed MDC1 foci by CPT treatment in cells with BANF1 and without BANF1, then cells were stained with anti-MDC1 antibody. As a result, it was confirmed that MDC1 foci was formed less when BANF1 was absent than when BANF1 was present (Figure12. A and B). Therefore, it is suggested

that BANF1 regulates MDC1 recruitment when DNA damage is generated by CPT.

When there is no BANF1 during CPT treatment, the recruitment of MDC1 as the DDR core protein is reduced, When BANF1 became knock down, I wanted to know if it affected cell viability during CPT treatment, so I conducted clonal survival. Control and BANF1-depleted cells were untreated or treated with 0.05, 0.1, 0.15 and 0.2 μM CPT during 3 hours. After 2 weeks, cells were fixed with Methyl alchohol and stained with methylene blue. As a result, it was confirmed that the cell's viability decreased in the absence of BANF1 (Figure13. A and B). This is thought to be due to the absence of BANF1, which reduces the recruitment of MDC1 to CPT-induced DNA damage sites, so CPT-induced DNA damage cannot be fully repaired and cell viability decreases.

To confirm whether BANF1 actually responds to CPT-induced DNA dam-age, we treated in Hela cells with 1 μM CPT, fixed at indication time, and then stained with anti-BANF1 antibody. As a result, BANF1 foci was found the most in

the 6 hours after CPT treatment and disappeared later, confirming that BANF1 played a role in the CPT-induced DNA damage (Figure14. A and B). The results of Figure 12~14 suggest that BANF1 can regulate MDC1-mediated repair pathways during CPT-induced DNA damage.

MDC1 is required to recruitment of TopBP1 when replication stress occurs, and TopBP1 also affects replication control and activation of Chk1, a cell cycle checkpoint-related protein..[15]. Therefore, in our experimental results, BANF1 can also affect the activation of Chk1 as the downstream function of TopBP1 because the recruitment of MDC1 as the upstream of TopBP1 is reduced without BANF1 during CPT treatment. Therefore, we confirmed whether the presence or absence of BANF1 affects Chk1 activity during CPT treatment. First, after transfecting siRNA-Control, BANF1 # 1, both BANF1 # 1 and Flag-BANF1 in U2OS cells, untreated or treated 1 μM CPT during indicated times, the Chk1 phosphorylation level was confirmed by Western blotting. As a result, it was confirmed that

Chk1's phosphorylation decreases when BANF1 is not present, and that Chk1's phosphorylation increases again when BANF1 is recovered (Figure 15).

