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NME1 regulates homologous recombination through interaction with BRCA2

조선대학교 대학원

의과학과

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국문초록

NME1의 DNA손상에 따른 상동재결합 활성조절연구

임 지 연

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BRCA2 단백질은 DNA 손상복구, 중심체의 조절, 세포질 분열, 전사, 세포주기 조절 및 DNA의 상동재조합 과정등을 조절함으로써 유전체 안정성 유지에 역할을 수행한다. 그러나 이런 BRCA2 자체의 기능을 조절하는 기전에 대한 연구는 미흡한 실정이다. 따라서, BRCA2 기능을 조절하는 단백질을 찾고자 BRCA2를 베이트로 yeast two-hybrid를 실시하였고, 그 결과 몇몇 새로운 결합단백질을 동정하였다. 본

연구에서는 동정된 단백질 중의 하나인 NME1이 BRCA2와 결합함으로써 BRCA2의 세포 내 여러 기전 중 상동재결합 활성에 미치는 영향을 조사하였다. 먼저, BRCA2 단백질이 생체 내에서 NME1과 결합함을 확인하였고, 두 단백질간의 상호작용이 방사선 조사와 DNA 복제손상 자극인 Hydroxyurea 처리에 의해 증가되는 것을 확인하였다. 세포 내 NME1의 손실은 BRCA2와 상동재결합 활성효소로 알려진 RAD51이 DNA 손상부위로의 recruitment를 저해하였고, 이는 BRCA2, RAD51의 면역염색과 염색체분리후 western blotting을 통해 관찰하였다. Clonal survival assay, comet assay, γ-H2AX 면역 염색법등을 통해 NME1이 결핍된 세포에서 DNA 손상복구 활성이 저하됨을 관찰하였다. 또한, NME1 결핍에 의한 DNA 손상복구 저하가 BRCA2의 상동재결합 활성 저하에 의한 것임을 DR-GFP 벡터 시스템을 통해 확인하였다. 따라서, 본 연구결과는 BRCA2의 상동재결합 활성을 조절하는 새로운 조절인자 NME1을 동정함과 동시에 상동재결합 활성을 조절하는 새로운 기전을 제시하였다.

INTRODUCTION

DNA double-strand breaks (DSBs), which are generated through ionizing radiation (IR) and through DNA-damaging chemicals, are the most dangerous DNA lesions, because if they are not repaired, they can results in mutations, genomic rearrangements, and cell death, which can leads to cancer[1]. DSBs are repaired by two major pathways, non- homologous end-joining(NHEJ) and homologous recombination(HR), whose relative contributions are strongly cell cycle dependent[2]. NHEJ is a pathway for DSB repair in which broken ends are healed without the requirement for significant sequence homolog. Therefore, NHEJ is error-prone pathway. The core NHEJ machinery includes the end-binding heterodimeric proteins-Ku70/Ku80, DNA-PKcs protein kinase, and the complex consisting of DNA ligase IV, XRCC4 and XLF[3]. However, for complex breaks, which have mismatched or covalently modified DNA ends, additional factors are required to modify the ends and facilitate their ligation. These factors

include Artemis, DNA polymerases λ and μ, terminal dinucletidyltrasferase, polynucleotide kinase-phosphatase, aprataxin (APTX) and APTX-polynucleotide kinase-phosphatase-like factor1[4].

On the other hands, HR uses homologous DNA sequences as a template for repair. So, HR is an error-free repair pathway. Compared to NHEJ, HR repair is a relatively slow process that involves a coordinated series of complex events[5]. Non-homologous end joining is the main mode of repair during G0/1 phases of the cell cycle, whereas HR only a modest portion of directs DSBs oc-curring in S and G2 cells. HR appears to be critical for repair in heterochroma-tin[6]. The DSBs in S and G2 cells that are repaired by HR repair are resected in multi-step processes that include MRN, CtIP, EXO1, and DNA2 nucleases to-gether with the BLM helicase. BRCA1 acts during the early stages of HR repair by facilitating initiation of end resection and also by recruiting PALB2 and BRCA2, which initiates and regulates RAD51 filament formation on ssDNA by

displacing RPA[7]. These filaments undergo homology search and subsequent invasion into homologous duplex DNA to form a D-loop structure, where they serve as a primer for DNA synthesis. After the extended end is displaced from the D-loop, it anneals to its partner-end to complete DSB repair[8]. DNA repair is essential for maintaining genome integrity, but its dysfunction leads to tumor-igenesis. As such, many DNA damage repair proteins are tumor suppressor. Therefore, BRCA2 is typical example.

BRCA2 was originally identified as a tumor suppressor, as germline mutation of the BRCA2 gene results in a high risk of developing breast, ovarian, pancreatic, prostatic, and male breast cancer[9]. Mutations of BRCA2 are also associated with other types of cancer such as prostate and pancreatic cancers[10]. BRCA2 are involved in maintaining genome integrity, DNA repair, cell cycle checkpoint control and even the regulation of key mitotic or cell division steps. Functions of BRCA2 in controlling genome integrity, which are primarily mediated through its

binding to the Rad51 recombinase, an essential enzyme that plays central roles in faithful repair of DNA double-strand breaks (DSBs), which facilitate the recruitment of Rad51 to sites of DSBs and the subsequent stimulation of DSB repair by homologous recombination. BRCA2 comprises an N-terminal region, a central region with eight BRC (Breast cancer) repeats, three tandem oligonucleotide binding folds (OB-folds), and a C-terminal motif[11]. The N-terminal region of BRCA2 is known to associate with PALB2, whereas the eight BRC repeats and the C-terminal motif are important for the interaction with RAD51[5]. BRCA2 is able to enhance RAD51 presynaptic assembly on RPA-coated ssDNA, leading to RPA-RAD51 exchange by promoting RAD51 binding to ssDNA, limiting binding of RAD51 to dsDNA, increasing the rate of RPA displacement from ssDNA by RAD51, and inhibiting RAD51's ssDNA-dependent ATP hydrolysis activity[7, 12]. At filament formation process of RAD51 also requires each of the five RAD51 paralogs, DSS1 (deleted in split hand/split foot 1), and

BCCIP (BRCA2 and CDKN1A interacting protein). Strand invasion of a sister chromatid by the RAD51 filament, resulting in displacement loop (D-loop) formation and heteroduplex DNA, requires the concerted action of the RAD54 ATPase, RAD51AP1, and PALB2[7].

NME1 is a metastasis suppressor whose expression is reduced in highly metastatic melanoma and breast carcinoma cells, and which possesses the ability to suppress metastatic growth without significant impact on the transformed phenotype[13]. This protein exhibits a nucleoside diphosphate kinase (NDPK) activity that propose to maintain balance in nucleotide pools which may limit pro-mutagenic mismatches during DNA repair[14]. NME1 also exhibits a histidine kinase activity that implicated as inhibitor of cell motility[15]. Furthermore, this protein described its 3′ -5′ exonuclease activity and these DNA cleaving molecules are predominantly involved with maintaining genomic fidelity during DNA synthesis and repair[16, 17]. Recently, it was reported that NME1 was

regulated cytoskeletal and focal adhesion dynamics, and promoted genomic stability through functional interactions with nucleotide excision repair (NER) and DNA double-strand break repair (DSBR) [18].

