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The Regulatory Mechanism of DNA Damage Checkpoint Protein MDC1

Graduate School of Chosun University

Department of Bio-Materials

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유전자손상체크단백질 MDC1의 조절기전 연구

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유전자손상체크단백질 MDC1의 조절기전 연구

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생물신소재학과

MDC1은 DNA double strand breaks 같은 DNA 손상 자극시 손상복구에 관여하는 중요한 단백질로 알려져 있다. 그러나 MDC1 기능을 조절하는 조절단백질과 그 자세한 기전에 대한 연구는 미흡한 실정이다. 따라서 본 연구논문에서는 MDC1의 활성을 조절하는 새로운 조절단백질을 밝히고자, MDC1의 yeast two hybrid assay를 실시하여 MDC1와 결합하는 몇몇 단백질 (KPNA2, ZNF114, PHB2, FHL2)들을 동정하였고, 그들 후보 조절유전자들의 결핍을 통해 MDC1활성에 미치는 영향을 조사하였다. 각 후보 결합유전자들이 결핍된 세포에서 MDC1의 DNA손상 foci, 상동재결합 활성이 감소함을 확인하였다. 또한 clonal survival

assay를 통해 후보유전자들이 결핍된 세포는 방사선조사에 더 민감함을 확인하였다. 따라서 본 연구결과는 MDC1에 결합해 MDC1 활성을 조절하는 새로운 단백질들을 동정하였고, MDC1 활성조절의 새로운 분자적 기전을 제시한다.

I. Introduction

DNA damage caused by range of agents including ionizing radiations, mutagens, thermal disruption, reactive oxygen species, replication errors or stress, affects the genome integrity and increase the chances of cancer formation (J. Lukas, Lukas, & Bartek, 2011; Rouse & Jackson, 2002; Zhou & Elledge, 2000). DNA damage either affects only one strand, single-strand breaks (SSBs) or both strands of DNA double-helix, double strand breaks (DSBs). DSBs are considered as most dangerous and prone to inaccurate repair due to lack of complementary strand to use as template to repair damaged strands. If left unrepaired, accumulation of DSBs leads to lethal mutations, loss of genetic information, chromosomal abnormality and ultimately cell death (Jackson, 2002; Kaina, 2003; Roos & Kaina, 2006). DSBs are primarily repaired by two major DNA repair pathways, non-homologous end joining (NHEJ) and homologous recombination (HR) (Kanaar, Hoeijmakers, & van Gent, 1998; Lieber, 2010; Shrivastav, De Haro, & Nickoloff, 2008). Among these two, HR maintains high degree of accuracy and fidelity by using undamaged sister chromatid as a template to repair DSBs (Harper & Elledge, 2007; Pardo, Gomez-Gonzalez, & Aguilera, 2009; Thompson & Schild, 2001).

Mediator of DNA damage checkpoint protein 1(MDC1, also known as NFBD1) plays a vital role in DNA damage repair, especially in DSBs repair, through DNA damage response (DDR) pathway signal transduction. It acts as

a mediator of DDR pathway by facilitating the recruitment of other repair proteins to the site of DNA repair (Lou et al., 2006; Stucki & Jackson, 2004; Xu & Stern, 2003a, 2003b). It contains a FHA domain at amino-terminal and two BRCT domains at the carboxy-terminal. At the central region, it contains 14 repeats of nearly 41-amino acids containing DNA-PKcs/Ku binding region (Stucki & Jackson, 2004; Xu & Stern, 2003a, 2003b). Upon DNA damage, MDC1 is hyperphosphorylated in ATM dependent manner and relocalizes to the damaged area. It interacts with phosphorylated H2AX through its C-terminal BRCT domain and recruits other DDR proteins to the site of action (Bekker-Jensen, Lukas, Melander, Bartek, & Lukas, 2005; Goldberg et al., 2003; C. Lukas et al., 2004; G. S. Stewart, Wang, Bignell, Taylor, & Elledge, 2003; Stucki et al., 2005). MDC1 is also required for the downstream events subsequent to the recruitment of repair proteins including phosphorylation and activation of repair proteins (Kim, Minter-Dykhouse, & Chen, 2006) (shu-Chun, 2006). Any delay or impairment in the recruitment of MDC1 to the nucleus at the time of DSB repair leads to deficient DDR signal transduction.

Karyopherin α-2 and cytoplasmic-nuclear transport of proteins

Cytoplasmic-nuclear transport of proteins occurs through nuclear pore complex (NPC) of the nuclear membrane (Nigg, 1997; Pante & Aebi, 1995). Micromolecules easily shuttle through NPC passively, whereas macromolecules larger than about 50kDa require karyopherins (Gorlich,

Prehn, Laskey, & Hartmann, 1994; M. Stewart, 2007; Zannini et al., 2003). Karyopherins are group of proteins involved in the transport of cargo proteins through NPC. Karyopherins can carry out both import (importins) and export (exportins) of cargoes from nucleus, more than 20 members of this karyopherin family have been described (Chook & Blobel, 2001; Goldfarb, Corbett, Mason, Harreman, & Adam, 2004; Schaller, Pollpeter, Apolonia, Goujon, & Malim, 2014). Nucleocytoplasmic shuttling takes place through various pathways, the classical nuclear protein import pathway is regarded as one of the best characterized pathways. In this pathway the cargo proteins transported to the nucleus by the heterodimeric complex consisting of a karyopherin/importin-β and a member of karyopherin/importin-α family. Karyopherin-α family member act as an adapter protein, whereas karyopherin-β involved in docking of protein to the nuclear membrane and transport it through NPC (Goldfarb et al., 2004; Moroianu, 1997; Moroianu, Blobel, & Radu, 1996).

Karyopherin α-2 (KPNA2, also known as importin α-1 or RAG cohort 1) is one of seven described member of the karyopherin/importin-α family. KPNA2 consists of an N-terminal hydrophilic domain, a central hydrophobic region and a short acidic C-terminus. The N-terminal domain binds to importin-β. The central hydrophobic region comprises of 10 armadillo (ARM) repeats, which binds to the cargo's nuclear localization sequence (NLS). Function of an acidic C-terminal region is not clear. KPNA2 recognizes cargo

proteins with NLS and transport them to nucleus through NPC by forming a heterodimeric complex with importin-β (Cingolani, Petosa, Weis, & Muller, 1999; Gorlich, Henklein, Laskey, & Hartmann, 1996; Gorlich et al., 1994; Nishinaka et al., 2004; Schaller et al., 2014; Teng, Wu, Tseng, Wong, & Kao, 2006). KPNA2 can recognize various types of NLSs including monopartite NLSs, consisting of a single cluster of basic amino acids, bipartite NLSs consisting of multiple clusters as well as additional non-classical NLSs (Goldfarb et al., 2004; Kosugi et al., 2009; Leung, Harreman, Hodel, Hodel, & Corbett, 2003).

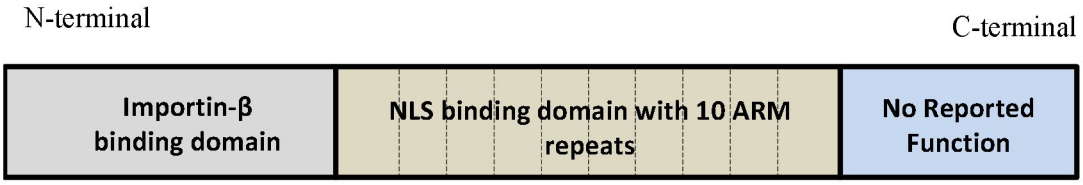


Figure 1. Schematic representation of KPNA2 domain structure

KPNA2 along with NLS bearing cargo and importin-β enters into nucleus through NPC. This process of nuclear transport takes place in two steps. First, the energy-independent docking of cargo proteins to the nuclear membrane and next the energy-dependent transport of cargo proteins through NPC (Macara, 2001; Rexach & Blobel, 1995). In the nucleus, RanGTP binds to the heterodimeric complex with cargo, triggers dissociation of complex and results in release of cargo into the nucleus. The RanGTP bound importin-β recycled directly to the cytoplasm, whereas KPNA2 binds to RanGTP and

exportin factor (CSA) then shuttled back to cytoplasm. In cytoplasm, KPNA2 bound RanGTP hydrolyzed by RanGAP and RanBP1 to release KPNA2 (Moroianu et al., 1996; Rexach & Blobel, 1995; M. Stewart, 2007). The "free"KPNA2 is now ready for another cycle of cytoplasmic-nuclear transport of NLS bearing cargo. It was previously reported that KPNA2 is critical for the nuclear translocation and nuclear foci formation of NBS1, a key regulator of MRE11-RAD50-NBS1 (MRN) complex which plays a principle role in DSB repair pathway (Tseng, Chang, Wu, & Teng, 2005).

In this report, we showed that KPNA2 plays an important role in IR induced DSB repair activity through transporting a mediator of DDR pathway, MDC1 to the site of action. In absence of KPNA2 expression, cells were not able to carry out DSB repair efficiently as in case of normal cells. Our results suggest that KPNA2 is required for nuclear translocation of MDC1 after IR induced DSB, which is a key factor in the process of DSB repair. KPNA2 depletion leads to impaired HR repair activity.

We also investigated the role of three more proteins namely Zinc Finger protein 114 (ZNF114), Prohibitin 2 (PHB2) and Four and a half LIM-only protein 2 (FHL2) in response to DNA damage. ZNF114 consists of one Krüppel associated box (KRAB) and four Zinc-finger double domains. It may be involved in the regulation of transcription since it has transcription repression domain, KRAB. It may also have the ability to bind to DNA

through its Zinc-finger double domains (Umeyama, Iwadate, & Taguchi, 2014). Other two proteins PHB2 and FHL2 may also plays a role in transcriptional regulation (Fimia, De Cesare, & Sassone-Corsi, 2000; S. J. Lee et al., 2008; Mishra, Murphy, & Murphy, 2006; Morlon & Sassone-Corsi, 2003).

Here we showed the novel functions of these three proteins in response to DNA damage. Till now no functions were established for these proteins in the field of DNA damage repair.

II. Materials and Methods

A. Cell culture and treatment

Human cervix adenocarcinoma cell line HeLa and human embryonic kidney cell line HEK293T were cultured in Dulbecco's modified Eagle's medium (Gibco-BRL, Grand Island, NY, USA). In all cases, the media was supplemented with 10% heat-inactivated fetal bovine serum (Cambrex Corp., East Rutherford, NJ, USA), 100 units/ml penicillin, and 100 mg/ml streptomycin sulfate (Invitrogen, Carlsbad, CA, USA). All cells were maintained in a humidified incubator containing 5% CO2 at 37oC. HeLa cell line was obtained from the American Type Culture Collection (ATCC, Rockville, MD, USA), and the HEK293T cell line was obtained from the Cornell Institute for Medical Research (New York, NY, USA). To induce DNA breaks, exponentially growing cells were irradiated at 5 and 10 Gray (Gy) from 137Cs source (Gammacell 3000 Elan irradiator, Best Theratronics, Ottawa, Canada) and allowed to recover at 37^{o}C for various amounts of time.

B. Generation of stable KPNA2 knockdown clones

The pSilencer2.1–U6 neo vector was obtained from Ambion (Austin, TX, USA). Vectors for expression of hairpin SiRNA's were constructed by

inserting corresponding pairs of annealed DNA oligonucleotides into the pSilencer 2.1–U6 vector between the BamHI and HindIII restriction sites according to the manufacturer's instructions. The KPNA2-specific target sequence was selected based on an online shRNA application from Invitrogen (http://www.ambion.com/techlib/nisc/psilencer-converter.html) using the human KPNA2 sequence as the reference sequence (GenBank Accession No. NM_002266.2). The target sequences were 5'-GCAUCAUGAUGAUCCAGAA dTdT-3' (sense) and 5'-UUCUGGAUCAUCAUGAUGC dTdT-3' (antisense) for KPNA2 siRNA #B A non-targeting sequence, 5'-CCUACGCCACCAAUUUCGU dTdT-3' and 5'-ACGAAAUUGGUGGCGUAGG dTdT-3', was used as a negative control. To generate single knockdown clones, HeLa cells were transfected with pSilencer2.1–U6, pSilencer2.1–U6 KPNA2 siRNA #B. Twenty-four hours after transfection, 400 mg/ml G418 was added to the culture medium for selection. After selection, stable clones were analyzed by real-time RT-PCR and western blotting to confirm down regulation of KPNA2.

C. RNA interference

The sequence of KPNA2 siRNA # A, B, C, E, F and G are GCAGCUAAGAAAGUACAUA, GCATCATGATGATCCAGAA, ACGAATTGGCATGGTGGTGAA, CCGGGUGUUGAUUCCGAA, CAGAUACCUGCUGGGCUAUUUCCUA, and

ACCUGCUGGGCUAUUUCCUACCUUA, respectively. The sequences of FHL2 siRNA#1 and siRNA#3 are CGAAUCUCUCUUUGGCAAG and CAACGACUGCUUUAACUGU, respectively. The sequence of ZNF114 siRNA#3 is GCAAAGUCGUCAGGACUUA. The sequence of PHB2 siRNA#2 is CUGAACCCCUCUUGGAUUAAGUU.

D. Plasmid constructs

The full-length MDC1 cDNA was amplified from human fibroblast cells by RT-PCR using the MDC1 primers 5'-GCCTCTAGAATGGCAATGCAGATG-3' (sense) and 5'-AATGGGCCCTCAGTCTTTGGCATC-3' (antisense). The amplified MDC1 cDNA construct was cloned into the mammalian expression vector pcDNA3 in-frame with the hemagglutinin (HA) tag. The MDC1 sequence was confirmed by automated DNA sequencing. The human KPNA2 full length cDNA was amplified from GM00637 human fibroblast cells by RT-PCR using the KPNA2 primers 5`-TTCGAATTCATGTCCACCAACGAG-3`(sense) and 5`-ACCGGATCCAA AGTTAAAGGTCCC-3`(antisense). The amplified KPNA2 cDNA construct was cloned into the mammalian expression vector pcDNA3 in-frame with the GFP tag. The KPNA2 sequence was confirmed by automated DNA sequencing.

E. Antibodies

The following antibodies were used: mouse monoclonal anti-KPNA2, rabbit polyclonal anti-RAD51, rabbit polyclonal anit-53BP1, rabbit polyclonal anti-BRCA1, mouse monoclonal anti-α-tubulin (TU-02), mouse monoclonal anti-β-actin, mouse monoclonal anti-HA tag, rabbit polyclonal anti-HA tag, mouse monoclonal anti-GFP tag and rabbit polyclonal anti-GFP tag (Santa Cruz Biotechnology), two MDC1 rabbit polyclonal antibodies (R1 and R2) were prepared in our lab, mouse monoclonal anti-γH2AX (JBW301, Upstate Biotechnology), rabbit monoclonal anti-histone H3 (D1H2, Cell Signaling), rabbit polyclonal anti-BRCA2 (A303-434A, Bethyl), mouse monoclonal anti-RPA34-19 (Ab-2, Millipore), rabbit polyclonal anti-PHB2 (ab15019, abcam), rabbit polyclonal anti-ZNF114 (24974-4AP, Protein tech) and mouse monoclonal anti-FHL2 (K0055-3, MBL Life Science).