Figure 12

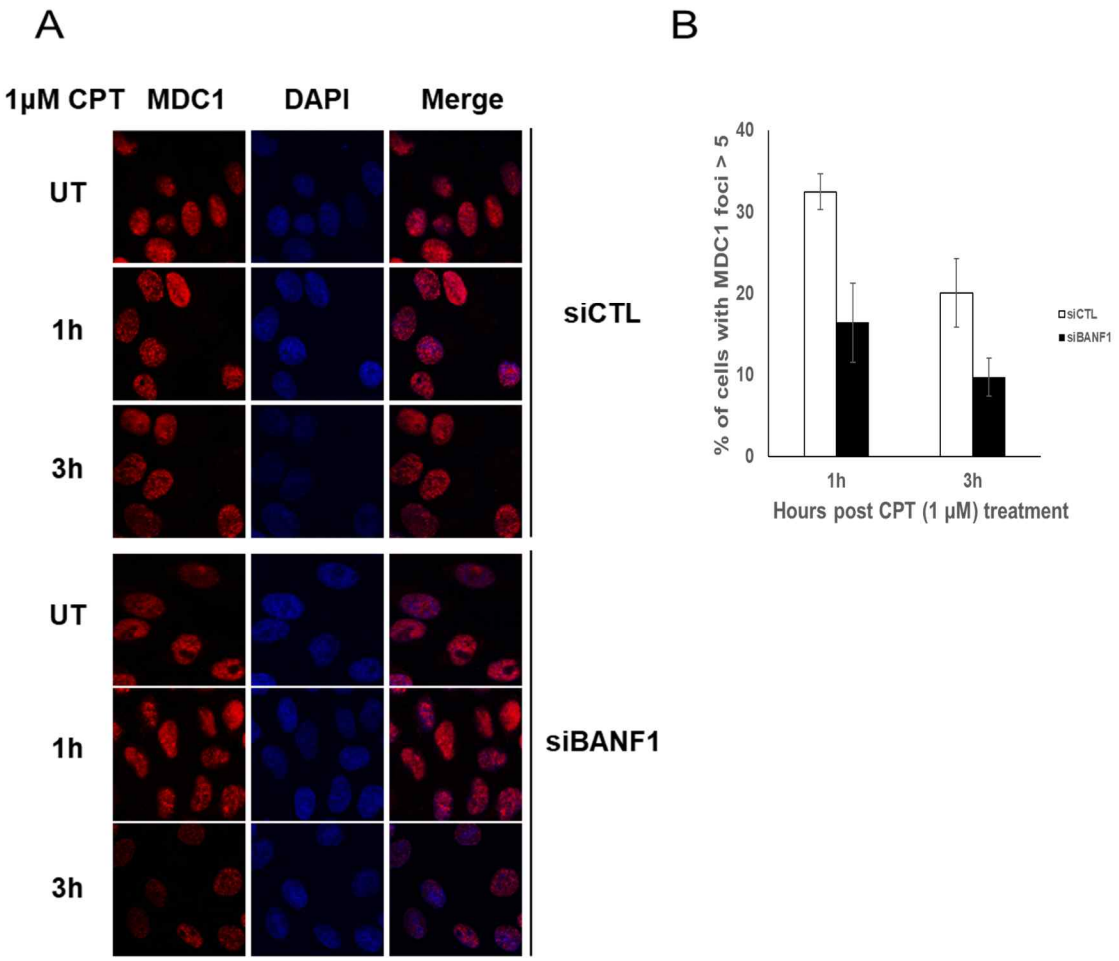

Figure 12. Depletion of BANF1 decrease MDC1 foci recruitment to CPT.

(A) Control and BANF1 siRNA were transfected in Hela cells, and then incubated for 48 hours. Next, the cells were untreated or treated following indicated time 1 μM CPT, and then fixed. MDC1 protein and nuclei in Hela cells were stained with each anti-MDC1 antibody and DAPI solution. (B) Quantification of Fig12A.

Figure 13

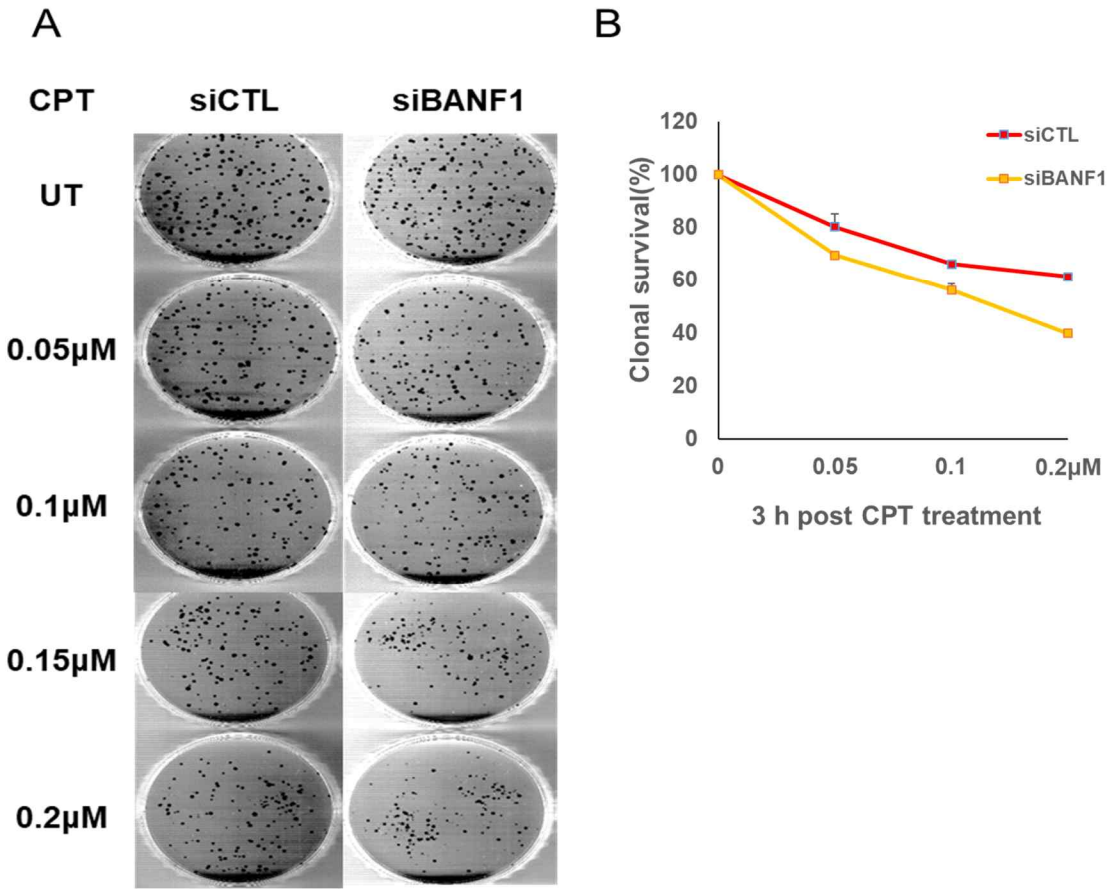

Figure 13. Depletion of BANF1 decrease clonal surviving to CPT.