In this study, we identified a novel BRCA2-interacting protein NME1 using yeast-two hybrid screening. We find that NME1 is associated with BRCA2 *in vivo*. Our results indicate that NME1-deficient cells affect recruitment of BRCA2 and RAD51 at DSB sites. Moreover, depletion of NME1 have a major DSB repair defect, shown by comet tail analysis and late γ-H2AX immunostaining. Based on the results, we purpose a critical role of NME1 in homologous recombination through association with BRCA2.

MATERIALS AND METHODS

1. Cell culture and treatment

The human cervix adenocarcinoma cell line HeLa, human osteosarcoma bone morphogenetic cell line U2OS and human embryonic kidney cell line HEK293T were cultured in DMEM medium supplemented with 10 % fetal bovine serum(FBS) and streptomycin (0.1 mg/ml), penicillin (100 units/ml). Cell growth was monitored under an inverted microscope. Upon reaching 70-80 % confluency, cells were digested with 0.5 % trypsin-EDTA before being passaged. All cell lines were maintained at 37 ° C and in 5 % CO2 at saturated humidity. 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 (Grammacell 3000 Elan irradiator, Best Theratronics) and allowed to recover at 37 ℃ incubator for various times. To induced replication stress, exponentially growing cells were exposed to 1 mM Hydroxyurea and al-

lowed to recover at 37 ° C for various times.

2. Plasmid construct

The full length NME1 cDNA was amplified from cDNA of HeLa cells by PCR using the NME1 primers 5′-ACC GAA TTC ATG GCC AAC TGT GAG-3′ (Forward primer) and 5′-GGG CTC GAG TCA TTC ATA GAT CCA-3′ (Reverse primer). The amplified NME1 cDNA construct was cloned into the expression vector pcDNA3 in frame with the HA tag. The NME1 sequence was confirmed by automated DNA sequencing.

3. Immunoprecipitation assay and Western blot analysis

Cells were lysed in RIPA buffer (50 mM Tris-HCl (pH 7.5), 150 mM NaCl, 1 % NP40, 0.5 % sodium deoxycholate(NaDOC), 0.1 % sodium dodecyl sulfate(SDS), 1 % TritonX-100) with protease inhibitors (Roche Diagnostic Corp.). 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 1hr with TBS-t (10mM Tris-HCl (pH 7.4), 150 mM NaCl and 0.1% Tween-20) containing 5 % skim milk and then in-cubated at 4° C 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 2hrs with peroxidase-conjugated secondary antibodies (1:4000) at RT. The membranes were washed six times for 10min, and developed using an enhanced chemiluminescence detection system (ECL; GE Healthcare, Buckinghamshire, UK). For the immunoprecipitation assay, aliquots of soluble cell lysates were precleared with G sepharose bead (GE Healthcare) and then incubated at 4° C for 3hrs. Next, the appropriate antibody was added, and incubated at 4° C for 12hrs. After the addition of fresh protein G sepharose beads, the reaction was incubated for 3hrs at room temperature. The beads were washed four times in RIPA buffer without protease inhibitor, resuspended in SDS sample buffer and boiled for 5min. The samples were then 1analyzed by western blotting using the

appropriate antibodies.

4. Immunofluorescence microscopy

To visualize nuclear foci, cells were grown on glass coverslips and were ir-radiated with ionizing radiation or Hydroxyurea. Cells were then washed twice with PBS, fixed with 4 % paraformaldehyde for 10min and ice-cold 98 % metha-nol for 5min, followed by permeabilization with 0.3 % Triton X-100 for 15min at room temperature. Next, the cover slips were washed three times with PBS and then blocked with 5 % BSA in PBS for 1hr. Whereas BRCA2 and Rad51 fixing is a little different. Cells were washed in PBS and fixed in 50 % methanol: 50 % acetone for 10min at -20° C, and then for 10min incubation in PBS with 5 % BSA and 0.1 % Tween 20[19]. The cells were single or double immunostained with primary antibodies against various proteins overnight at 4° C. Next, the cells were washed with PBS and then stained with Alexa Fluor 488 (green, Mo-lecular Probes) or Alexa Fluor 594 (red, Molecular Probes) conjugated second-

ary 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.

5. Chromatin Fractionation

HeLa and 293T cells were treated with 10 Gy IR or 1 mM HU and harvested. The pellet was resuspended and incubated for 10min in ice-cold buffer containing 10 mM Hepes-KOH (pH 7.9), 1.5 mM MgCl2, 10 mM KCl, 0.2 % triton and complete protease inhibitor cocktail (Roche Diagnostic Corp). The suspension was homogenized and the supernatant containing the cytoplasmic fraction was collected after centrifugation 15min at 400g at 4° C. The pellet was washed in ice-cold PBS, resuspended in cold buffer containing 420 mM NaCl, 20 mM Hepes-KOH (pH 7.9), 20 % glycerol, 2 mM MgCl2, 0.2 mM EDTA, 0.1 % triton,

0.5 mM DTT, and complete protease inhibitor cocktail (Roche Diagnostic Corp) and incubated on ice for 1hr. The supernatant containing the nuclear fraction was collected after 15min centrifugation at 18000g at 4° C. The chromatin-containing pellet was resuspended in cold PBS supplemented with 600 mM NaCl, 1 % N-octyl glucoside, and 125 units of DNase, incubated for 30min in an ultra-sonic bath; and centrifuged for 15min at 18000g at 4° C. Chromatin proteins were collected with the supernatant.

6. Clonal survival assay

After treatment with IR or HU, $1x10^3$ cells were immediately seeded onto a 60 mm dish in duplicated and grown for 2weeks at 37° C to allow colony for-mation. Colonies were fixed with 95 % methanol for 10min and stained with 2 % methylene blue in 50 % ethanol. The fraction of surviving cells was calculated as the ratio of the plating as efficiencies of treated cells to untreated cells.

7. comet assay

The alkaline version of the comet assay (single cell agarose gel electrophoresis) was performed as described previously. For the comet assay, HeLa cell was treated with 10 Gy IR by followed by incubation in culture medium at 37° C. Cells were then harvested ($1x10^5$cells/ml), homogenized with low-melting point agarose, spread on a microscope slide pre-coated with normal-melting-point agarose and covered with a coverslip. After 30min at 4° C, the coverslip was removed from the slides. Slides were submerged in cold lysis solution (2.5 M NaCl, 0.1 M EDTA, 10 mM Tris-HCl (pH 10), 10 % dimethylsulfoxide and 1 % Triton-X100) for 1hr. After lysis, the slides were placed in an electrophoresis chamber, covered with electrophoresis buffer (300 mM NaOH, 200 mM EDTA, pH >13) and left for 30min for the DNA to unwind. The electrophoresis ran for 30min (1 V/cm tank length), after which the slides were and fixed in 70 % ethanol for 5min. air-dried and neutralized slides were stained with 30-50 ul ethidi-

um bromide (20 mg/ml). Cells were screened per sample in a fluorescent microscope. Average comet tail moment was scored for 40-50 cells/slide using a computerized image analysis system (Komet 5.5; Andor Technology, South Windsor, CT, USA).

8. Homologous recombination assay (DR-GFP assay)

To measure the HR repair, stable cell lines expressing DR-GFP reports were generated by transfection using lipofectamine 2000 (Invitrogen). U2OS-DR-GFP cells were transfected with control or NME1 siRNA, and then transfected with 3 μg of I-SceI-expressing vector. After 43hr, cells were fixed 4 % paraformaldehyde and stained 5μg/ml Hoechst (Sigma) for 2hrs. The images were shown at x10 magnification using an inverted fluorescence microscope (Nikon). The data are presented as the mean ± SD. value in three independent experiments.