F. Yeast two-hybrid analysis

The two-hybrid analysis of the full length MDC1-fragment interaction was carried out using a Matchmaker two-hybrid cDNA library from human testis (Clontech, Mountain View, CA, USA). The human KPNA2 gene was cloned from a human testis cDNA pool (purchased from Clontech) by polymerase chain reaction (PCR). The DNA fragments containing sequences derived from MDC1 and KPNA2 were ligated into the pACT2 and pAS2-1 vectors (Clontech), respectively. The pAS2-1 and pACT2 plasmids contain

the GAL4 DNA-binding domain and the GAL4 activation domain, respectively, just upstream of their cloning sites. The plasmids for the positive control experiment (pGBKT7-53 and pGADT7-T) and for the negative control experiment (pGBKT7-Lam and pGADT7-T) were supplied by the manufacturer (Clontech). The plasmids containing the MDC1 and KPNA2 sequences were introduced into the yeast strain AH109. The two-hybrid interaction between MDC1 and KPNA2 was then tested according to the manufacturer's protocol (Clontech Matchmaker GAL4 protocol). The interaction between MDC1 and KPNA2 induced the expression of the URA3, ADE2and LacZ reporter genes, which allow the yeast strain to grow on a synthetic dextrose minimal medium plate without uracil and adenine, and to produce a blue color in the presence of X-Gal.

G. Preparation of subcellular fractions

Cells were harvested and then lysed with cytosol extraction buffer (CEB; 10mM HEPES (pH 7.5), 3mM MgCl2, 14mM KCl, 5% glycerol, 1mM DTT) with protease inhibitors (Roche Diagnostic corp.) for 10min in ice. For complete lysis, 0.2% NP-40 was added and vortexed for 10sec. After centrifugation at 8600g for 2min, the supernatant was collected and labelled as cytosol fraction. The pellet was washed with CEB without inhibitors for three times and lysed with nuclear extraction buffer (NEB; 10mM HEPES (pH 7.5), 3mM MgCl2, 400mM NaCl, 5% glycerol, 1mM DTT) with protease

inhibitors for 30min at 4ºC, followed by centrifugation at 13200 r.p.m for 30 mins. The supernatant containing nuclear extracts were collected in new tubes. Total fraction was prepared by lysed the cells with RIPA buffer [50mM Tris–HCl (pH 7.5), 150mM NaCl, 1% Nonidet P-40, 0.5% sodium deoxycholate, 0.1% sodium dodecyl sulfate] containing ethylenediaminetetraacetic acid (EDTA)-free protease inhibitor cocktail (Roche, Basel, Switzerland).

H. Immunoprecipitation assay and western-blot analysis

Cells were lysed in RIPA buffer [50mM Tris–HCl (pH 7.5), 150mM NaCl, 1% Nonidet P-40, 0.5% sodium deoxycholate, 0.1% sodium dodecyl sulfate] containing ethylenediaminetetraacetic acid (EDTA)-free protease inhibitor cocktail (Roche, Basel, Switzerland). Equal amounts of proteins were then resolved on 6–15% SDS-PAGE gels, followed by electrotransfer to polyvinylidene difluoride membranes (Millipore, Bedford, MA, USA). The membranes were blocked for 2 h in TBS-t [10mM Tris–HCl (pH 7.4), 150 mM NaCl, 0.1% Tween 20] containing 5% fat-free milk at room temperature and then incubated with the indicated primary antibodies overnight at 4oC. After incubation for 2 h with appropriate peroxidase conjugated secondary antibodies [1:4000, Jackson ImmunoResearch Inc., West Grove, PA, USA], developed using enhanced chemiluminescence detection system. For the immunoprecipitation assay, aliquots of soluble cell lysates were precleared

with protein A/G plus- agarose (Santa Cruz Biotechnology) or G sepharose (GE Healthcare) bead as indicated and then incubated at 4oC for 3 h. Next, the appropriate antibody was added and incubated at 4oC for 12 h. After the addition of fresh protein A/G plus-agarose or G sepharose bead, the reaction was incubated overnight at 4oC with rotation. The beads were washed five times in RIPA buffer without protease inhibitors, re-suspended in SDS sample buffer and boiled for 5 min. The samples were then analyzed by western blotting using the appropriate antibodies.

I. Immunostaining

To visualize γ-ray-induced foci, untreated cells or cells treated with 10Gy γ-ray were cultured on coverslips coated with poly-L-lysine (Sigma). Cells were washed twice with PBS and fixed with 4% paraformaldehyde for 10 min and ice cold 98% methanol for 5 min, followed by permeabilization with 0.3% Triton X-100 for 15 min at room temperature (J. H. Lee et al., 2010). Next, the coverslips were washed three times with PBS, followed by blocking with 0.1% bovine serum albumin in PBS for 3 h at room temperature. The cells were double-immunostained using primary antibodies directed against the indicated proteins overnight at 4oC. The cells were then washed with PBS and stained with the appropriate Alexa Fluor 488- or Alexa Fluor 594-conjugated secondary antibodies (green and red fluorescence, respectively; Molecular Probes, Eugene, OR, USA). After washing, the

coverslips were mounted onto slides using Vectashield mounting medium containing 4, 6-diamidino-2-phenylindole (DAPI; Vector Laboratories, Burlingame, CA, USA). Fluorescence images were taken under a confocal microscope (Zeiss LSM 510 Meta; Carl Zeiss, Jena, Germany) and analyzed with Zeiss LSM Image Examiner software (Carl Zeiss).

J. Clonal survival assay

After treatment with IR, 5 ×102 cells were immediately seeded onto a 60-mm dish in duplicate and grown for 2–3 weeks at 37oC to allow colony formation (Shahi et al., 2011). Colonies were stained with 2% methylene blue in 50% ethanol and counted. The fraction of surviving cells was calculated as the ratio of the plating efficiencies of treated cells to untreated cells. Cell survival results are reported as the mean value ± standard deviation for three independent experiments.

K. Neutral Comet assay (single-cell gel electrophoresis)

Single cell gel electrophoresis assay was carried out as described previously with modification (Chowdhury et al., 2005). Cells were left untreated or treated with 10Gy γ-irradiation, trypsinized after specific time points and resuspended in PBS. Aliquots of the cell suspension (20μl, 1x105 cells) were transferred to 1.5ml tubes and then mixed with 200μl of low-melting temperature agarose and distributed onto conventional microscope

slides that had been pre-coated with standard agarose (0.5% in PBS). The agarose was allowed to solidify at 4ºC for 1 h. The slides were then immersed in lysis solution (Trivigen) for 1 h at 4ºC. Then slides were drained for excess buffer and gently immersed in pre-cooled neutral electrophoresis buffer (60.57g Tris Base, 204.12g NaOAc for 500ml of 10x buffer, pH=9) for 30 min. Electrophoresis done in a horizontal electrophoresis apparatus filled with fresh neutral electrophoresis buffer for 30 min at 300mA. Slides were air-dried and stained with 30-50µl of SYBR green 1 nucleic acid gel stain (Lonza). The slides were analyzed at 400X magnification using a fluorescence microscope (Nikon). The microscope images revealed circular shapes indicating undamaged DNA, or comet-like shapes indicating the DNA had migrated out from the head to form a tail (damaged DNA). Average comet tail moment was scored for 40-50 cells/slide using a computerized image analysis system (Komet5.5, Andor Technology, USA).

L. Analysis of Homologous Recombination activity

To measure the HR repair activity, stable U2OS cell lines expressing DR-GFP reporters were generated by transfection using lipofectamine 2000, Invitrogen. DR-GFP-U2OS cells were transfected with control or KPNA2 siRNA, at 6 h cells were again transfected with Isce-1 expression vector. After 48 h cell were fixed with 4% paraformaldehyde and nuclear stained with 5µg/ml Hoechst (Sigma) for 1 h. The images were shown at x10

magnification using an inverted florescence microscope, Nikon. The data are presented as the mean + SD.

M. Statistical analysis

Data are presented as means ± SD. Statistical comparisons were carried out using unpaired t- tests, and values of $P<0.01$ were considered to be statistically significant.

III. Results

Chapter 1 – KPNA2

A. Identification of MDC1-interacting proteins

MDC1 is a well-known mediator of DNA DSB repair (Goldberg et al., 2003; G. S. Stewart et al., 2003). To identify unknown MDC1 interacting proteins yeast two-hybrid screening for human MDC1 (1721-2089 amino acids) as bait was carried out. Through this screening we identified KPNA2, ZNF114, PHB2 and FHL2 as most relevant and novel binding partners of MDC1.

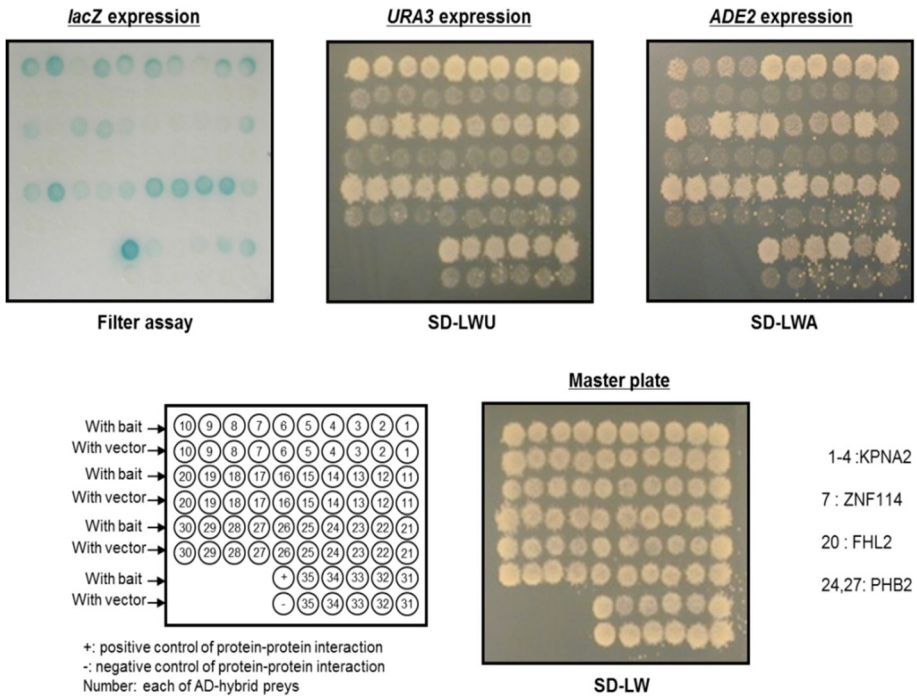


Figure 2. Protein interaction study by Yeast two-hybridization assay

B. Endogenous binding of MDC1 and KPNA2

It has been reported that KPNA2 drives the translocation of various cargo proteins from the cytoplasm to the nucleus through binding with NLS domain. These cargo proteins includes cell cycle regulators, cancer related proteins and DNA-double strand break repair proteins (Christiansen & Dyrskjot, 2013; Teng et al., 2006; Zannini et al., 2003). The interaction we observed between KPNA2 and MDC1 in yeast two-hybrid was confirmed by immunoprecipitation assay in cultured mammalian cells.

HEK293T cells were treated with 10Gy γ-irradiation to induce DSBs and harvested after 3hrs. The cells were lysed, and endogenous MDC1 was immunoprecipitated with specific antibody. Immunoprecipiates were then subjected to western blotting with an anti-KPNA2 or an anti-MDC1 antibody (Figure 3A). Immunoprecipitation results through western blotting showed us KPNA2 interacts with MDC1. The reciprocal experiment was also performed, KPNA2 was immunoprecipitated and western-blot analysis done with an anti-KPNA2 or an anti-MDC1 antibody (Figure 3B). These results confirmed the interaction between MDC1 and KPNA2. To confirm that the interaction between KPNA2 and MDC1 is specific, rabbit or mouse IgG was coimmunoprecipitated with MDC1 or KPNA2. The results showed us binding were specific and it's not due to antibody non-specific interaction.

Immunoprecipitation for both MDC1 and KPNA2 antibody was also performed (Figure 3).

C. Exogenous binding of full length MDC1 and KPNA2

Our endogenous binding result was further confirmed by exogenous co-Immunoprecipitation using full length MDC1 tagged with HA and GFP tagged full length KPNA2. HEK 293T cells were co-transfected with HA mock and GFP mock or HA-MDC1 and GFP mock or HA-MDC1 and GFP-KPNA2. Immunoprecipitation assay were performed by using an anti-HA antibody and immunoblotting was done with an anti-GFP or an anti-HA antibody (Figure 3C). Immunoprecipitation results through western blotting showed us HA-MDC1 interacts with GFP-KPNA2, results similar to endogenous Co-Immunoprecipitation. The reciprocal experiment was performed as immunoprecipitation with GFP-KPNA2 and immunoblotting was done with HA-MDC1 (Figure 3D). Inputs were loaded in the same order as immunoprecipitates. These results further confirmed interaction between HA-MDC1 and GFP-KPNA2.

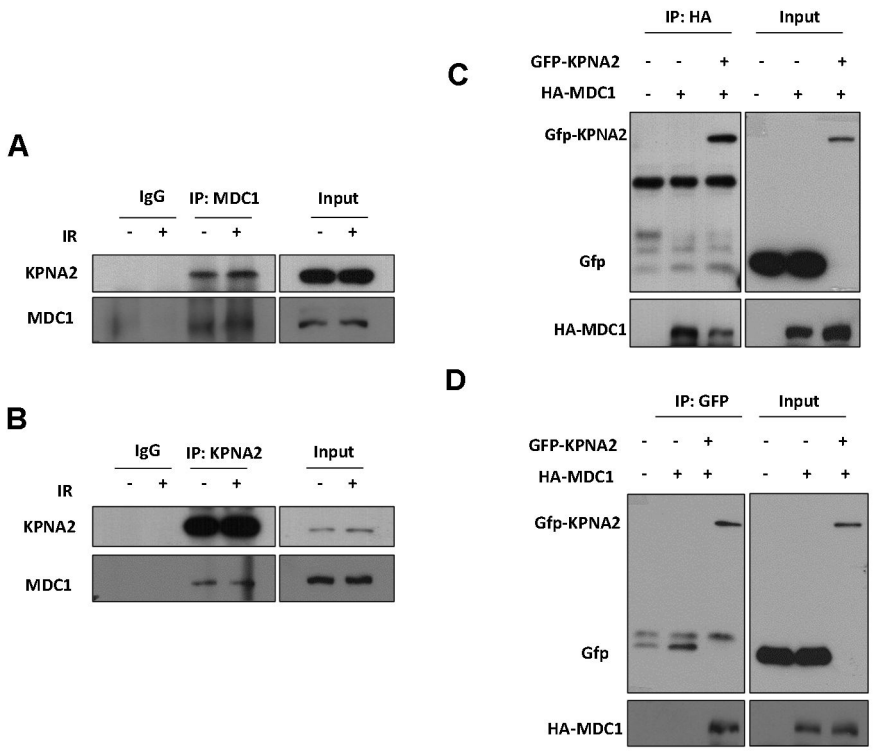


Figure 3. KPNA2 interacts with MDC1.

(A) HEK293T cells were untreated or treated with 10Gy γ-irradiation. After 3 h cells were extracted and lysed. Proteins were immunoprecipitated from the lysates using an anti-MDC1 antibody and then subjected to western-blot analysis using antibodies specific for KPNA2 or MDC1. The fifth and sixth lane contains corresponding inputs and Rabbit IgG was used for negative control immunoprecipitations. **(B)** HEK293T cells were untreated or treated with 10Gy γ-irradiation. After 3 h cells were extracted and lysed. Proteins were immunoprecipitated from the lysates using an anti-KPNA2 antibody and

then subjected to western-blot analysis using antibodies specific for KPNA2 or MDC1. The fifth and sixth lane contains corresponding inputs and normal mouse IgG was used for negative control immunoprecipitations. **(C)** HEK293T cells were co-transfected with HA mock and GFP mock or HA-MDC1 and GFP mock or HA-MDC1 and GFP-KPNA2 expression vectors. After 48 h cells were extracted and lysed. Proteins were immunoprecipitated from the lysates using an anti-HA antibody and then subjected to western-blot analysis using antibodies specific for HA or GFP. Inputs were loaded in the same order as immunoprecipitates. **(D)** HEK293T cells were co-transfected with HA mock and GFP mock or HA-MDC1 and GFP mock or HA-MDC1 and GFP-KPNA2 expression vectors. After 48 h cells were extracted and lysed. Proteins were immunoprecipitated from the lysates using an anti-GFP antibody and then subjected to western-blot analysis using antibodies specific for HA or GFP. Inputs were loaded in the same order as immunoprecipitates.