(A) Control and BANF1-depleted Hela cells were untreated or treated with 0.05 μ, 0.1 μ, 0.15 μ and 0.2 μM CPT during 3 hours. After 2 weeks, cells were fixed with Methyl alcohol. Next, the colonies were stained with methylene blue. (B) Quantification of cell viability Fig13A.

Figure 14

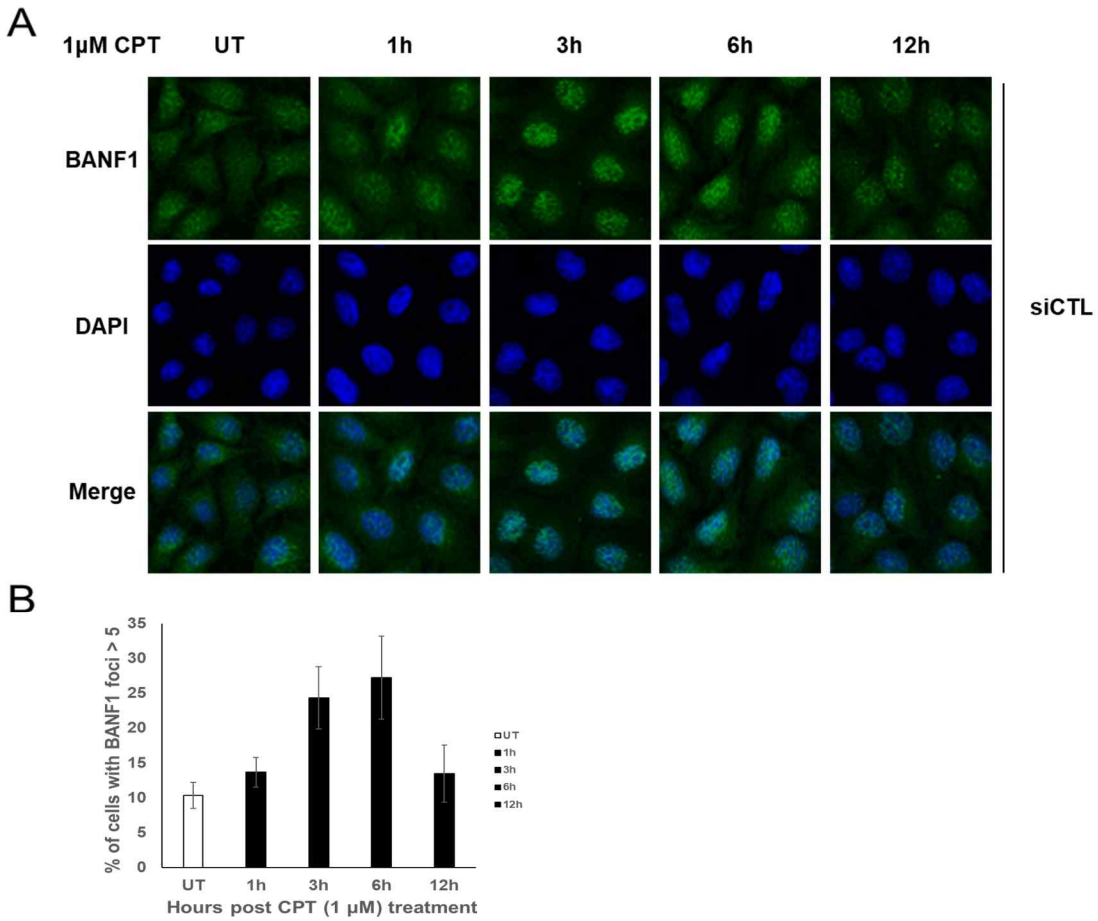

Figure 14. BANF1 responds to Camptothecin (CPT).

(A) Hela cells were grown in 60mm dish for 2days, then induced DNA damage by 1μM CPT treatment. Next, the cells were fixed following indicated time after CPT treatment. BANF1 proteins and nuclei in Hela cells were stained with each anti-BANF1 antibody and DAPI solution. (B) Quantification of Fig14A.