RESULT

1. Identification of NME1 as a BRCA2-associated protein

To explore the molecular mechanism of BRCA2 in homologous recombination (HR), we performed the yeast two-hybrid screen using the C-terminal fragment (2100-3418 amino acids) of human BRCA2 as the bait for novel BRCA2-interacting proteins (Figure 1A). Through this screen we identified novel putative BRCA2-associated proteins including NME1 (NM_000269), SIAH (NM_003031) and TRAF2 (NM_021138) (Figure 1B). Among these, NME1 was most relevant because NME1 is transcription factor and regulates genomic stability.

To confirm that BRCA2 and NME1 interact with each other under physiological conditions, we performed reciprocal co-immunoprecipitation experiments using endogenous proteins. HeLa cells were treated with 10 Gy IR or 1 mM HU to make DSB. After 3hrs, undamaged and damaged cells were lysed and extract-

ed by centrifugation. The whole cell lysates was immunoprecipitated with an anti-BRCA2 or NME1 antibody. Immunoprecipitates were subjected to western blotting with an anti-NME1 or BRCA2 antibody. As result shown in Figure 1C, BRCA2 and NME1 were indeed immunoprecipitated with each other. There was a stronger association between BRCA2 and NME1 after exposure to IR or HU, indicating a DNA damage-inducible association of these two proteins. These results suggest that the interaction between BRCA2 and NME1 may play role a DNA damage response.

Figure 1

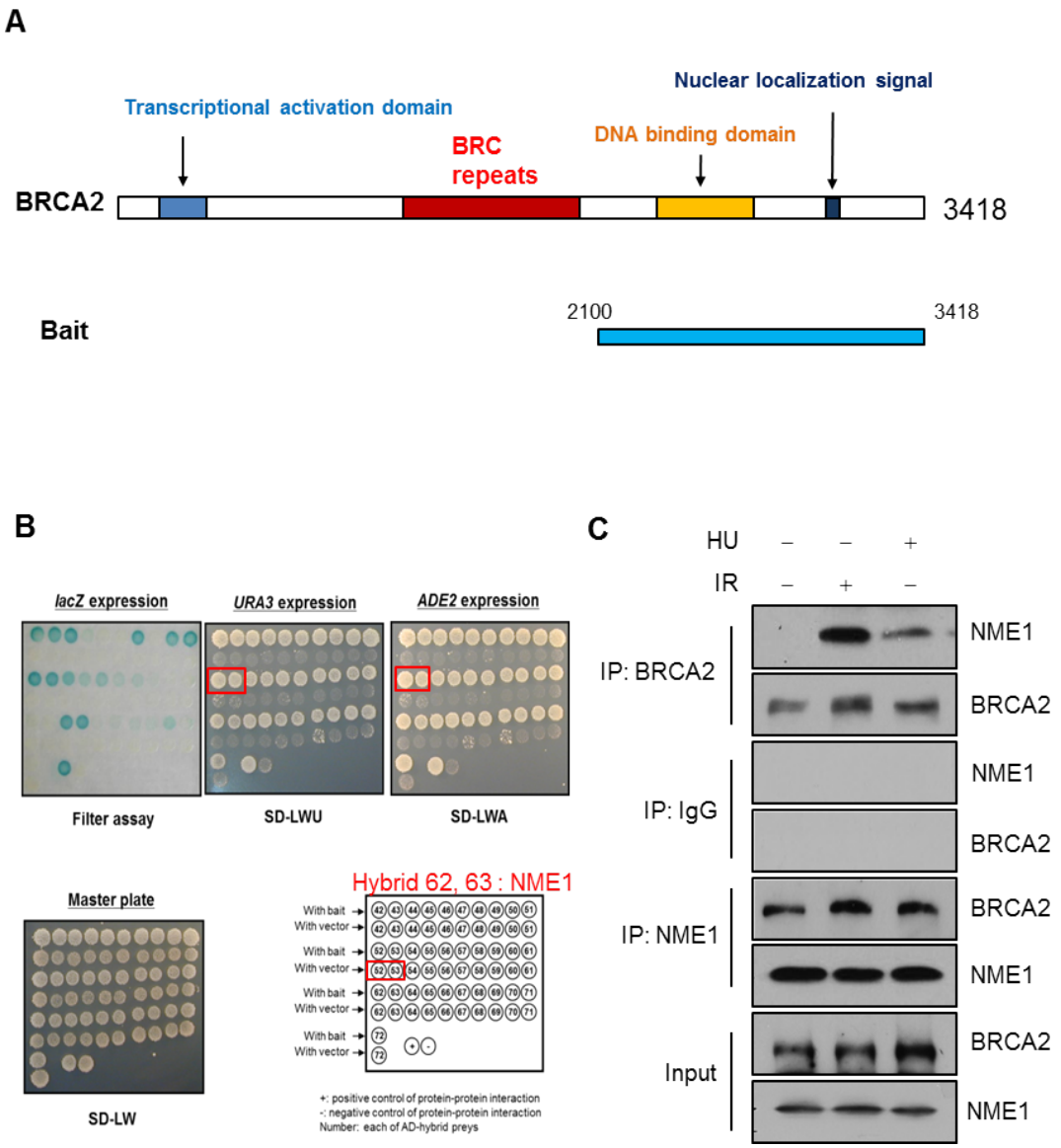


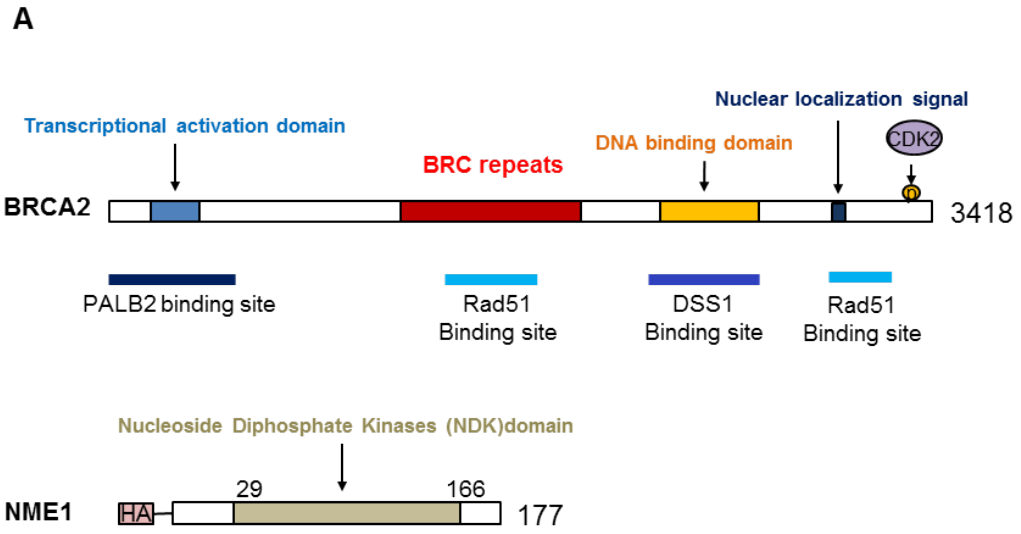
Figure 1. Endogenous NME1 interacts with BRCA2.

(A) Schematic representation of BRCA2 and bait construct for yeast two-hybrid assay. (B) Yeast two-hybrid assay using BRCA2 as bait. (C) HeLa cells was treated with 10 Gy IR or 1 mM HU to make DSB and harvested after 3hrs, un-treated cells was used as control. Total cell lysates were subjected to immuno-precipitation for NME1 or BRCA2 antibody followed by western-blot analysis using antibodies specific for BRCA2 or NME1. Normal rabbit IgG was used for negative control immunoprecipitations.