D. KPNA2 knockdown results in impaired nuclear translocation and IR induced nuclear focus formation of MDC1

Since previous results confirmed that KPNA2 directly binds to MDC1 and KPNA2 being a transporter protein, we thought of investigating the role of KPNA2 in MDC1 translocation from cytosol to DNA break site to execute repair after IR induced DSBs. To inspect this, control and KPNA2 stably knockdown HeLa cells were IR treated to induce DNA DSBs, and then nuclear and total fractions were extracted to detect MDC1 and KPNA2 protein expression through western blotting technique. In control cells, endogenous MDC1 expression level was increased in nuclear fraction after IR treatment. Whereas in KPNA2 stably knock-down cells, MDC1 level was found to be the same after IR induced DSB (Figure 4A). Our endogenous result was further confirmed by using full length MDC1 tagged with HA. HEK 293T cells were transiently transfected with HA tagged full-length MDC1 and then transfected with control or KPNA2 siRNAs. In this experiment design, HA-MDC1 level was found to be less after IR treatment in siKPNA2 treated cells compared to control (Figure 4B). Exogenous experiment results were in accordance with endogenously confirmed results. These results showed that in KPNA2 abolished cells MDC1 was not translocated to the nucleus after IR induced DSBs. It reveals that KPNA2 as a transporter protein is required for normal DNA DSB repair through regulating MDC1 translocation to the site of DNA repair.

We next measured MDC1 focus formation after DSB in control and KPNA2 stably knockdown HeLa cells in time dependent manner. Control and KPNA2 knockdown HeLa cells were treated with 10Gy γ-irradiation to induce DSBs and incubated for different time intervals. Cells were then fixed with 4% paraformaldehyde and immunofluorescence staining performed. Under confocal microscopy, MDC1 staining pattern was observed and number of intense nuclear foci was counted. After analyzing our immunofluorescence results, we observed two main differences between KPNA2 knock down and control cells. First, MDC1 staining intensity was more in cytoplasm in control cells compared to KPNA2 stable cells after IR treatment. Secondly, the MDC1 foci number was found to be decreased in KPNA2 stably knock down cells compared to control cells (Figure 5A). The decrease in KPNA2 expression results in impaired nuclear translocation of MDC1 and reduced MDC1 nuclear focus formation after IR induced DSB.

Our endogenous result was further confirmed by using full length MDC1 tagged with HA. KPNA2 stably knock down and Control cells were seeded on glass slides and transiently transfected with HA tagged full length MDC1. After 48 hours of transfection cells were treated with 10Gy γ-irradiation time dependently and immunostained with anti-HA antibody. As we observed in endogenous experiment, number of HA-MDC1 nuclear foci

was found to be decreased in KPNA2 stable cells compared to control (Figure 5B). These results denoted that KPNA2 drives the cytosol to nuclear translocation of MDC1 and DSB induced MDC1 nuclear focus formation.

E. KPNA2 mediated MDC1 translocation is necessary for nuclear focus formation of MDC1 downstream repair proteins after DSB

It is already reported that MDC1 is a critical upstream mediator in cellular response to DSBs, which works with H2AX to promote requirement of repair proteins to DNA repair site (Minter-Dykhouse, Ward, Huen, Chen, & Lou, 2008; G. S. Stewart et al., 2003). Since MDC1 nuclear focus formation in KPNA2 abolished cells were found to be decreased in previous experiment, we thought of checking two main MDC1downstram repair proteins 53BP1 and BRCA1. We also investigated one MDC1 upstream protein, γH2AX focus formation in early time after DNA damage. So we investigated nuclear focus formation of these three proteins in control and KPNA2 knockdown cells in the same method as we did for detecting MDC1 foci. Our results designated that both 53BP1 and BRCA1 foci formation was decreased in KPNA2 knock down cells compared with those of control cells (Figure 6A & 6B). Whereas, no change was detected between KPNA2 abolished and control cells in terms of early time γH2AX foci (Figure 6C). It shows that reduced MDC1 nuclear translocation and foci formation affected only downstream repair proteins. Thus, we concluded that KPNA2 mediated

translocation of MDC1 to the site of DNA repair is significantly important to carry out DNA damage repair since it affects the requirement of other repair proteins to the site of action.

F. KPNA2 knockdown leads to increased IR sensitivity and impaired DNA DSB repair

We examined Control and KPNA2 knockdown cells for sensitivity to IR by clonal survival assay. The survival of cells was measured by colony formation after treatment to IR. The colonies were observed in control and KPNA2 knockdown cells after 0, 1, 2 and 5 Gy IR treatments for 2 and 3 weeks of incubation. The observed results shown us, KPNA2 knockdown cells showed decreased number of colonies compared to control cells after 2 and 3 weeks of IR (Figure 7). This result showed that KPNA2 depletion leads to increased IR sensitivity.

Since KPNA2 knockdown resulted in increased IR sensitivity in cells, we intended to investigate the effect of KPNA2 knock down in DNA DSB repair. For this we assessed the late time focus formation of DSB marker γH2AX after IR treatment. The histone variant H2AX is phosphorylated at Ser-139 residue, forming γH2AX, as early cellular response to induction of DNA double strand breaks (Mah, El-Osta, & Karagiannis, 2010; Rogakou, Boon, Redon, & Bonner, 1999). Therefore, quantification of γH2AX foci in

late time after IR treatment can be used as the measure of DNA DSB repair activity. Control and KPNA2 knockdown cells were IR treated and incubated for 24hrs. After incubation cells were fixed and immunofluorescent stained for γH2AX foci detection. Control cells were found to have less number of γH2AX foci, whereas KPNA2 knockdown cells were counted with high number of foci (Figure 8). This result indicated us KPNA2 deficient cells accumulated more DSBs than control cells as a result of inefficient DNA DSB repair activity.

Next, we used single-cell gel electrophoresis (comet assay) under neutral condition to measure IR induced DSB repair activity in KPNA2 knockdown cells. Neutral comet assay is very sensitive method which can measure even very low level of DNA breaks. For this, HeLa cells transiently transfected with control and KPNA2 siRNAs were treated 10Gy IR to make DSBs and harvested in different time points. Based on DNA mobility or comet tail movement DSB repair will be measured. The tail movement was found to be less in control compared to KPNA2 siRNA treated HeLa cells (Figure 9). This increased tail movement in KPNA2 knock-down cells indicates the impaired DNA DSB repair in absence of KPNA2 expression. Taken together, results from functional analysis provide strong and clear evidence that KPNA2 is involved in the efficient DNA DSB repair and is required for optimal cell survival upon IR- induced DNA damage.

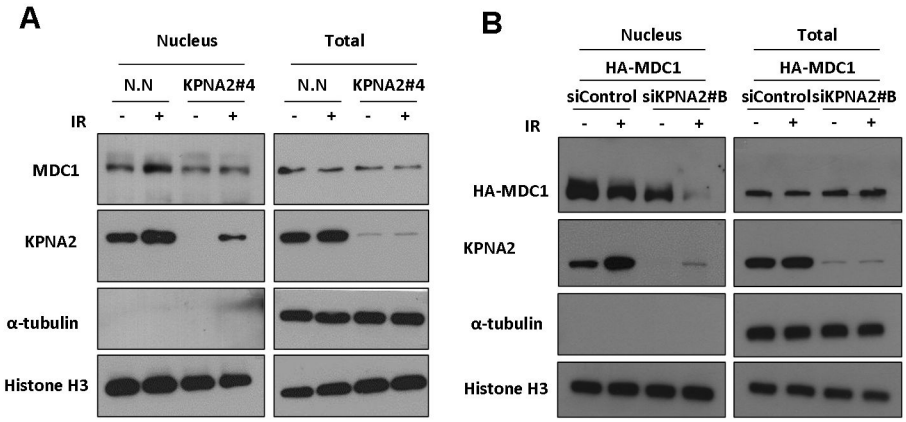
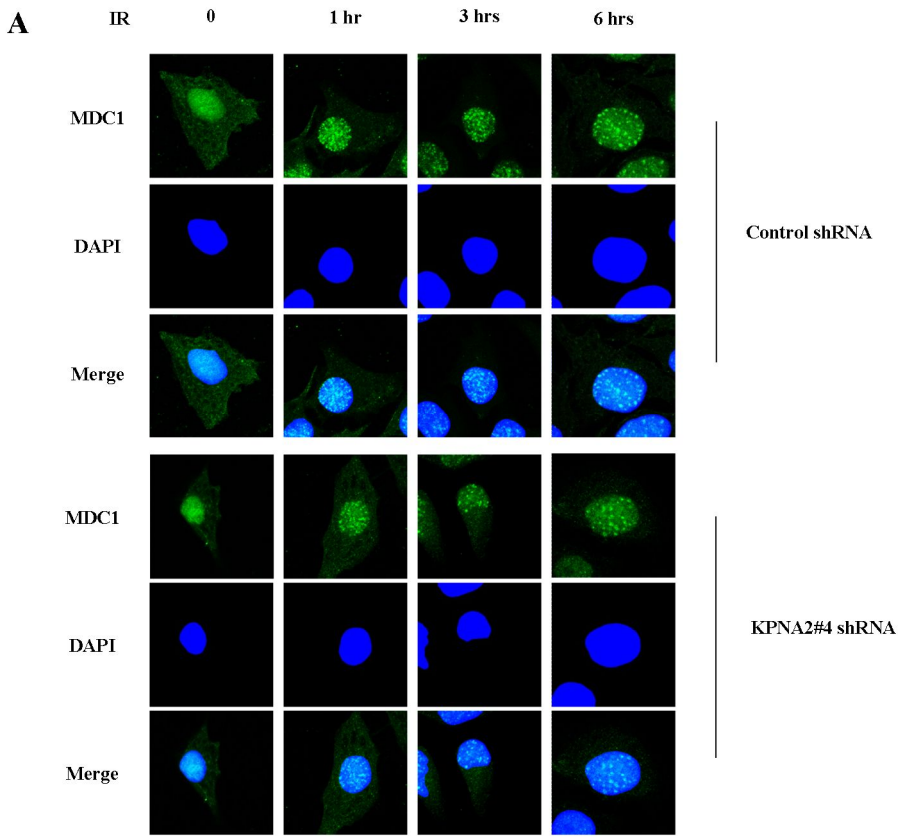
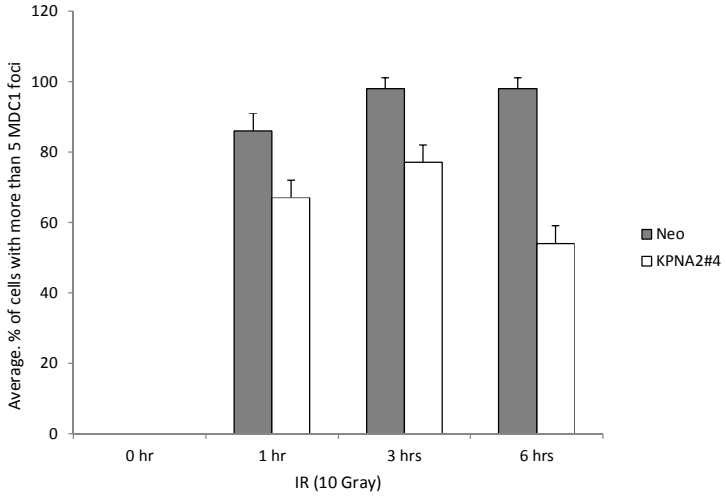
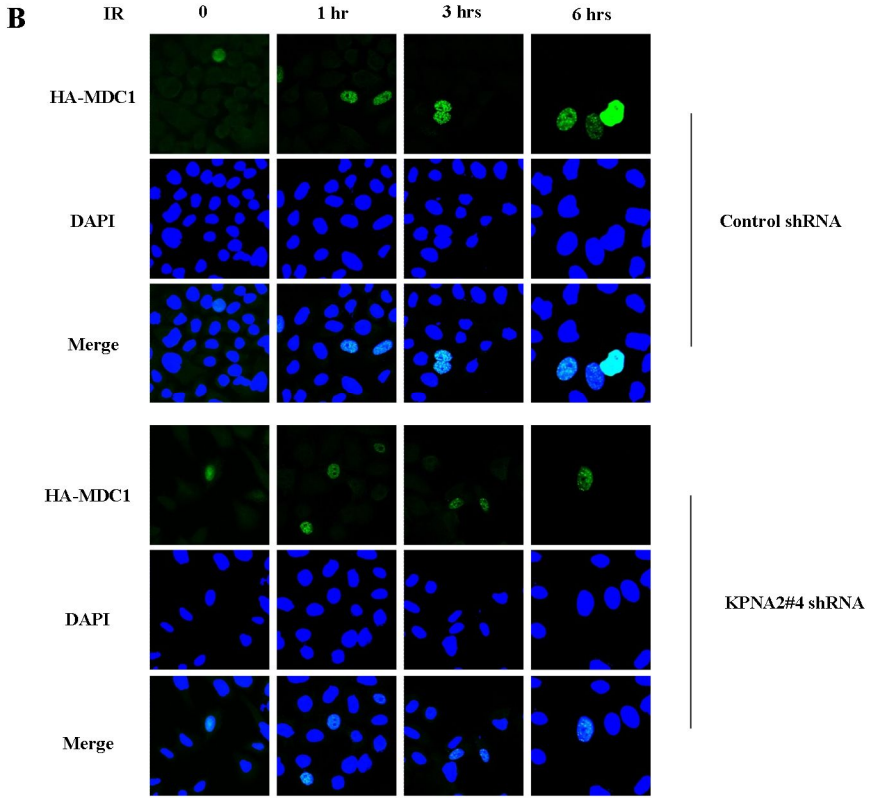


Figure 4. KPNA2 mediates MDC1 nuclear transport in response to γ-irradiation induced DNA DSBs.

(A) Control and KPNA2 stably knock-down HeLa cells were untreated or treated with 10Gy γ-irradiation. At 3 h after irradiation, the cells were fractionated into nuclear and total extracts as described in the experiments procedures. The fractionated nuclear and total extracts were subjected to western blotting using antibodies against MDC1 and KPNA2. Histone H3 and α-tubulin proteins were detected as positive controls for nuclear and total fractions respectively. **(B)** HEK293T cells were transfected with Control or KPNA2 siRNAs. After 6 h cells were transfected with HA-MDC1. After 3 h of irradiation, the cells were fractionated into nuclear and total extracts as described in the experiments procedures. The fractionated nuclear and total extracts were subjected to western blotting using antibodies against HA and KPNA2. Histone H3 and α-tubulin proteins were detected as positive controls for nuclear and total fractions respectively.