Figure 15

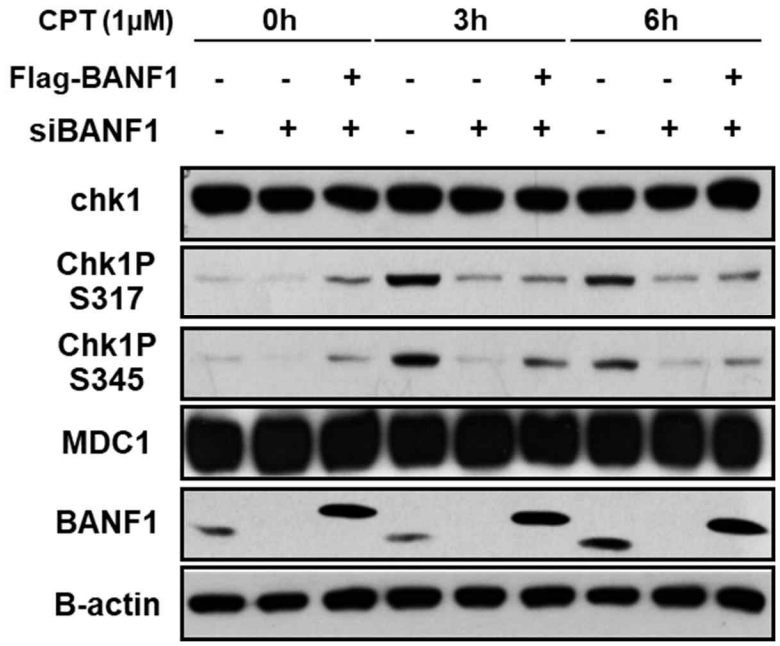

Figure 15. BANF1 regulates Chk1 phosphorylation in CPT treated cells.

SiRNA-control and siRNA-BANF1 and siRNA-BANF1 with Flag-BANF1 ex-pression vector were transfected in U2OS, and then untreated or treated with 1 μM CPT during indicated times. U2OS cells were subjected to western blotting using antibodies anti-Chk1, anti-Chk1p S317, anti-Chk1p S345, anti-MDC1, anti-BANF1, anti-β-actin.

DISCUSSION

DNA damage occurs during radiation, chemical reagents, or cell replication. This DNA damage induces genome instability and causes cell death or cancer. Therefore, cells use DNA damage response (DDR) systems to maintain genome stability. DNA damage response (DDR) system refers to DNA damage sensing by ATM and ATR, histone H2AX is phosphorylated by ATM and ATR to γ-H2AX, and MDC1 is then recruited to γ-H2AX. This early signaling always occurs regardless of cell cycle[18]. In mammalian cells, Typical repair pathways in the event of a double strand break (DSB) include homologous recombination (HR) and non-homologous end-joining (NHEJ). After MDC1 / γ-H2AX, HR repair pathway occurs when BRCA1 is recruited in the downstream, and NHEJ occurs when 53BP1 is recruited[19]. In DNA damage such as Replication Stress, early signaling occurs, MDC1/γ-H2AX, and BRCT5 of TopBP1 interacts with MDC1. TopBP1 is required to coat ssDNA with RPA. After that, TopBP1 and

ATR/ATRIP are interacted, and ATR kinase is activated. Activated ATR kinase is required for Chk1 phosphorylation and activation. Control the replication checkpoint in the process[15].

MDC1, one of the core DDR proteins, is a scaffold protein known for its involvement in DNA damage recognition, DNA repairing, and cell cycles[20]. We identified BANF1 interacting with MDC1 by Yeast-two-hybrid. BANF1 is a highly conserved DNA-binding protein that has been reported to be involved in various pathways, including mitosis, nuclear assembly, viral infection, chromo-some and gene regulation, and DNA damage response[6, 7]. It is not known how the interaction between the DDR core protein MDC1 and BANF1 is related to DDR. We treated IR and HU and CPT to investigate if BANF1 interacts with MDC1 can regulate DDR functions of MDC1.

First, the expression vector was used to confirm with Exogenous that BANF1 was interactive with MDC1 (Figure1. A). Check which domain BANF1

interacts with MDC1. As a result, it was confirmed that BANF1 interacts with PST domain(1130-1661bp) of MDC1 (Figure1. B and C).