The interaction between BRCA2 and NME1 was further confirmed by ex-ogenous co-immunoprecipitation. The full length NME1 cDNA was amplified from HeLa cells by PCR using the NME1 primers. The amplified NME1 cDNA construct was cloned into expression vector pcDNA3 in frame with the HA tag (Figure 2A). The BRCA2 cDNA construct was cloned into the untagged expres-sion vector pcDNA3. HeLa cells were transiently co-transfected with BRCA2 and HA-NME1. Cells were harvested and lysed at 48hrs after transfection, was immunoprecipitated with a BRCA2 specific antibody. Immunoprecipitates were subjected to western blotting with an anti-HA antibody. HA-tagged NME1 co-immunoprecipitated with BRCA2 Immunoprecipitation results through western blotting showed us the BRCA2 binding to HA-NME1 (Figure 2B). The reciprocal experiment was performed, HA antibody was co-immunoprecipitated with BRCA2. The rabbit IgG was used for negative control immunoprecipitations. This protein interaction did not require the presence of DNA and interaction of two

proteins occurred in nucleus (data not shown). These results suggested that BRCA2 forms a physiological complex with NME1 *in vivo*.

Figure 2



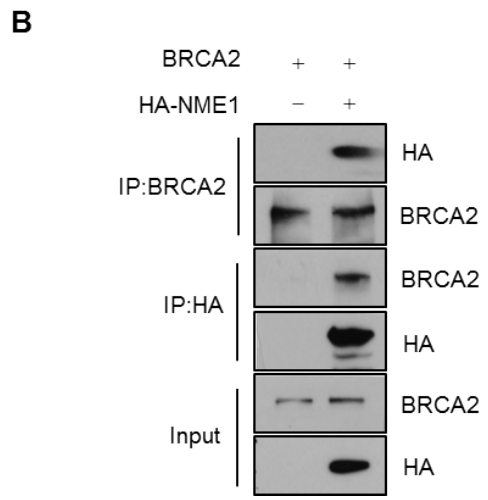


Figure 2. Exogenous NME1 interacts with BRCA2.

(A) Schematic representation of BRCA2 full length (upper) and NME1 with the HA tag (lower). The position of transcriptional activation domain (sky blue), the BRC motif (red), the DNA –binding domain (yellow) and the nuclear localization signal (dark blue). The indicated BRCA2-interacting proteins are shown under the corresponding region of BRCA2 required for their association. NDK domain of NME1 (29-166 aa) is noted. (B) Co-Immunoprecipitation of BRCA2 and HA-NME1. HeLa cells were co-transfected with BRCA2 and HA-NME1, and after 48hr harvested. Indicated total lysates were immunoprecipitated using a BRCA2 or HA antibody. Immunoprecipitates were then subjected to western-blot analysis using antibodies specific for HA or BRCA2. Normal rabbit IgG was used for negative control immunoprecipitations.

2. NME1 is required for the recruitment of BRCA2 at DNA damage site.

Because an interaction was founded between BRCA2 and NME1, we reasoned that NME1 might control DNA-damage induced BRCA2 foci formation. To address this point, we deleted U2OS cells for NME1 by siRNA and examined the localization of BRCA2 at sites of DNA damage. Immunoblotting confirmed that the expression of NME1 was reduced in cells transiently transfecting with NME1 siRNA, as compared to that of control siRNA-transfecting cells (Figure 3A). We investigated with BRCA2 foci formation after DSB in control and NME1-depleted U2OS cells. Control and NME1-depleted cells were treated with 10 Gy IR to make DSB and harvested after 12hrs. Cells were fixed with 50 % methanol: 50 % acetone and immunofluorescence assay performed. Our observation showed that depletion of NME1 decreased the BRCA2 foci formation as compared with control cells (Figure 3B and C). Together these finding suggest that NME1 is required for the recruitment of BRCA2 to sites of DSB after DNA damage occurs.

Figure 3

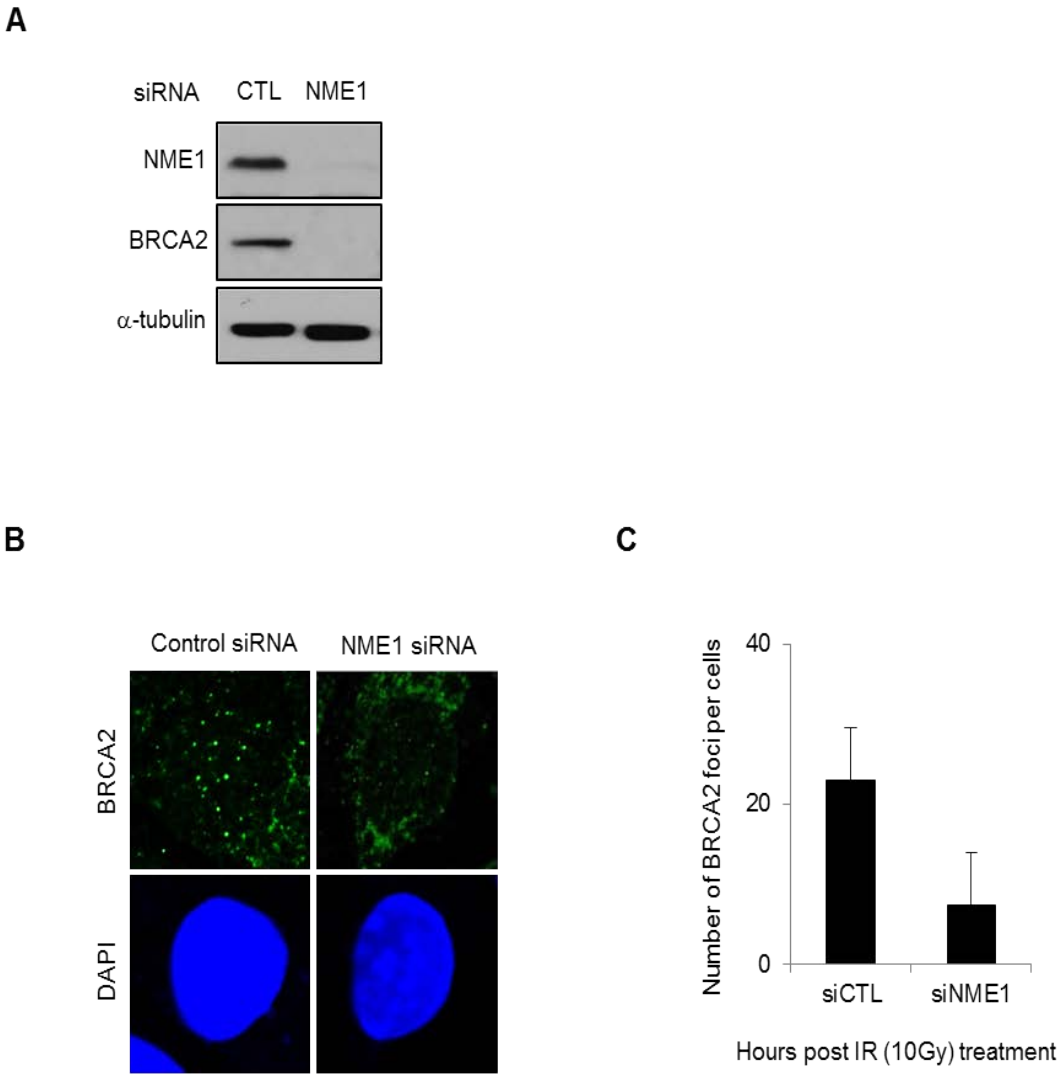


Figure 3. Depletion of NME1 impairs the formation of BRCA2 foci in DSB site.