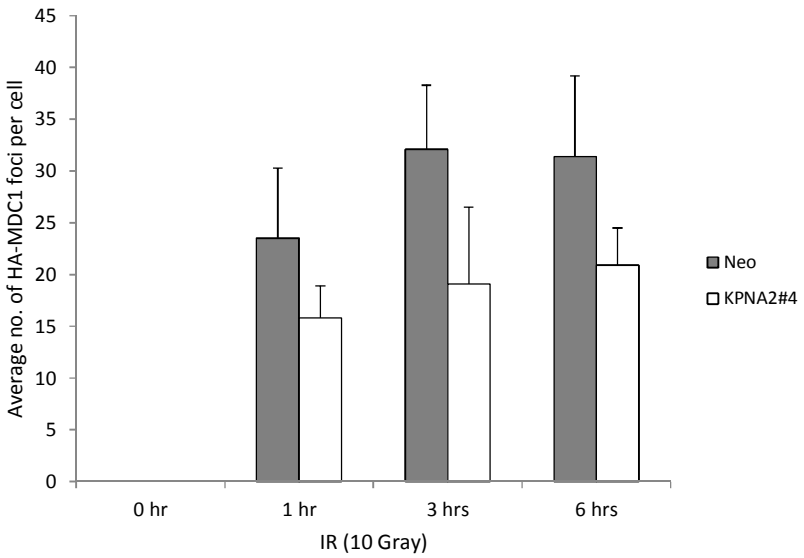
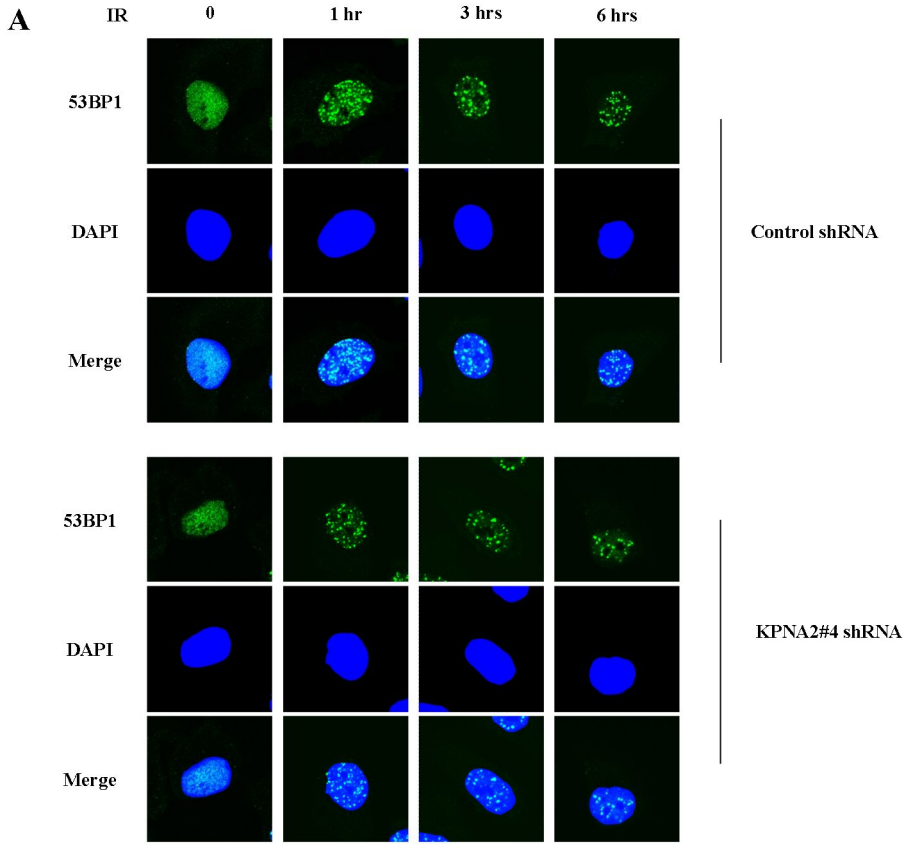
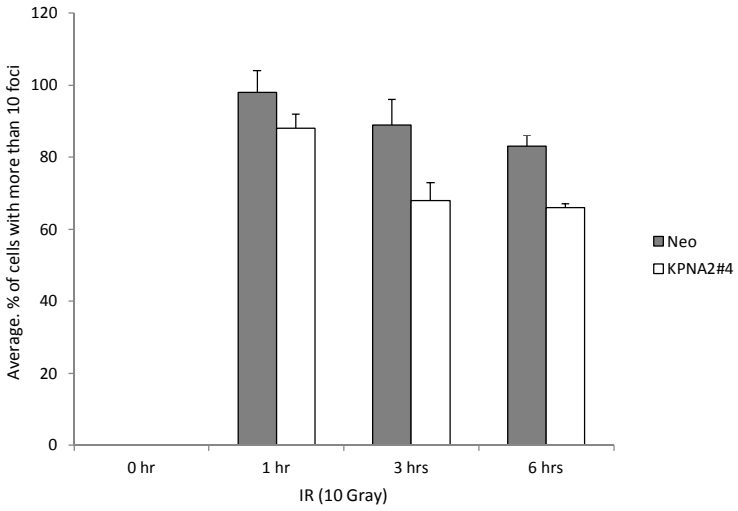
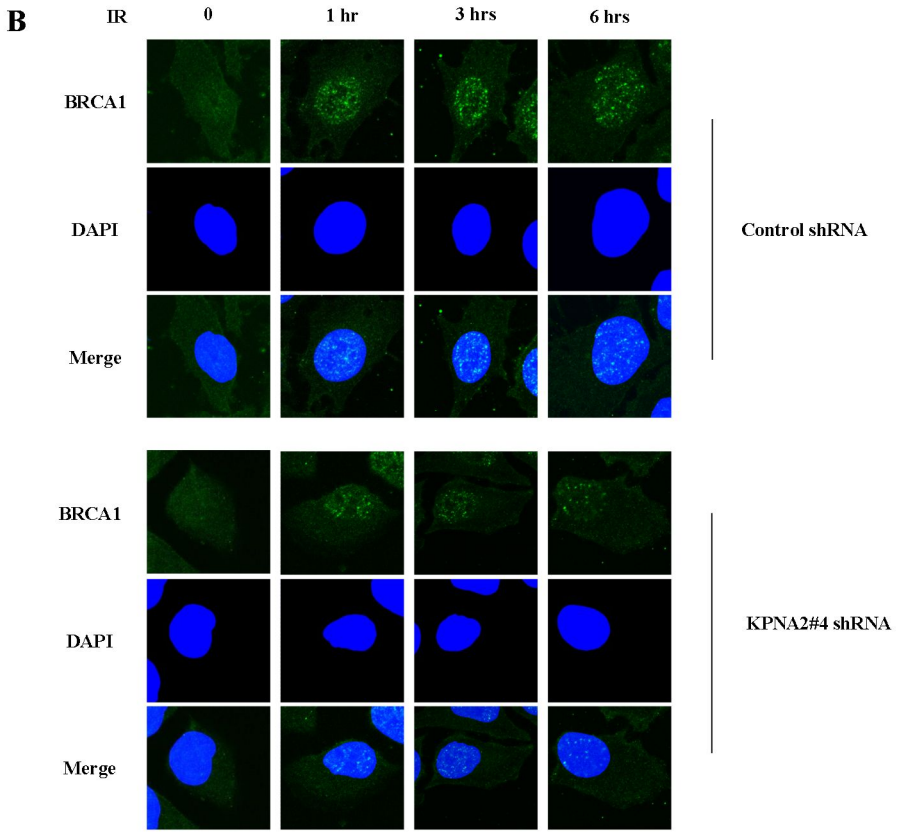


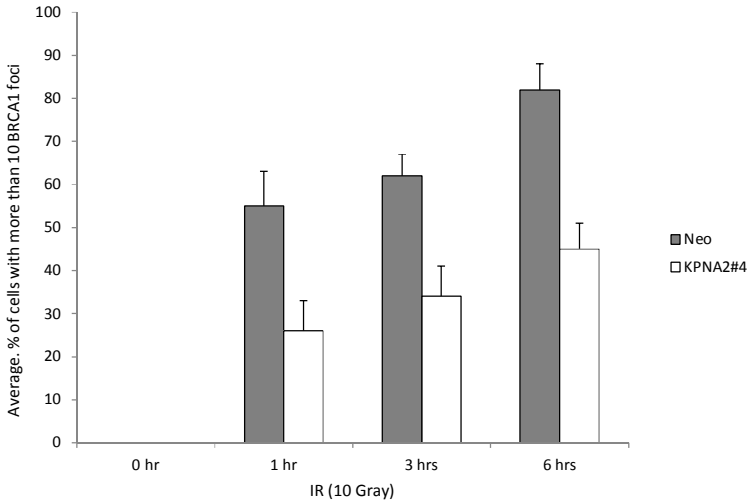
Figure 5. Down regulation of MDC1 foci formation in KPNA2-deficient HeLa cells after IR treatment.

(A) Control and KPNA2-depleted HeLa cells were untreated or treated with 10Gy γ-irradiation, fixed at the indicated time points and immunostained with anti-MDC1 antibody. 4'-6-Diamidino-2-phenylindole (DAPI) staining was performed to indicate the position of nucleus. Cells with more than 5 intense MDC1 foci were counted and graphically represented as average percentage of cells with more than 5 MDC1 foci. **(B)** Control and KPNA2-depleted HeLa cells were transfected with HA tagged full length MDC1 and after 48 h cells were untreated or treated with 10Gy γ-irradiation. At indicated time points cells were fixed and immunostained with anti-HA antibody. DAPI staining was performed to indicate the position of nucleus. HA-MDC1 foci were counted in each cell and graphically represented as average no of foci per cell.









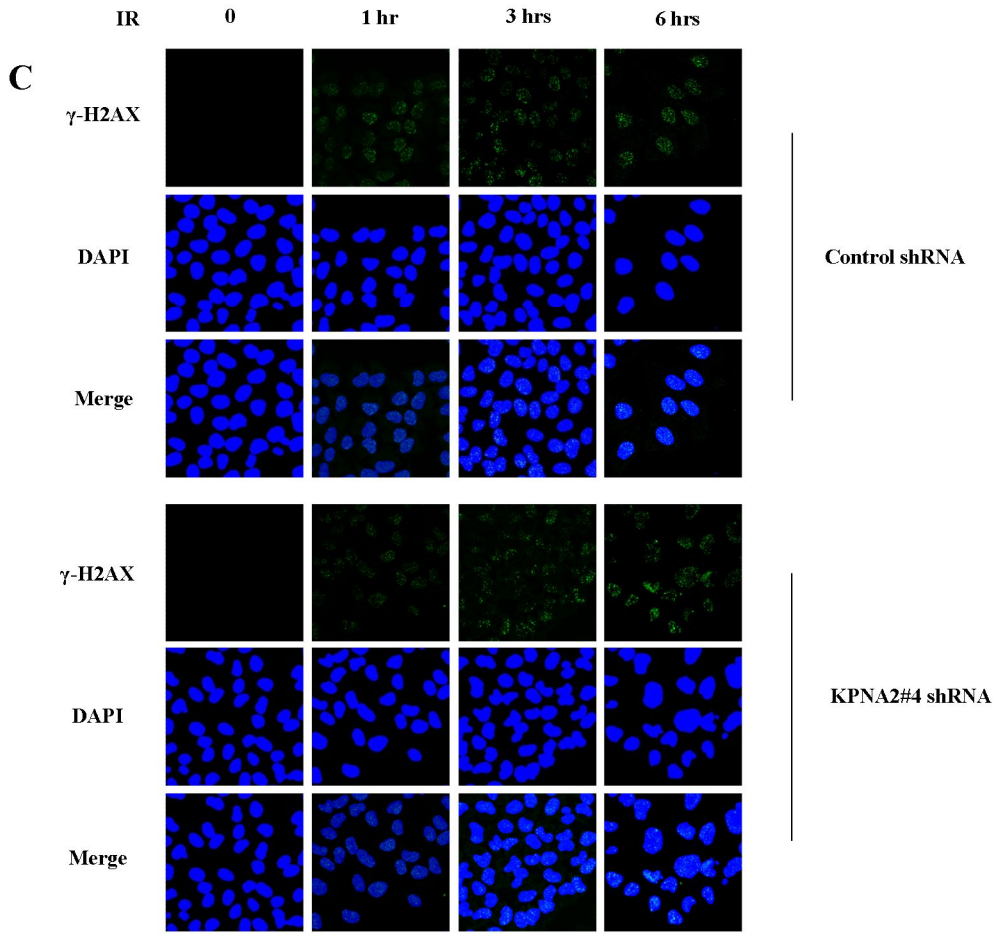


Figure 6. Down regulation of 53BP1 and BRCA1 foci formation in KPNA2-deficient HeLa cells after IR treatment.

(A) Control and KPNA2-depleted HeLa cells were untreated or treated with 10Gy γ-irradiation, fixed at the indicated time points and immunostained with anti-53BP1 antibody. DAPI staining was performed to indicate the position of nucleus. Cells with more than 10 intense 53BP1 foci were counted and

graphically represented as average percentage of cells with more than 10 53BP1 foci. (B) Down regulation of BRCA1 foci formation in KPNA2-deficient HeLa cells after IR treatment. Control and KPNA2-depleted HeLa cells were untreated or treated with 10Gy γ-irradiation, fixed at the indicated time points and immunostained with anti-BRCA1 antibody. DAPI staining was performed to indicate the position of nucleus. Cells with more than 10 intense BRCA1 foci were counted and graphically represented as average percentage of cells with more than 10 BRCA1 foci. (C) No difference in IR induced γH2AX foci formation between KPNA2-deficient and control HeLa cells. Control and KPNA2-depleted HeLa cells were untreated or treated with 10Gy γ-irradiation, fixed at the indicated time points and immunostained with anti- γH2AX antibody. DAPI staining was performed to indicate the position of nucleus.

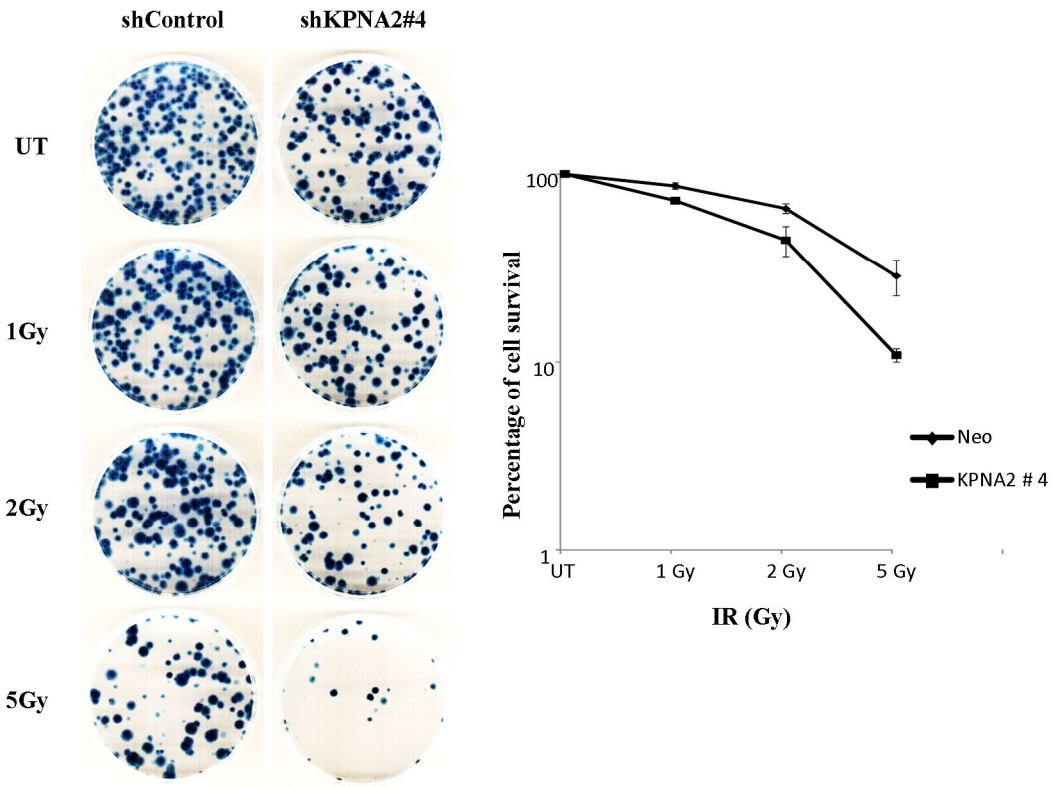


Figure 7. KPNA2 knockdown sensitized cells to IR treatment.

KPNA2 depletion affects survival of cells following exposure to γ-irradiation. Control and KPNA2- depleted HeLa cells were untreated or treated with 1, 2 and 5 Gy ionizing radiation, after 3 weeks colonies were stained with methylene blue and counted.