First, BANF1 does not affect MDC1 recruitment in γ-H2AX generated by IR (Figure2. A and B). Then, after MDC1/γ-H2AX, which is early signaling in IR, BRCA1 and 53BP1 foci were observed as Downstream. As a result, it was confirmed that BRCA1 and 53BP1 foci decreased when BANF1 was depleted (Figure3. A and B). Since BRCA1 and 53BP1 foci decreased, homologous recombination (HR) and non-homologous end-joining (NHEJ) were finally measured using DR-GFP cells and EJ5-GFP cells. As a result, the choice of HR and NHEJ pathways was not affected with or without BANF1 (Figure4. A ~ D). BANF1 regulates BRCA1 and 53BP1 foci recruitment, but BANF1 may have affected HR/NHEJ on MDC1 independent via unknown pathways. When IR was treated, there was no difference in clonal survival with or without BANF1 (Figure5. A and B). However, in the absence of BANF1, the DNA damage marker

γ-H2AX foci increased slightly (Figure6. A and B). This increased γ-H2AX foci from Hela cells at IR Treat, but eventually did not affect clonal survival, so BANF1 was considered to have affected the final effect, clonal survival, through MDC1-independent unknown athways. In IR Treat, BANF1 recruited into the nucleus (Figure7. A and B) but does not significantly affect the DDR function of MDC1, so it can be inferred that BANF1 functions as an MDC1-independent pathway in IR.

BANF1 did not affect MDC1 foci recruitment to γ-H2AX by interacting with MDC1 during HU Treat (Figure8. A and B). In addition, BANF1 did not affect RPA foci recruitment after MDC1/γ-H2AX signaling in HU (Figure9. A and B). It didn't affect the final effect, clonal survival (Figure10. A and B). However, it can be inferred that HU-induced MDC1 DDR pathway plays an independent role as BANF1 foci gathers in the nucleus during HU Treat (Figure11. A and B).

It was confirmed that BANF1-depleted Hela cells reduced MDC1 foci to

γ-H2AX during CPT treatment (Figure12. A and B). This indicates that BANF1 can regulate MDC1 recruitment through CPT-induced DNA damage through interaction with MDC1. Finally, it was confirmed that clonal survival decreased when BANF1 was depleted (Figure13. A and B). It can be thought that when BANF1 is depleted, MDC1 recruitment decreases, CPT-induced MDC1 DDR function decreases, and thus cell viability decreases. In fact, BANF1 recruited into the nucleus during CPT treatment (Figure14. A and B), suggesting that BANF1 interacts with MDC1, which may affect MDC1 DDR function. One of the CPT-induced MDC1 DDR functions is to control the cell cycle checkpoint. Chk1 regulates cell cycle by becoming phosphorylation as a cell cycle checkpoint factor, but when BANF1 is depleted, Chk1 phosphorylation decreases, and when BANF1 is recovered, Chk1 phosphorylation recovers (Figure 15). This suggests that BANF1 regulates MDC1-mediated DDR function through interaction with

MDC1 during CPT treatment. These results show that the BANF1-MDC1 interaction does not significantly affect the DDR function of MDC1 in the DNA damage type for IR and HU, but can regulate the MDC1 DDR function by regulates the recruitment of MDC1 in the CPT-induced DNA damage.

ABSTRACT

BANF1 regulates MDC1-mediated DNA damage response

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MDC1, one of the core proteins in DNA damage response, is known to be involved in damage recognition, recovery, and cell cycle regulation when DNA damage occurs. In this study, we sought to elucidate molecular mechanism of MDC1-related DNA damage response. We identified a novel binding protein of MDC1, BANF1, through yeast two-hybrid screening assay. BANF1 is a highly

conserved DNA-binding protein that has been reported to be involved in mitosis, nuclear assembly, viral infection, chromatin and gene regulation, and DNA damage response. However, it is not clear the mechanism of DNA damage. In this study, it was determined whether BANF1 regulates MDC1 mediated DNA damage response by interaction of MDC1. First, we showed that depletion of BANF1 not influence to MDC1 recruitment, DSB repair including NHEJ/HR, sensitivity in IR treatment. To the next, effect of BANF1 in MDC1-mediated response after Hydroxyurea treatment is investigated. BANF1-depleted cells show no difference in MDC1 and RPA recruitment, sensitivity of HU, compared control cells. Therefore, it is found that BANF1 is independent of MDC1 function in IR and HU treated cellular re-sponse. However, we showed that knockdown of BANF1 results in hypersensi-tivity and decrease the MDC1 damage foci to Camptothecin (CPT). Moreover, BANF1 depleted cells show reduced phosphorylation of Chk1 after CPT treatment, which may contribute to DNA damage checkpoint bypass. We also found that

BANF1 is recruited to damage site to CPT treatment until 6 hours and then de-creased. Collectively, our findings demonstrate a crucial role for BANF1 in CPT damage-mediated response through interaction of BANF1 and MDC1.

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