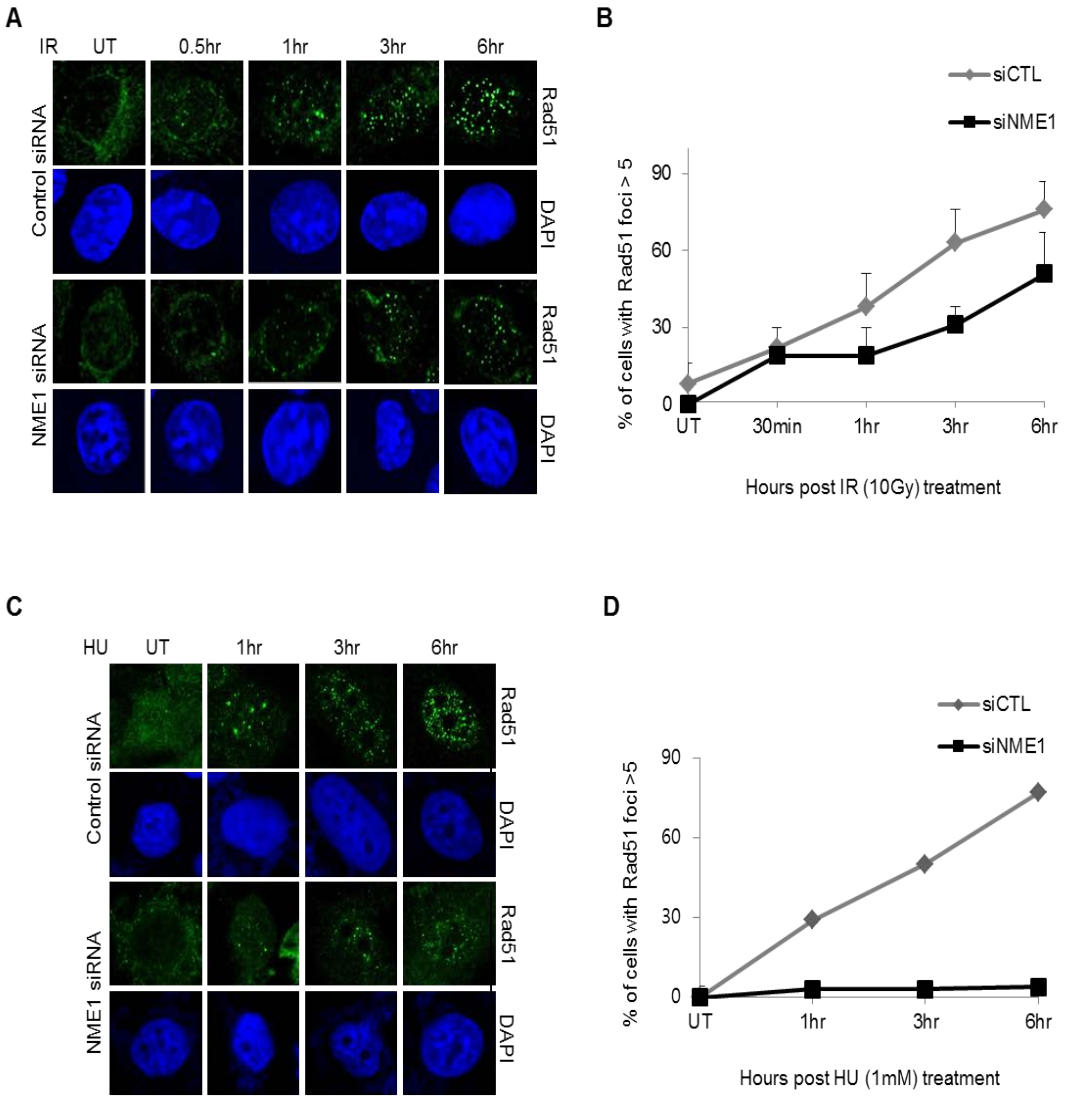
(A) U2OS cells were transfected with control or NME1 siRNA. After 48hrs, the expression level of NME1 was confirmed by western blot analysis using anti-NME1 and anti-BRCA2 antibodies. α-tubulin was used as loading control. (B) Control and NME1-depleted U2OS cells were untreated or treated with 10 Gy IR for 12hr, and then fixed. Cells were stained with an anti-BRCA2 antibody. DAPI staining was performed to indicate the position of nuclei. (C) The number of respective BRCA2 foci is indicated. At least 20 cells were analyzed. Data are presented as mean ±SD.

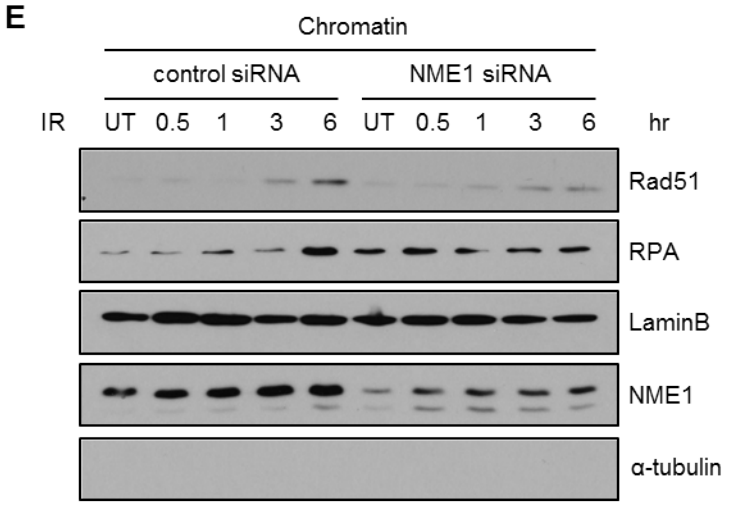
3. Depletion of NME1 impairs Rad51 recruitment to DNA damage site.

BRCA2 has been reported to bind Rad51, a protein essential for homologous recombination and the recombinational repair of DNA double-strand breaks[21]. In normal cells, a redistribution of Rad51 protein, manifested as formation of Rad51 nuclear foci, is seen upon IR treatment[22]. Here, we investigated whether NME1-depleted cells impairs the Rad51 foci formation in response to DNA damage. Control and NME1-depleted cells were treated with 10 Gy IR or 1 mM HU to make DSB. Cells were fixed with 50 % methanol: 50 % acetone and stained with anti-Rad51 antibody. Immunofluorescence microscopy showed that NME1 depletion impaired the Rad51 foci formation upon IR or HU-induced DSB (Figure 4A, C). After observing our results, in contrast to, the Rad51 foci for-mation was reduced significantly in NME1-depleted cells as compared with con-trol cells (Figure 4B, D). Since RAD51, a critical component of the HR process, was recruited to chromatin in response to DNA damage, we examined effects of

NME1 on chromatin association of Rad51. Control and NME1-depleted HeLa cells were fractionated into chromatin fraction. Anti-RAD51 antibody was used to detect endogenous Rad51, and LaminB was used as specific makers for chromatin fractions (Figure 4E, F). Importantly, RAD51 recruitment to DNA damage chromosome was decreased in the absence NME1. BRCA2 and RPA, as regulation protein of HR, also decreased in depletion of NME1. Collectively, these results imply that NME1 is required for the loading of Rad51, RPA and BRCA2 to DNA damage sites. These data indicated that NME1 might relate with homologous recombination repair.