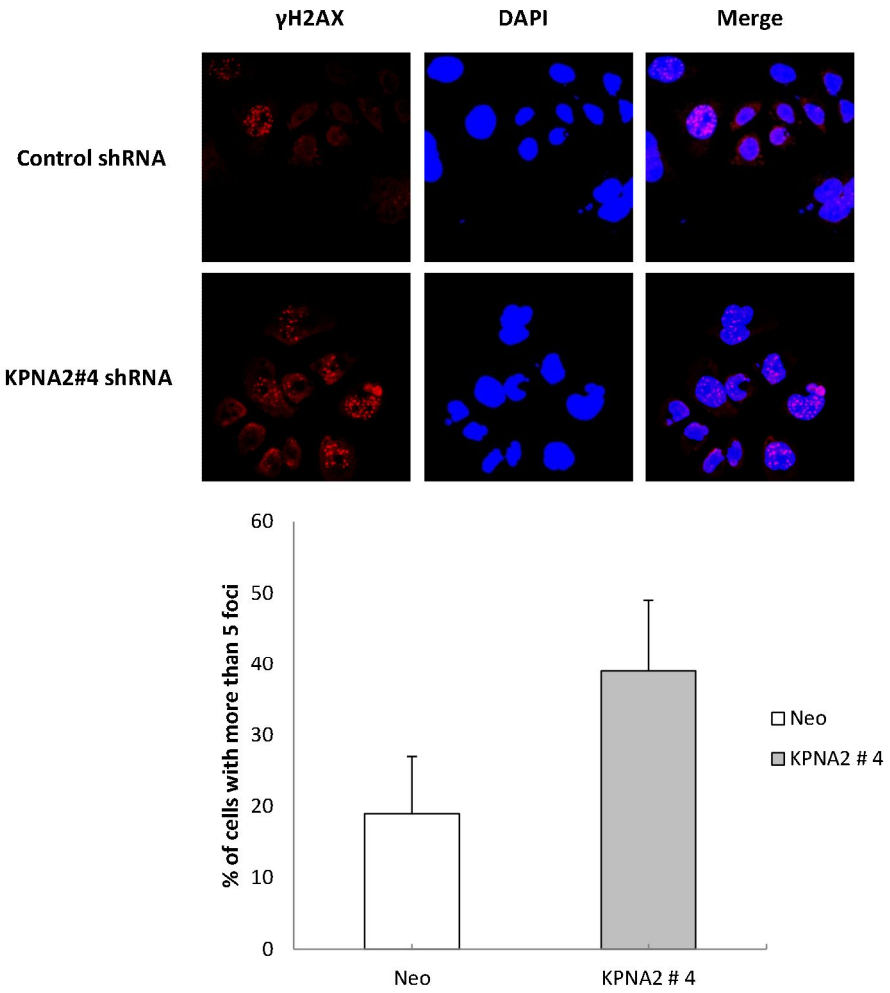


Figure 8. KPNA2 knockdown cells exhibits prolonged γ-H2AX foci formation after IR.

Control and KPNA2-depleted HeLa cells were treated with 10Gy γ-irradiation. Cells were fixed at 24 h after irradiation and analyzed by immunofluorescence with γ-H2AX antibody. Cells with more than 5 intense γ-H2AX foci were counted and graphically represented as average percentage of cells with more than 5 γ-H2AX foci.

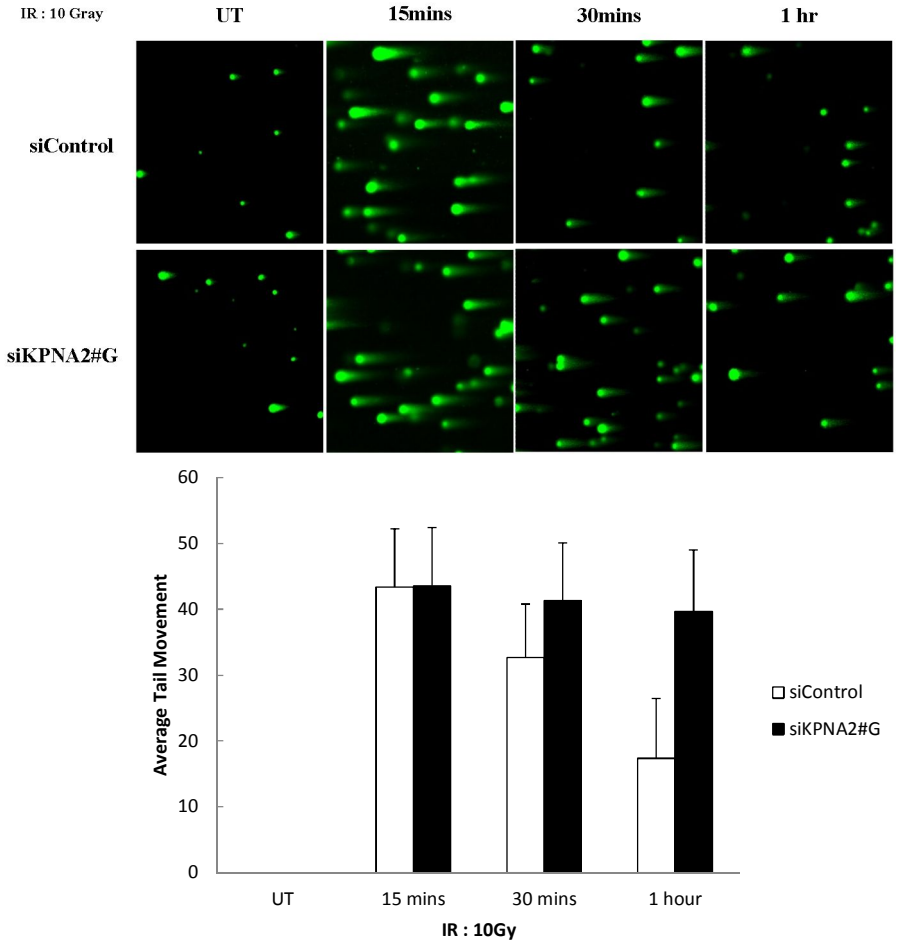


Figure 9. KPNA2 knockdown results in decreased DSB repair.

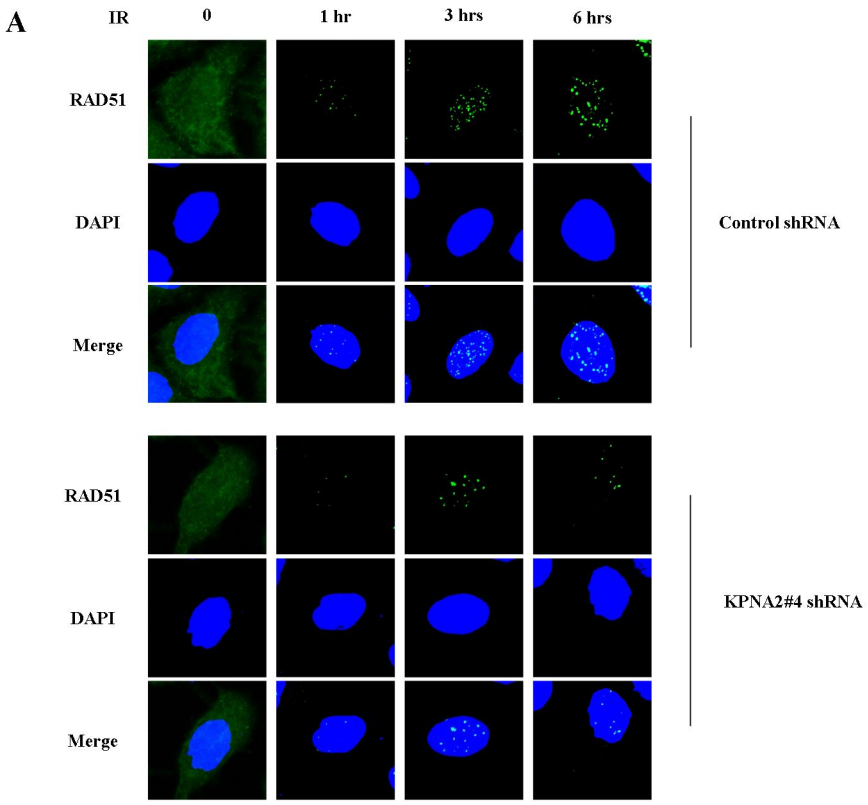
Control and KPNA2-depleted HeLa cells were untreated or treated with 10Gy γ-irradiation. At the indicated time points cells were harvested to carry out comet assay under neutral condition. Comet images were captured using fluorescence microscopy, and tail moment was analyzed using Komet 5.5 analysis software. Representative comet images obtained at different time points are shown. Changes in the tail moments between control and KPNA2 knockdown cells after IR treatment are represented in histogram.

G. KPNA2 Knockdown leads to impaired homologous recombination repair

To understand the effect of KPNA2 knock-down over homologous recombination (HR) repair efficiency we first thought of checking a primary HR repair marker, Rab51 foci formation in control and KPNA2 knock-down cells. Control and KPNA2 shRNA knockdown HeLa cells were 10Gy IR treated to induce DNA DSB and incubated for different time intervals for recovery. Then fixed with 4% paraformaldehyde and immunostaining procedure carried out to detect Rad51 foci formation. The result showed that Rad51 nuclear focal formation was found to be decreased in KPNA2 knock-down cells in comparison to control (Figure 10).

Since Rab51 foci formation was found to be impaired in absence of KPNA2 expression, we intended to investigate any involvement of KPNA2 in HR activity. To this purpose, GFP-based chromosomal reporter assay in DR-GFP-U2OS cells were used. In this system DSB generated through the expression of I-Sce1 endonuclease, which cleaves a specific recognition site located in the GFP gene and cells were incubated for recovery. Repair efficiency via HR is monitored through measuring the percentage of cells expressing GFP using fluorescence microscope. To assess the HR repair efficiency in KPNA2 knock-down cells, we measured the level of HR repair activity in KPNA2 impaired cells in comparison to control cells. For this, DR-

GFP-U2OS cells were transfected with control and KPNA2 siRNAs, and then second transfection with 3μg of I-Sce1 expression vector. After 48 hrs, cells were fixed with 4% paraformaldehyde and nucleus stained with Hoechst. GFP expressions in repaired cells were monitored using inverted florescence microscope. The analysis showed nearly 5-fold decreased GFP expression in KPNA2 abolished cells when compared to control (Figure 11B & C). This result indicates the involvement of KPNA2 in DSB repair through HR pathway.



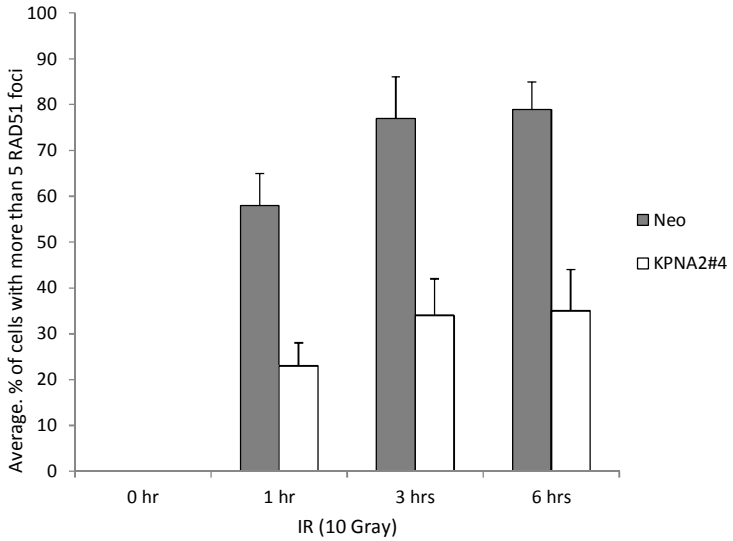


Figure 10. Down regulation of RAD51 foci formation in KPNA2-deficient HeLa cells after IR treatment.

Control and KPNA2-depleted HeLa cells were untreated or treated with 10Gy γ-irradiation, fixed at the indicated time points and immunostained with anti-RAD51 antibody. DAPIstaining was performed to indicate the position of nucleus. Cells with more than 5 intense RAD51 foci were counted and graphically represented as average percentage of cells with more than 5 RAD51 foci.

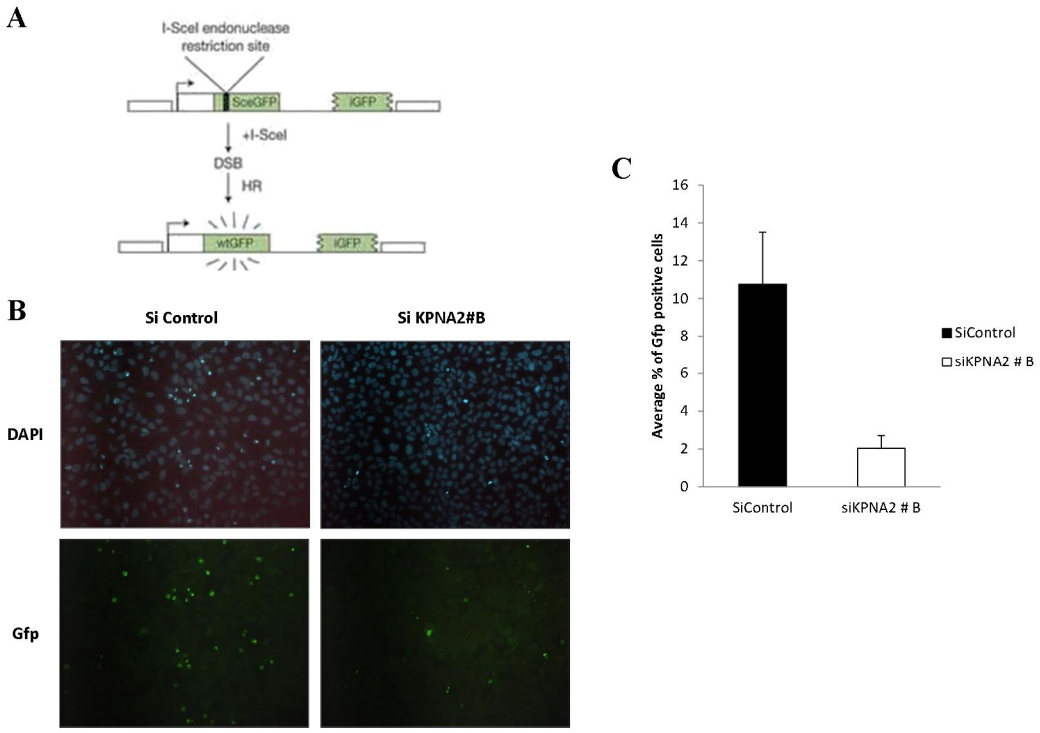


Figure 11. Impaired HR activity in KPNA2 abolished cells.

(A) Schematic representation of GFP-based chromosomal reporter assay. (B) DR-GFP-U2OS cells were transfected with siControl or siKPNA2, at 6 h after incubation cells were again transfected with Isce-1 endonuclease expression vector. After 48 h cells were fixe with 4% paraformaldehyde and nucleus stained with Hoechst. (C) GFP positive cells were counted and graphically represented as average percentage of GFP positive cells.

Chapter 2 – ZNF114

A. ZNF114 abolishment leads to increased IR sensitivity of cells due to inefficient DNA DSB repair

First of all we investigated IR sensitivity in control and ZNF114 knockdown cells though colony survival assay. Sensitivity of cells towards IR is measured by colony formation ability after irradiation. The colonies were observed in control and ZNF114 knockdown cells after 0, 1, 2 and 5 Gy IR treatments for 2 and 3 weeks of incubation. The observed results shown us, ZNF114 knockdown cells showed decreased number of colonies compared to control cells after 2 and 3 weeks of IR (Figure 12). This result showed that ZNF114 depletion leads to increased IR sensitivity.

Since ZNF114 knockdown resulted in increased IR sensitivity in cells, we intended to investigate the effect of ZNF114 knockdown in DNA DSB repair. For this we assessed the late time focus formation of DSB marker γH2AX after IR treatment. Control and ZNF114 knockdown cells were IR treated and incubated for 24hrs. After incubation cells were fixed and immunofluorescent stained for γH2AX foci detection. Control cells were found to have less number of γH2AX foci, whereas ZNF114 knockdown cells were counted with high number of foci (Figure 13).

Next, we used single-cell gel electrophoresis (comet assay) under neutral condition to measure IR induced DSB repair activity in ZNF114 knockdown cells. For this, HeLa cells transiently transfected with control and ZNF114 siRNAs were treated 10Gy IR to make DSBs and harvested in different time points. Based on DNA mobility or comet tail movement DSB repair will be measured. The tail movement was found to be less in control compared to ZNF114 siRNA treated HeLa cells (Figure 14). This increased tail movement in ZNF114 knockdown cells indicates the impaired DNA DSB repair in absence of ZNF114 expression.

Taken together, these initial results provide strong and clear evidence that ZNF114 is involved in the efficient DNA DSB repair and is required for optimal cell survival upon IR- induced DNA damage.

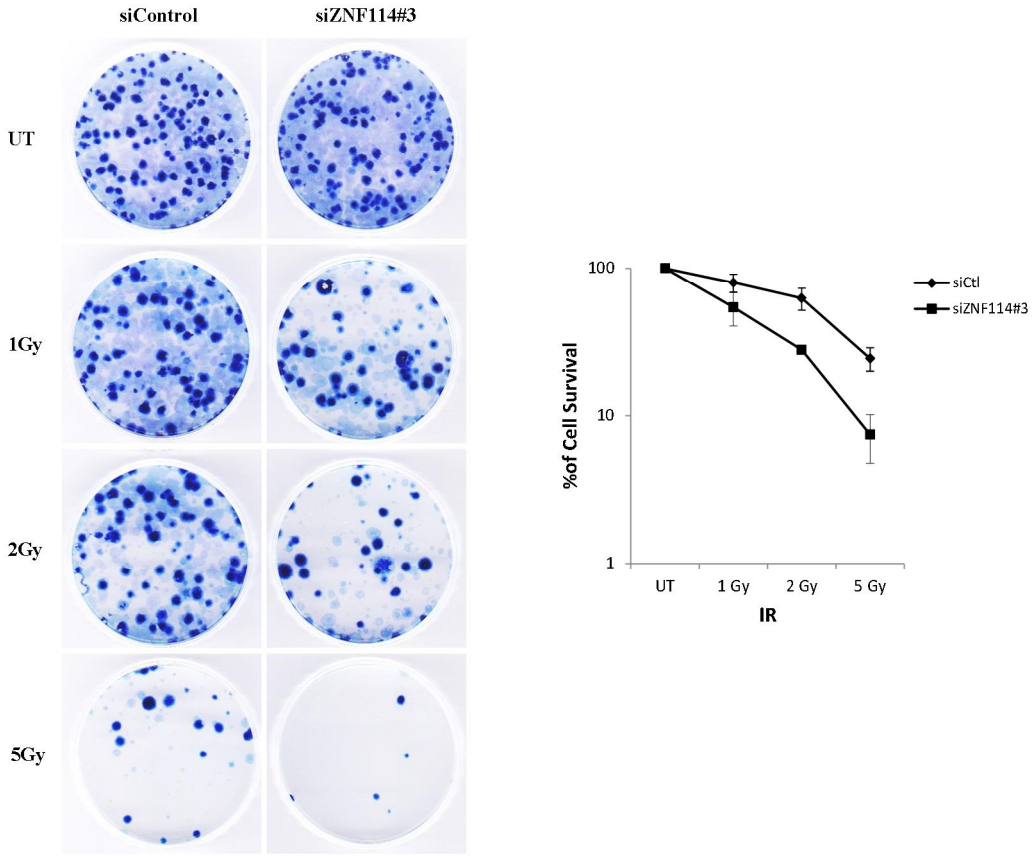


Figure 12. ZNF114 knockdown sensitized cells to IR treatment.

ZNF114 depletion affects survival of cells following exposure to γ-irradiation. Control and ZNF114- depleted HeLa cells were untreated or treated with 1, 2 and 5 Gy ionizing radiation, after 3 weeks colonies were stained with methylene blue and counted.

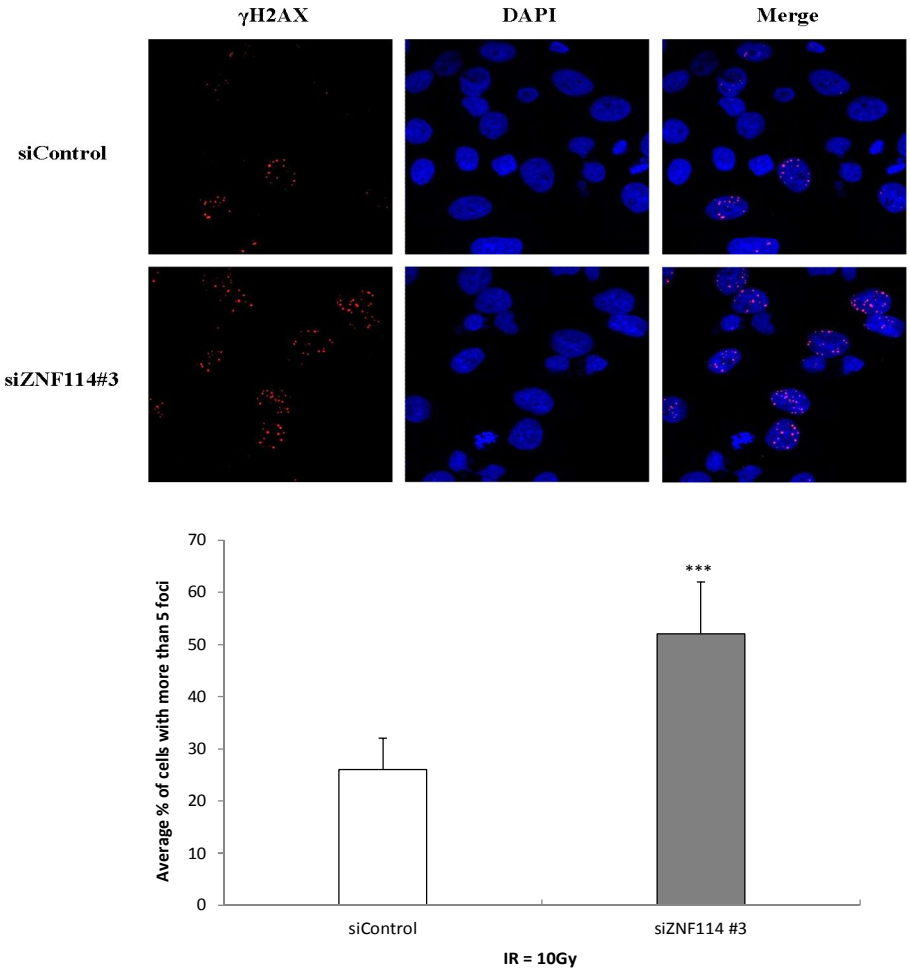


Figure 13. ZNF114 knockdown cells exhibits prolonged γ-H2AX foci after IR treatment.

Control and ZNF114-depleted HeLa cells were treated with 10Gy γ-irradiation. Cells were fixed at 16 h after irradiation and analyzed by immunofluorescence with γ-H2AX antibody. Cells with more than 5 intense foci were counted and graphically represented as average percentage of cells with more than 5 γ-H2AX foci.

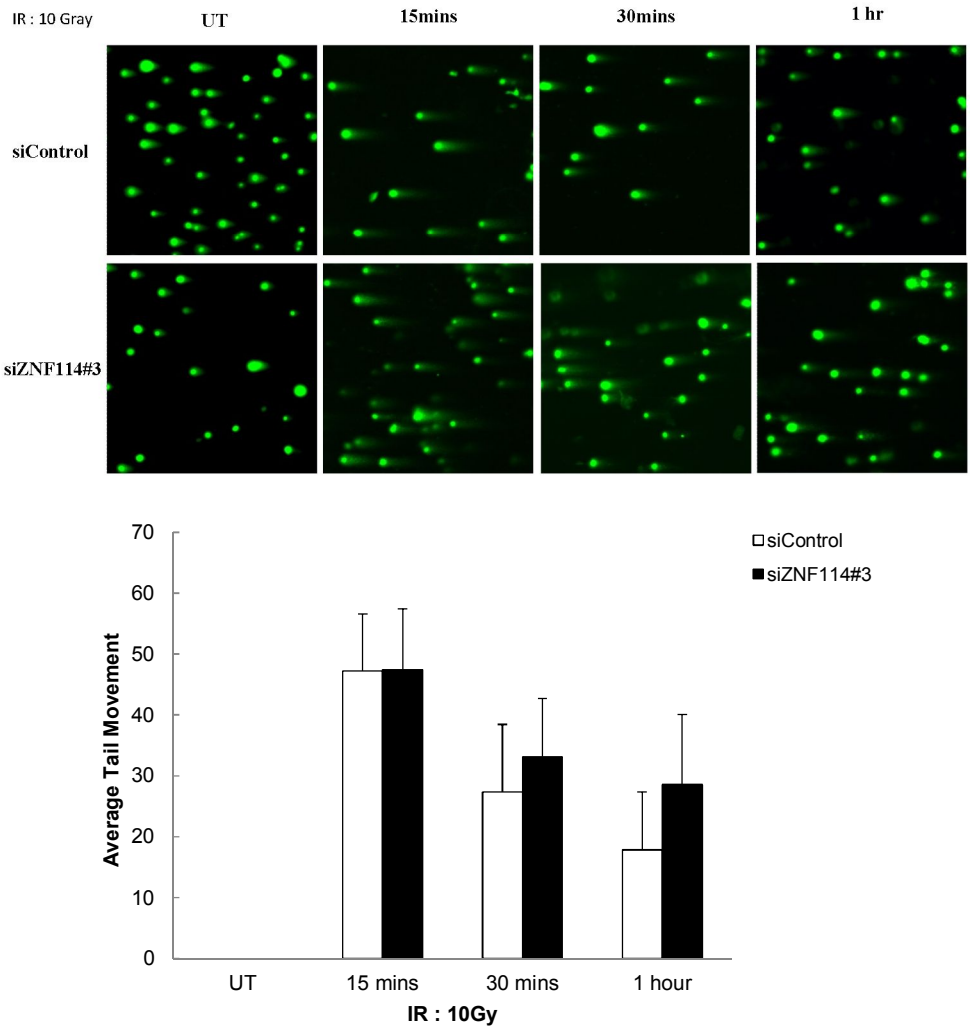


Figure 14. ZNF114 knockdown results in decreased DSB repair.

Control and ZNF114-depleted HeLa cells were untreated or treated with 10Gy γ-irradiation. Comet images were captured using fluorescence microscopy, and tail moment was analyzed using Komet 5.5 analysis software. Changes in the tail moments between control and ZNF114 knockdown cells after IR treatment are represented in histogram.

B. ZNF114 plays a role in DNA DSB repair through Homologous Recombination (HR)

Previous results provided enough evidences that ZNF114 is involved in the process of DNA damage repair. We wanted to determine thought which pathway it regulates DSB repair. For this we tested ZNF114 function in two important DNA damage repair pathways, HR and NHEJ. We studied the effect of ZNF114 knockdown over HR repair activity through DR-Gfp reporter assay. DR-Gfp U_2OS cells were seeded and transfected with control and ZNF114 siRNAs followed by Isce1 plasmid transfection after 6 hrs. Then cells were incubated for recovery. After 48hrs cells were analyzed through FACS (fluorescence activated cell sorting) machine to measure Gfp fluorescence as a measure of HR activity. Positive Gfp expressions in ZNF114 siRNA treated cells were found to be significantly decreased compared to control (Figure 15 & 16). To investigate ZNF114 involvement in NHEJ, same DR-Gfp assay was used by transfecting DR-Gfp HeLa cells. There is no difference in Gfp expression was detected between control and siZNF114 treated cells (Figure 17). These results clearly showed that ZNF114 plays a role in DNA damage repair though HR pathway.

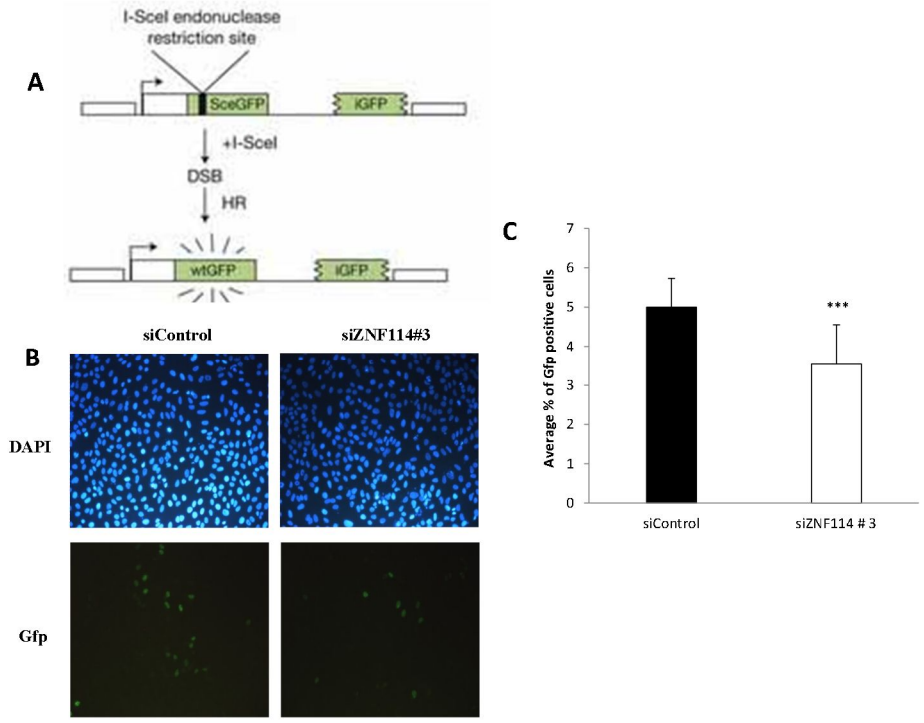


Figure 15. Impaired HR activity in ZNF114 abolished cells.

(A) Schematic representation of GFP-based chromosomal reporter assay. (B) DR-GFP-U2OS cells were transfected with siControl or siZNF114, at 6 h after incubation cells were again transfected with Isce-1 endonuclease expression vector. After 48 h cells were fixe with 4% paraformaldehyde and nucleus stained with Hoechst. (C) GFP positive cells were counted and graphically represented as average percentage of GFP positive cells.

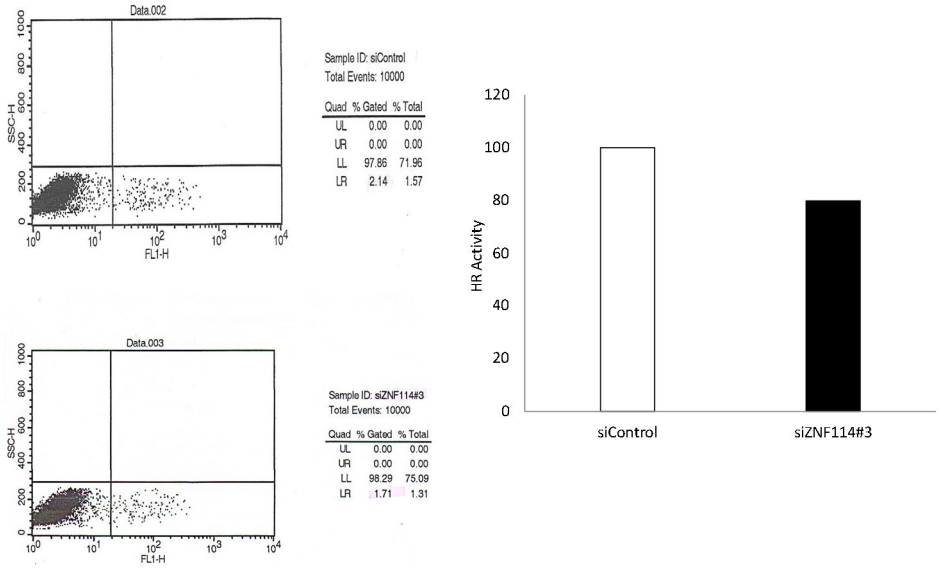


Figure 16. Impaired HR activity in ZNF114 abolished cells – FACS analysis.