Figure 4.





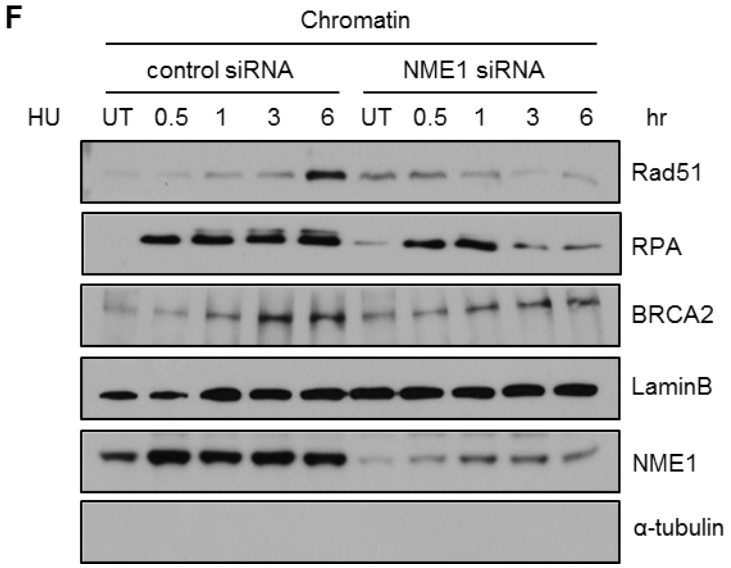


Figure 4. Depletion of NME1 impairs Rad51recruitment to DNA dam-age site.

(A) Control and NME1-depleted U2OS cells were untreated or treated with 10 Gy IR, and then fixed. Cells were stained with an anti-Rad51 antibody. DAPI was used for nuclear staining. (B) The histogram showed the percentage of cells with Rad51 foci number > 5 cells. At least 100 cells were analyzed for each treatment. Results are shown as means ±SD. (C) Control and NME1-depleted HeLa cells were untreated or treated with 1 mM HU, and then fixed. Cells were stained with an anti-Rad51 antibody. DAPI was used for nuclear staining. (D) The histogram showed the percentage of cells with Rad51 foci number > 5 cells. Results are shown as means ±SD. (E), (F) Indicated 293T cells were fractionated into chromatin extracts, and then fraction was subject to western blot using antibod-ies against Rad51, RPA, BRCA2 NME1, LaminB and α-tubulin.

4. Depletion of NME1 sensitize cells to IR.

To address the overall consequences of the diminished BRCA2 foci formation that occurs in NME1-depleted cells in response to DNA damage, we analyzed the sensitivity of cells to IR treatment and DSB repair. The effect of NME1 depletion on radiation sensitivity, Control and NME1 knockdown HeLa cells were treated with IR, and clonal cell survival assay was performed. We found that NME1 depleted cells exhibited a reduction of colony numbers after treatment with DNA damage, compare to control cells (Figure 5A, B). These results indicated that NME1 is required for cell survival in response to DSBs.

Figure 5

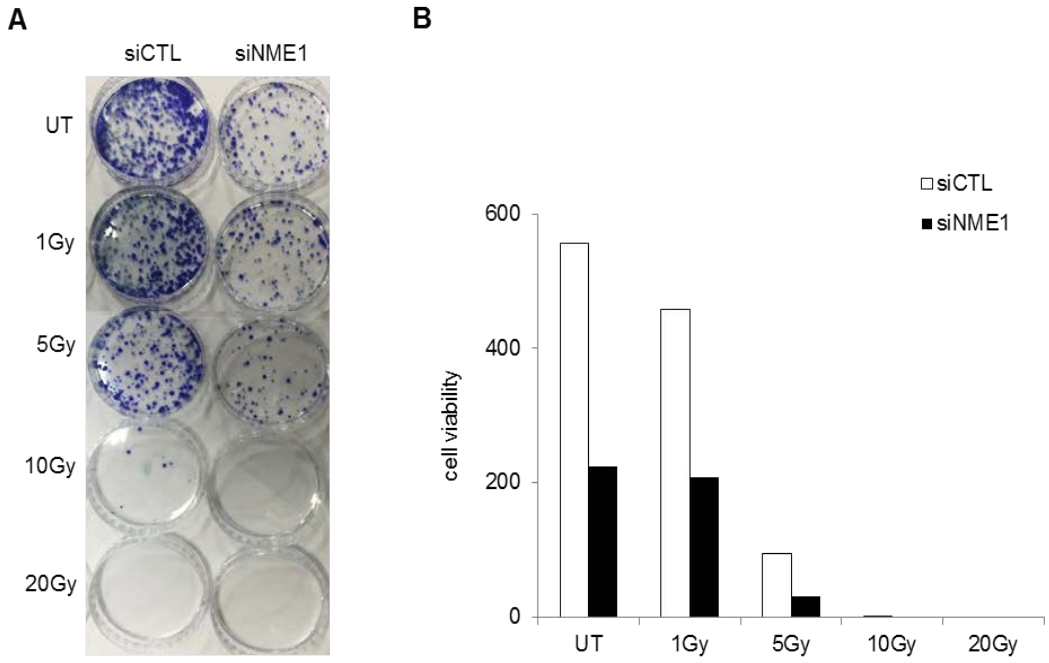


Figure 5. NME1-depleted cells are defective in viability.