DR-GFP-U2OS cells were transfected with siControl or siZNF114, at 6 h after incubation cells were again transfected with Isce-1 endonuclease expression vector. After 48 h cells were extracted and FACS analyzed for GFP expression. FACS analyzed results were graphically represented as HR activity.

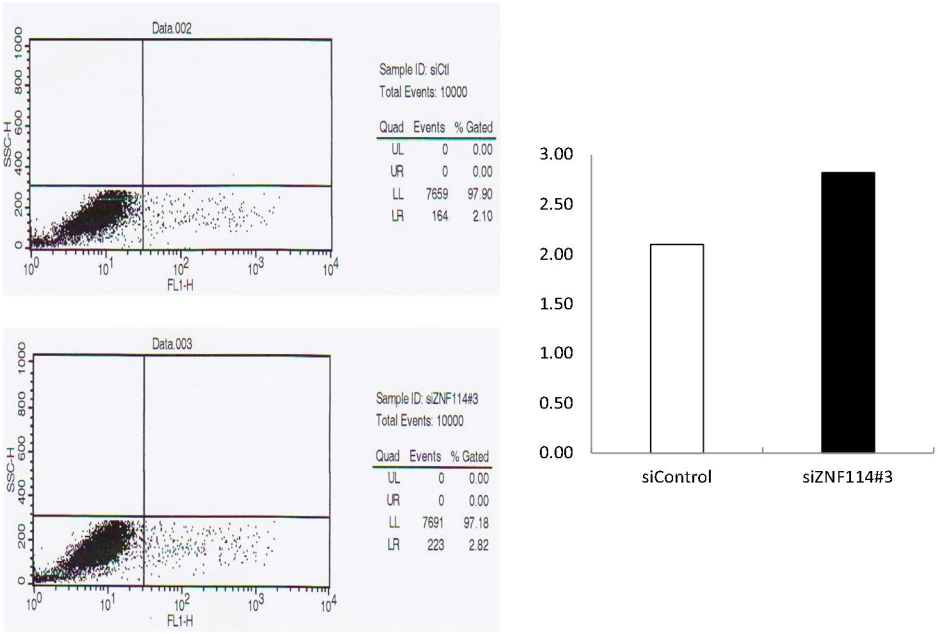
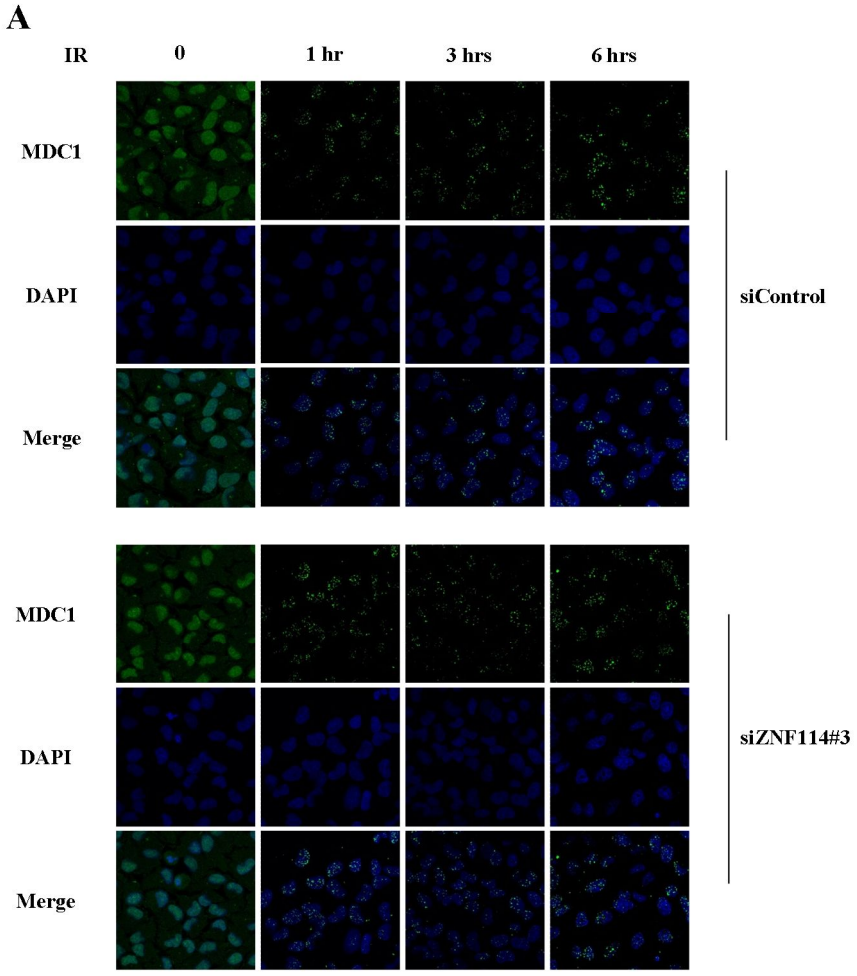


Figure 17. NHEJ activity not decreased in ZNF114 abolished cells – FACS analysis.

DR-GFP-HeLa cells were transfected with siControl or siZNF114, at 6 h after incubation cells were again transfected with Isce-1 endonuclease expression vector. After 48 h cells were extracted and FACS analyzed for GFP expression. FACS analyzed results were graphically represented as NHEJ activity.

C. Effect of ZNF114 knockdown over DDR proteins

ZNF114 was found to be involved in DNA damage repair specifically in HR pathway. So we intended to investigate the effect of ZNN114 knockdown over the ability of DDR proteins to form nuclear foci. Control and ZNF114 knockdown HeLa cells were treated with 10Gy γ-irradiation in order to induce DSBs and incubated at 37^{o}C with 5% CO_2 for different time intervals. Then cells were fixed and stained with MDC1, 53BP1, BRCA1, RAD51 and RPA antibodies using same method as mentioned earlier. Foci formation pattern and foci number were detected under confocal microscopy in control and siZNF114 cells. No significant difference was detected in terms of MDC1 (Figure 18A) and 53BP1 nuclear foci formation (Figure 18B). So, we concluded that ZNF114 knockdown do not have any effect on MDC1 and 53BP1 foci formation.



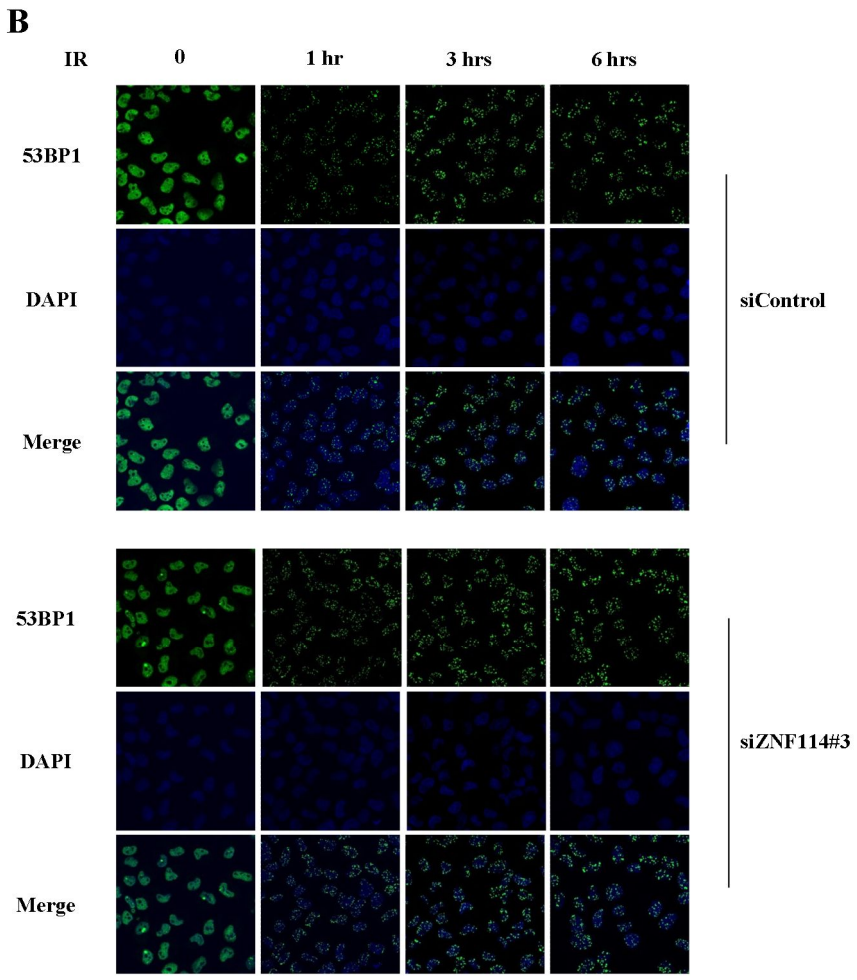
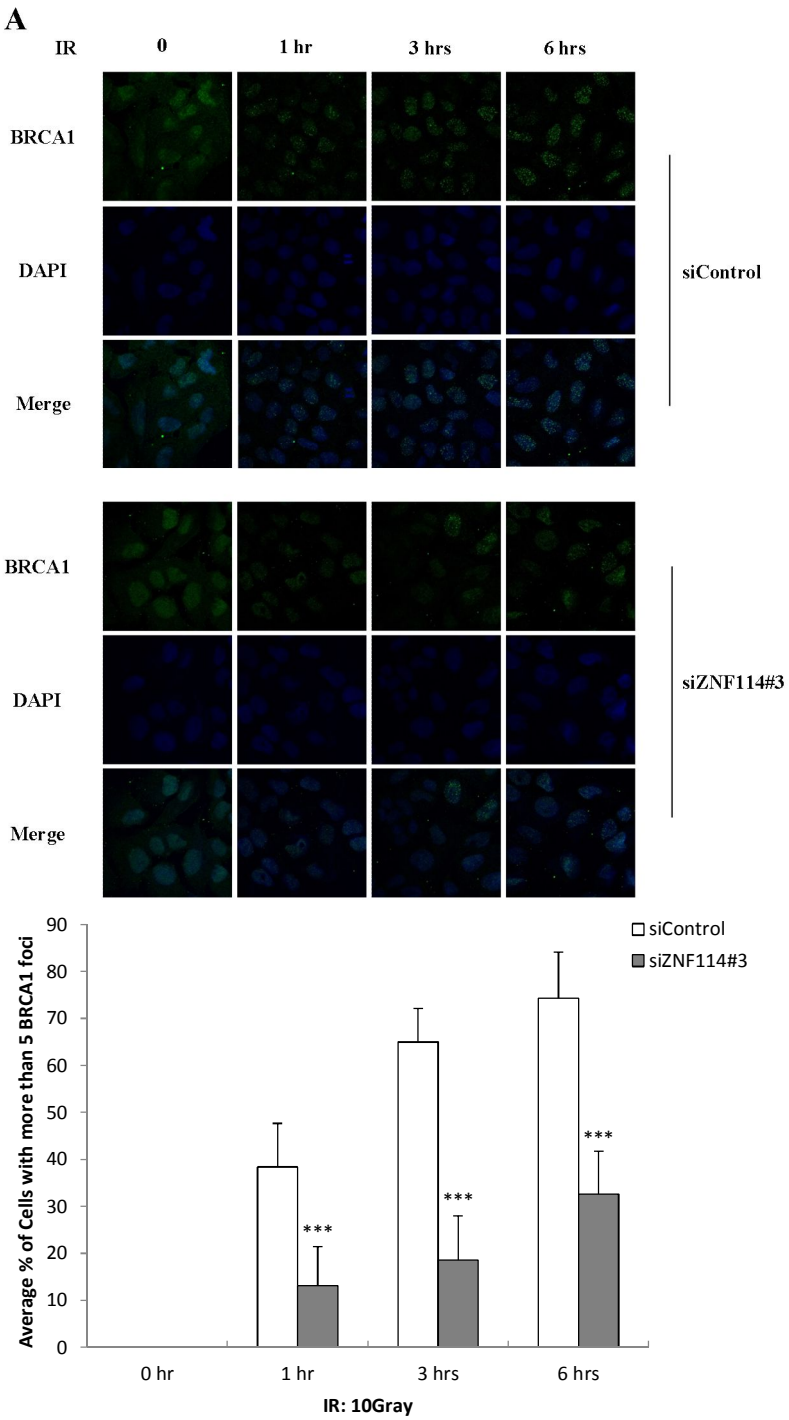


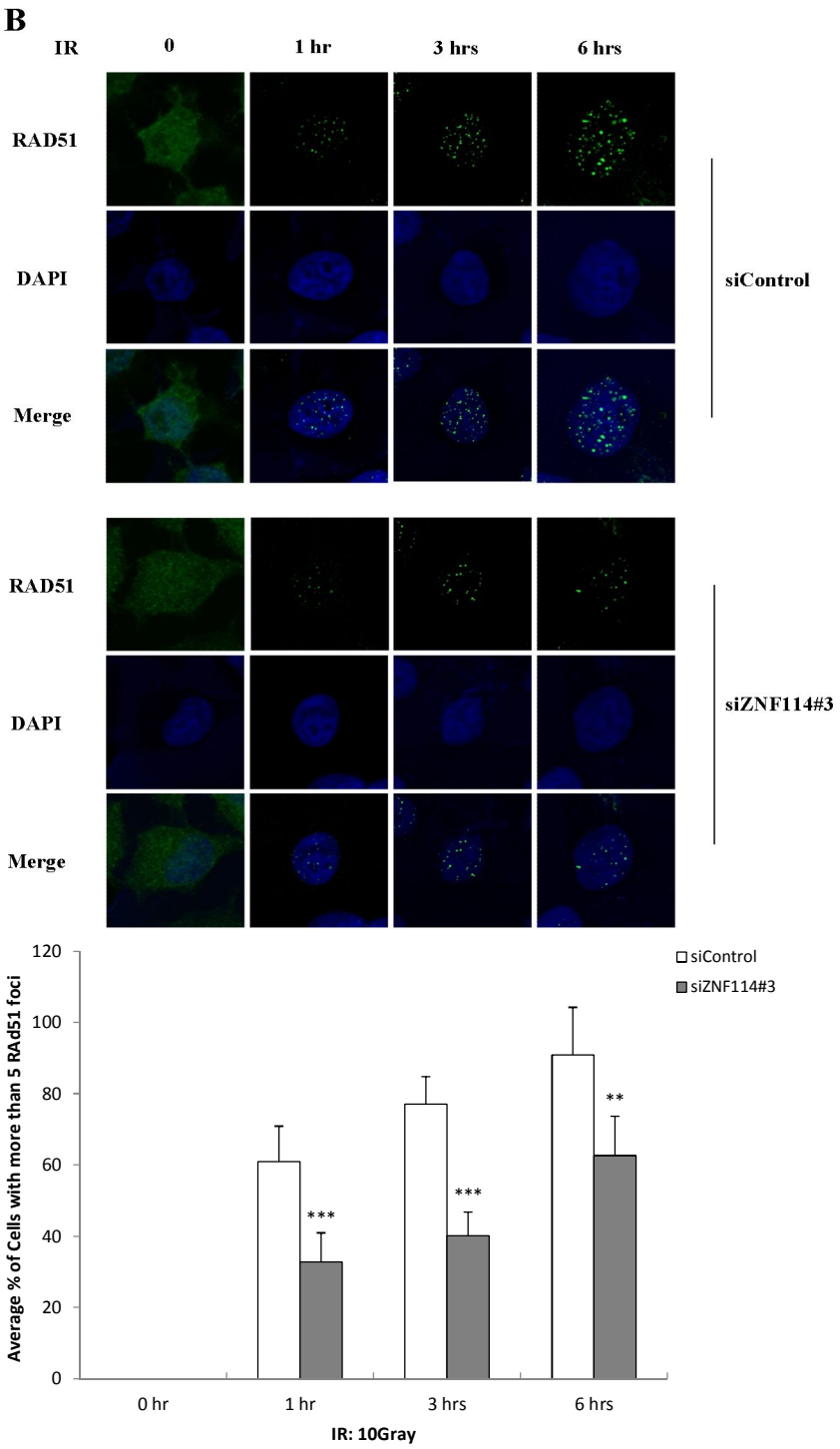
Figure 18. No difference in MDC1 and 53BP1 nuclear foci formation.