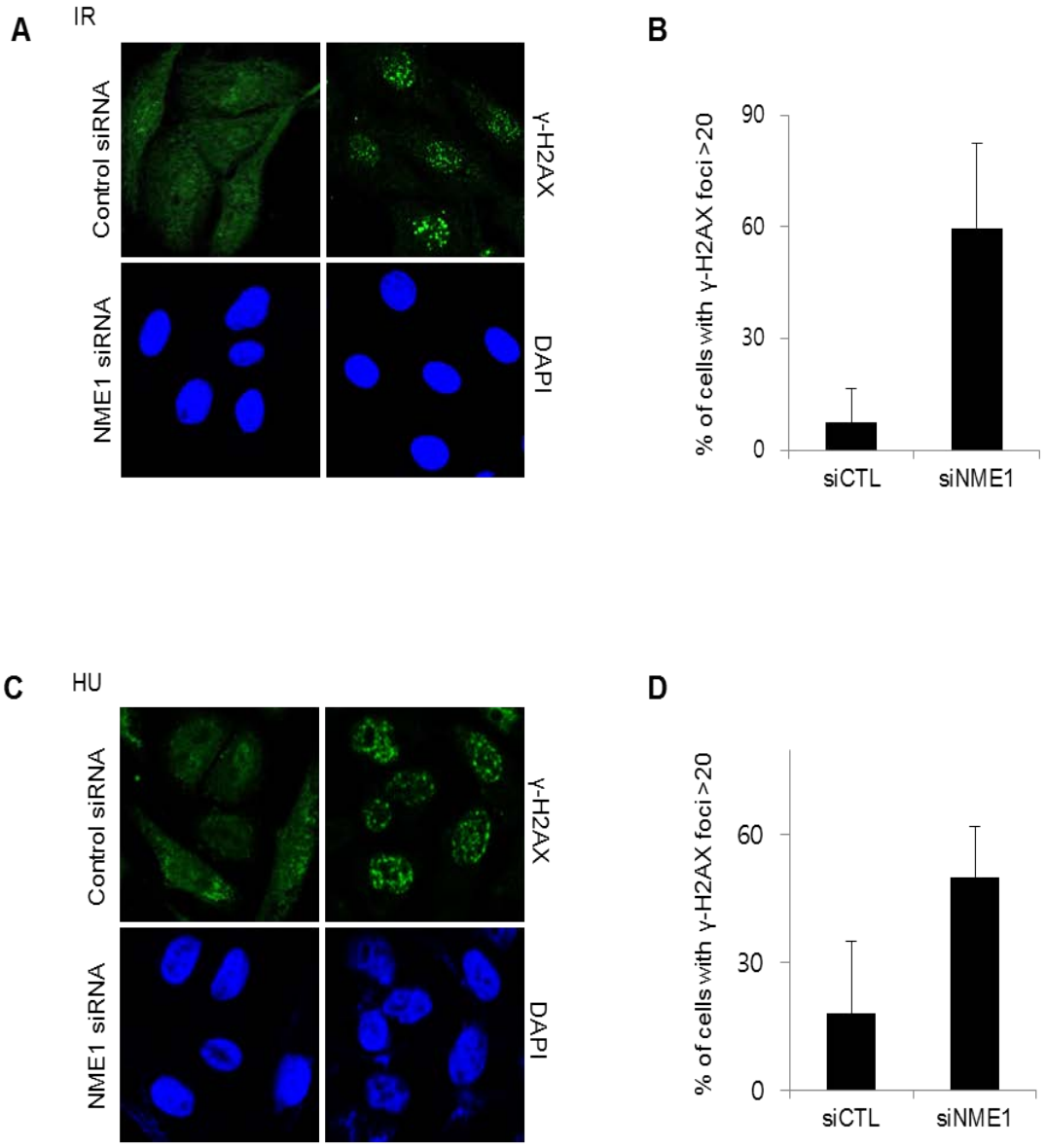
(A), (B) Control and NME1 knockdown HeLa cells were treated with IR or HU, and clonal cell survival assay was performed. After 2weeks, cells were stained methylene blue, and the number of surviving colonies was counted.

5. NME1 depletion decrease DNA repair.

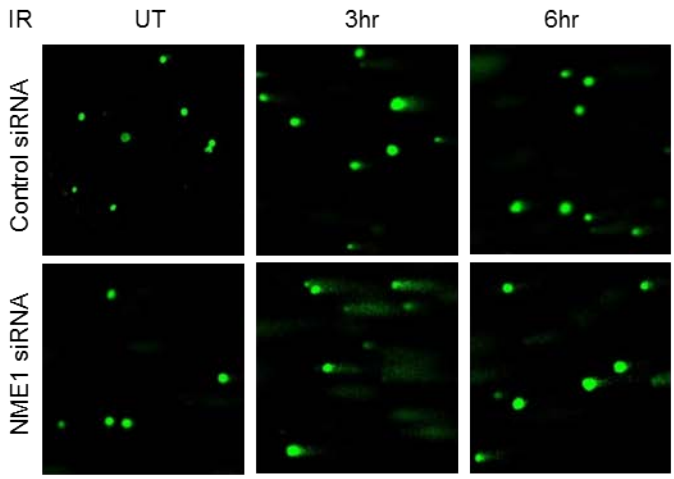
To further investigated whether suppression of NME1 in the cells causes a defects in DNA damage repair, the formation of γ-H2AX foci was analyzed by immunofluorescence microscopy. γ-H2AX is used as the indicator for identifying of DSB induction and DNA repair [23], suggesting that the cells with unrepaired DNA damage still remains a formation of γ-H2AX foci. Control and NME1-depleted cells were treated with 5 Gy IR or 1 mM HU to induce DSB and harvested after 24hrs. Cells were fixed with 4% paraformaldehyde and immunofluorescence assay performed. As a result Figure 6, the depletion of NME1 with siRNA led to increased level of unrepaired DSBs after treatment with IR or HU, as evidenced by the number of γ-H2AX foci remaining. These studies suggest that NME1 is required for DSB repair. In addition, we measured DNA repair activity in control or NME1 depleted HeLa cells using comet assays. The comet assay (single-cell gel electrophoresis) is a simple method for measuring DNA

strand breaks in eukaryotic cells. For this, control and both NME1 knockdown cells were treated 10 Gy IR to make DSB and harvested in different time points. The demonstrated that depletion of NME1 resulted in extensively longer comet tails after IR treatment than control cells (Figure 6E, F). The observation of long comet tails in NME1 deficient cells indicates impaired DNA DSB repair. These results proved that NME1 knockdown cells unable to repair IR induced DSBs.

Figure 6



E



F

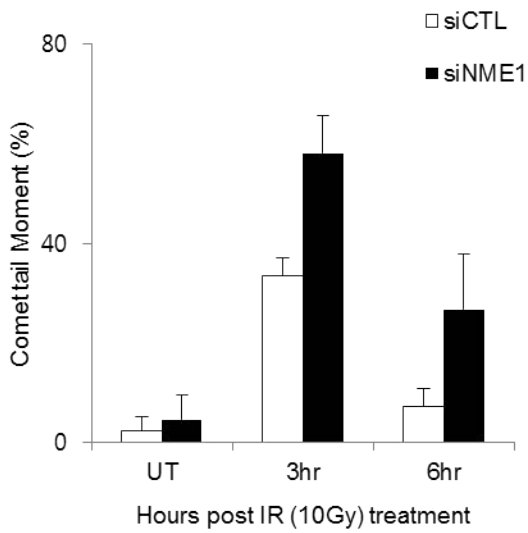


Figure 6. Depletion of NME1 leads to impaired DSB repair.

(A) Control and NME1 depleted U2OS cells were treated with 5 Gy IR 24hrs, and then fixed. Cells were stained with an anti-γ-H2AX antibody. DAPI staining was performed to indicate the position of nuclei. (B) Quantification of cells with γ-H2AX foci in control or NME1 siRNA cells. Results are shown as means ±SD. (C) Control and NME1 depleted U2OS cells were treated with 1 mM HU 24hrs, and then fixed. Cells were stained with an anti-γ-H2AX antibody. DAPI staining was performed to indicate the position of nuclei. (D) Quantification of cells with γ-H2AX foci in control or NME1 siRNA cells. Results are shown as means ±SD. (E) Influence of NME1 depletion on single cell comet assay of HeLa cells treated with IR. (F) The tail moments of cells for untreated, 3, and 6hrs after IR treatment were measured. The length and intensity of DNA tails relative to heads is shown as % of the relative tail moment. Results are shown as means ±SD.

6. NME1-depleted cells are defective in Homologous recombination (HR).

Cells have evolved a number of DSB repair pathways to address these lesions. Non-homologous end-joining (NHEJ) and homologous recombination (HR) comprise the two major pathways by which DSBs are repaired in cells[24]. BRCA2 is essential for efficient homology-directed repair, presumably in conjunction with the Rad51 recombinase[25]. Because BRCA2 directly mediates DSB repair through HR, and because NME1 regulates DSB repair via association with BRCA2, we examined whether NME1 depletion would lead to functional changes in HR of DSBs. To this purpose, GFP-based chromosomal reporter assay in DR-GFP-U2OS cells were used. DSB, are generated through the expression of I-SecI endonuclease, which cleaves a specific recognition site located in the GFP gene. In this system, repair efficiency via HR is monitored by measuring the percentage of cells expressing GFP using fluorescence microscope (Figure

7A).

To assess the involvement of NME1 in HR-mediated repair, we examined the levels of HR when control and NME1 depleted. The analysis of HR in DR-GFP U2OS cells showed a ~3-fold decrease in the NME1 knockdown cells compared with control cells (Figure 7B, C). These results indicate that NME1 contributes to DSB repair through regulation of HR.

Figure 7

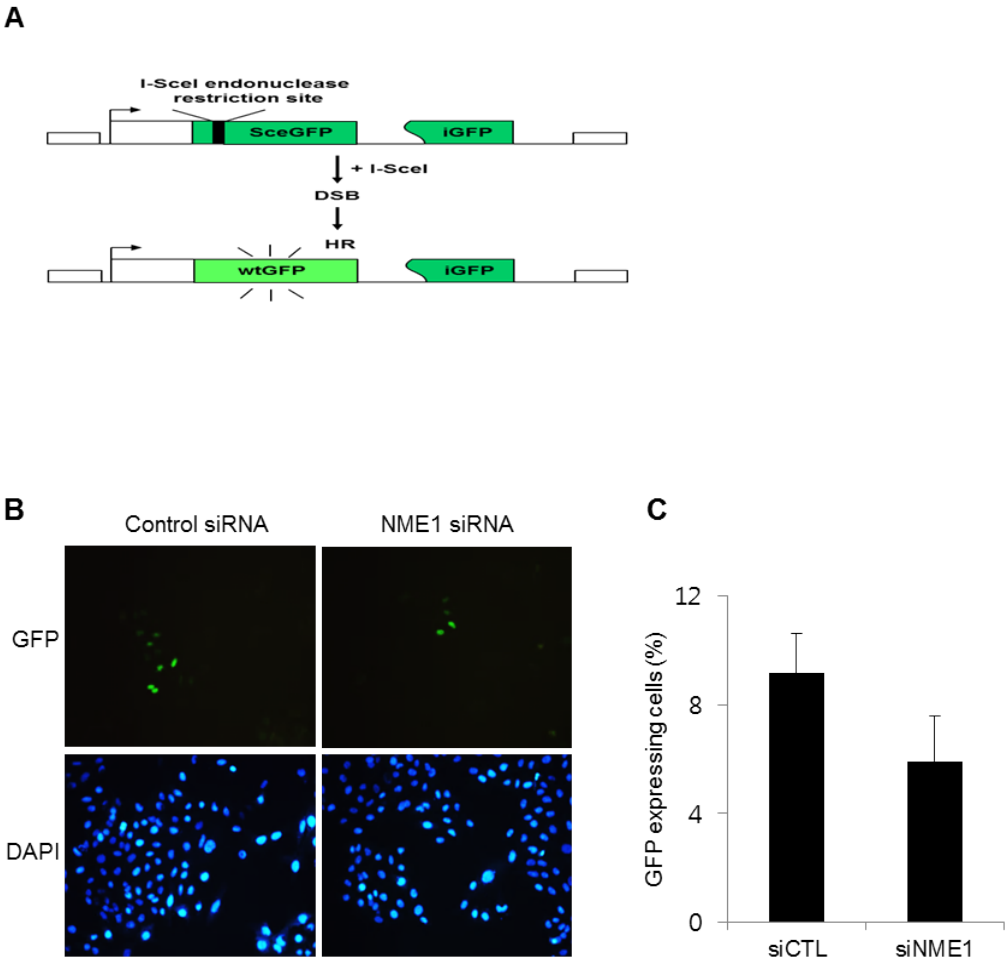


Figure 7. NME1-depleted cells are defective in Homologous recombination (HR).

(A) A schematic showing the assay for the fluorescence-based measurement of HR-mediated DSB repair. The percentage of an I-SecI site in the GFP reporter gene allows for the introduction of a DSB in the presence of the endonuclease I-SecI. When the DSB is repaired, the reporter construct will then express GFP that can be measured and quantitated. (B) DR-GFP U2OS cells were transfected with the control or NME1 siRNA for 24hrs and then transfected with the I-Sce1 expression vector to induce double strand breaks. After 43hrs, the GFP-positive cells were visualized using fluorescence staining was performed to indicate the visible cell. (C) Quantification of cells with GFP expression in control or NME1 siRNA cells. The percentage of the cells for GFP expression was measured by fluorescent microscope. Results are shown as means ±SD.

DISCUSSION

Previous reports have demonstrated that BRCA2 facilitate the recruitment of Rad51 to sites of DSBs and the subsequent stimulation of DSB repair by homologous recombination (HR) [26]. We performed the yeast two-hybrid screening method and found a novel BRCA2-interacting protein, NME1. We showed that the interaction between NME1 and BRCA2 *in vivo*, and association of NME1 with BRCA2 is enhanced in response to DNA damage, such as ionizing radiation (IR) and hydroxyurea (HU). Further, depletion of NME1 results in impaired foci formation of BRCA2 to DSB site. Our study also revealed that absence of NME1 impaired foci formation and chromatin recruitment of Rad51, a protein essential of DSB repair. In support of this idea, we found that cells depleted of NME1 accumulate high level of persistent DSBs, as detected by formation of γ-H2AX foci and comet assay. These studies suggest that NME1 is required for DSB re-

pair. We further demonstrated that absence of NME1 sensitizes cells to DNA damage, and impaired HR by homologous recombination assay.

It was reported that NME1 was regulated cytoskeletal and focal adhesion dynamics, and promoted genomic stability through functional interactions with nucleotide excision repair (NER) and DNA double-strand break repair (DSBR) [13]. Previous reports showed that NME1 played an important and direct role in DNA repair and the maintenance of genomic integrity through 3′-5′ exo-nuclease activity. Our results illuminate a novel interaction between NME1 and BRCA2 that is crucial for DSB repair, and may be relate with genomic stability.

In summary, the interactions between NME1 and the DNA damage BRCA2 suggest that NME1 participates in the homologous recombination. The exact contribution of NME1 to DNA damage response is not yet clear. Further study must have done to demonstrate with detailed mechanism of NME1. Moreover, effect of BRCA2 in function of NME1 must be investigated.

ABSTRACT

NME1 regulates homologous recombination through interaction with BRCA2

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BRCA2 plays a role in maintenance of genomic stability in response to DNA damage, centrosome regulation, cytokinesis, transcription, cell cycle regulation and DNA homologous recombination. However, the precise molecular mechanism of BRCA2 on the homologous recombinant remains unclear. Here, we identified a transcription factor NME1 as a novel interacting protein of BRCA2 though a yeast two-hybrid system. We show that the BRCA2 interacts with NME1 *in vivo*, and interaction between both proteins was enhanced under DNA damage, such as

ionizing radiation (IR) and hydroxyurea (HU). Depletion of NME1 results in impaired formation of DNA damage-induced BRCA2 and Rad51 foci and suppression of chromatin-bound Rad51. NME1-depleted cells exhibited an accumulation of double-strand breaks (DSBs), as determined by measurement of late γ-H2AX foci in nuclear and comet assay. We further demonstrated that absence of NME1 sensitizes cells to IR, and impaired HR by DR-GFP assay. Disruption of the NME1-BRCA2 interaction prevented accumulation of BRCA2 at sites of DSBs and suppressed HR. Taken together, our results suggest a novel regulation mechanism of BRCA2 in homologous recombination through interaction with NME1.

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