No difference in IR induced (A) MDC1 and (B) 53BP1 foci formation between ZNF114-deficient and control HeLa cells. Control and ZNF114-depleted HeLa cells were untreated or treated with 10Gy γ-irradiation, fixed at the indicated time points and immunostained with (A) anti-MDC1 and (B) anti-53BP1 antibodies. DAPI staining was performed to indicate the position of nucleus.

D. ZNF114 knockdown leads to decreased nuclear foci formation of MDC1 downstream proteins

Even though ZNF114 knockdown does not have any effect on MDC1 and 53BP1 foci formation, we detected a significantly decreased nuclear foci formation for MDC1 down-stream repair proteins BRCA1, RAD51, and RPA in siZNF114 condition (Figure 19A, B and C). Thus, we got strong evidence that ZNF114 is involved in DNA damage repair though HR and loss of ZNF114 expression leads to inefficient DNA damage repair due to improper or reduced DDR protein recruitment.





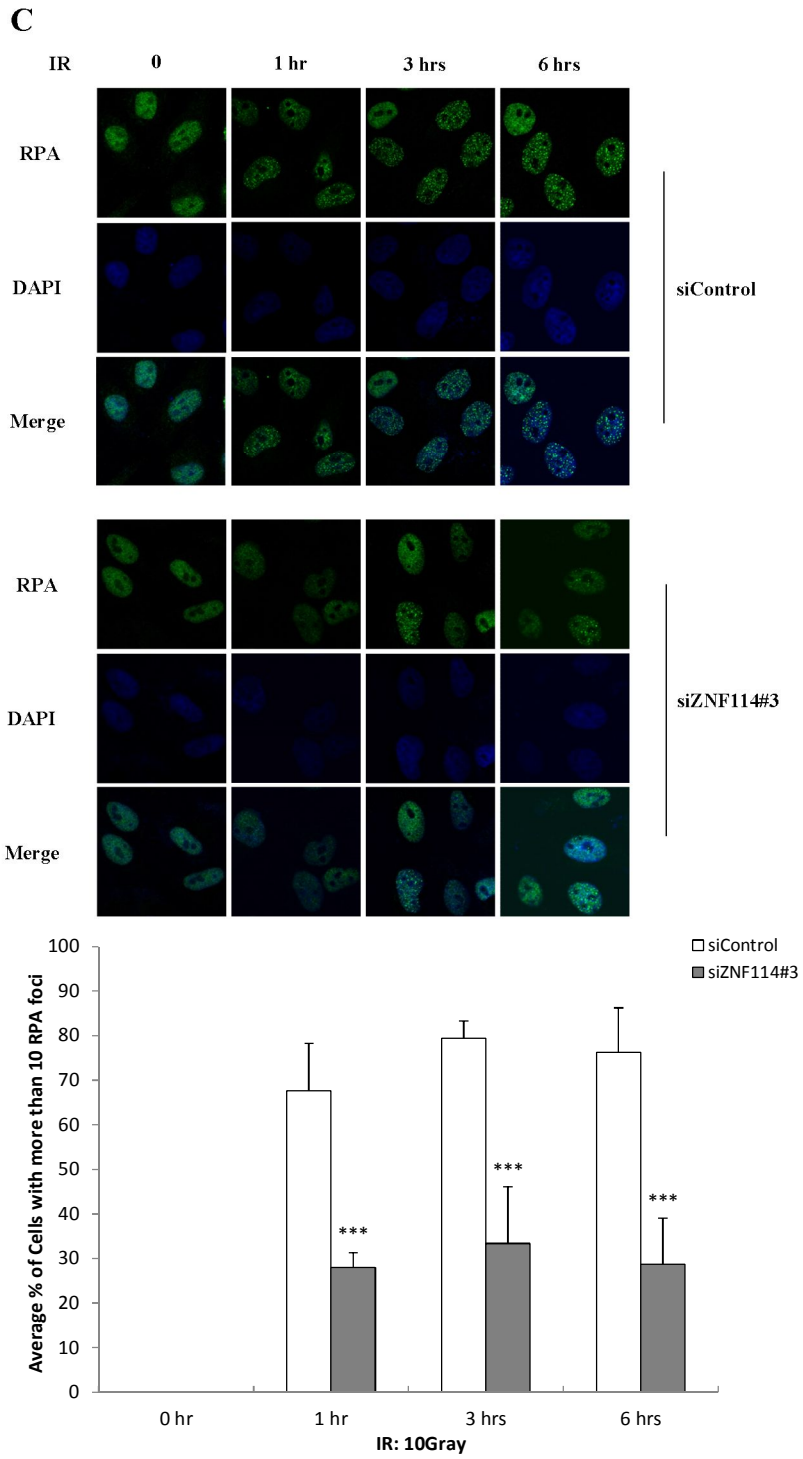


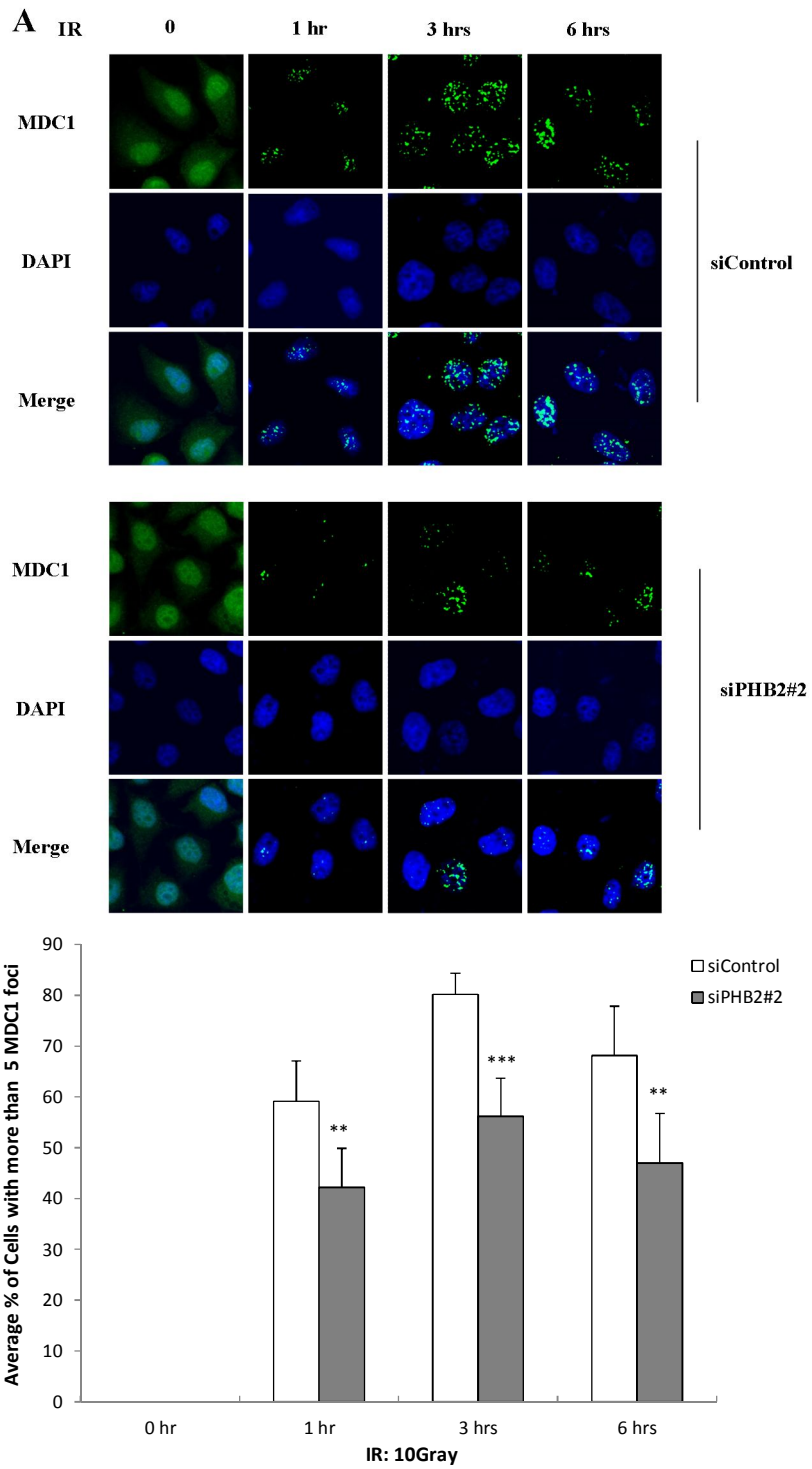
Figure 19. Down regulation of BRCA1, RAD51 and RPA foci formation in ZNF114-deficient HeLa cells after IR treatment.

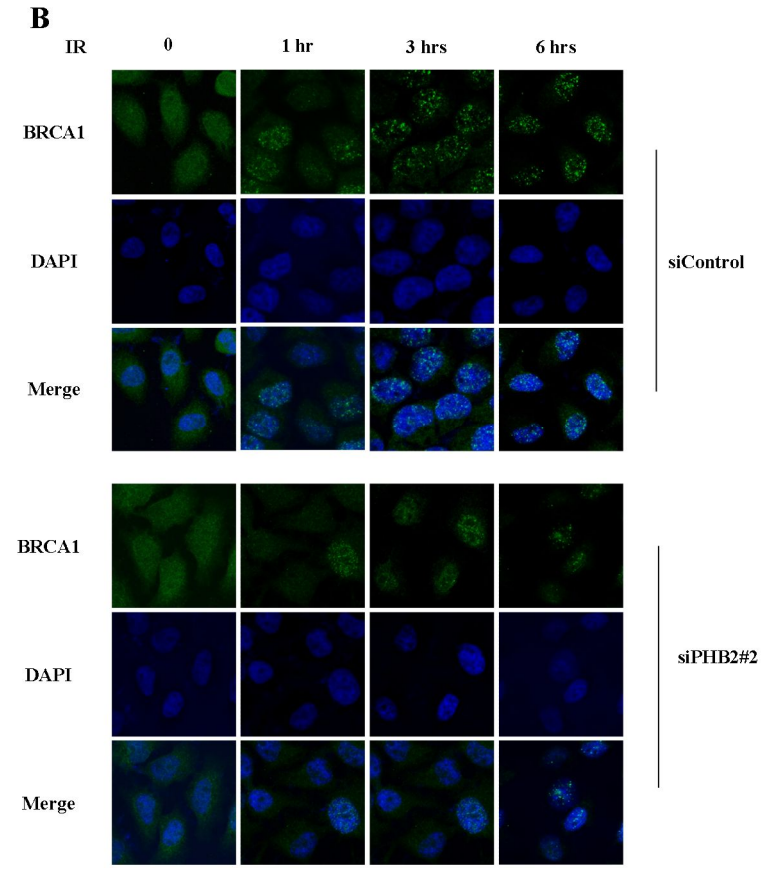
(A) Control and ZNF114-depleted HeLa cells were untreated or treated with 10Gy γ-irradiation, fixed at the indicated time points and immunostained with anti-BRCA1 antibody. DAPI staining was performed to indicate the position of nucleus. Cells with more than 5 intense foci were counted and graphically represented as average percentage of cells with more than 5 BRCA1 foci. **(B)** Down regulation of RAD51 foci formation in ZNF114-deficient HeLa cells after IR treatment. Control and ZNF114-depleted HeLa cells were untreated or treated with 10Gy γ-irradiation, fixed at the indicated time points and immunostained with anti-RAD51 antibody. DAPI staining was performed to indicate the position of nucleus. Cells with more than 5 intense foci were counted and graphically represented as average percentage of cells with more than 5 RAD51 foci. **(C)** Down regulation of RPA foci formation in ZNF114-deficient HeLa cells after IR treatment. Control and ZNF114-depleted HeLa cells were untreated or treated with 10Gy γ-irradiation, fixed at the indicated time points and immunostained with anti-RPA antibody. DAPI staining was performed to indicate the position of nucleus. Cells with more than 10 intense foci were counted and graphically represented as average percentage of cells with more than 10 RPA foci.

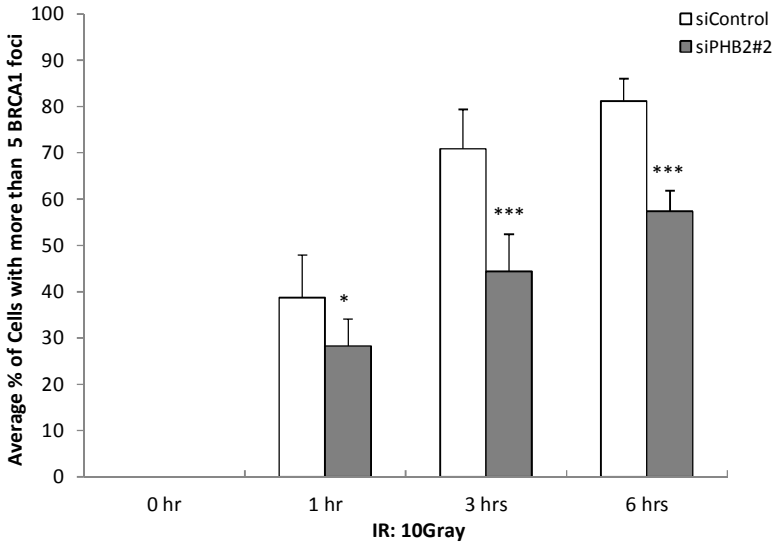
Chapter 3 – PHB2

A. Effect of PHB2 knockdown over MDC1 and its downstream proteins to form irradiation induced nuclear foci

We intended to investigate the effect of PHB2 knockdown over the ability of DNA damage checkpoint protein MDC1 and its downstream proteins to form irradiation induced nuclear foci. Control and PHB2 knockdown HeLa cells were treated with 10Gy γ-irradiation in order to induce DSBs and incubated at 37^{o}C with 5% CO_2 for different time intervals. Then cells were fixed and stained with MDC1, BRCA1 and 53BP1 antibodies using same method as mentioned earlier. Confocal microscopy analysis revealed that number of cells with more than 5 MDC1 foci was found be significantly decreased in siPHB2 treated cells compared to control (Figure 20A). The two MDC1 downstream proteins namely BRCA1 and 53BP1 also microscopically detected for foci number. We detected significantly decreased number of BRCA1 foci in siPHB2 cells compared to control (Figure 20B), whereas no difference was detected between siPHB2 and control cells in terms of 53BP1 foci number (Figure 20C). So, we concluded that PHB2 knockdown have an effect on MDC1 foci formation and its downstream protein BRCA1.







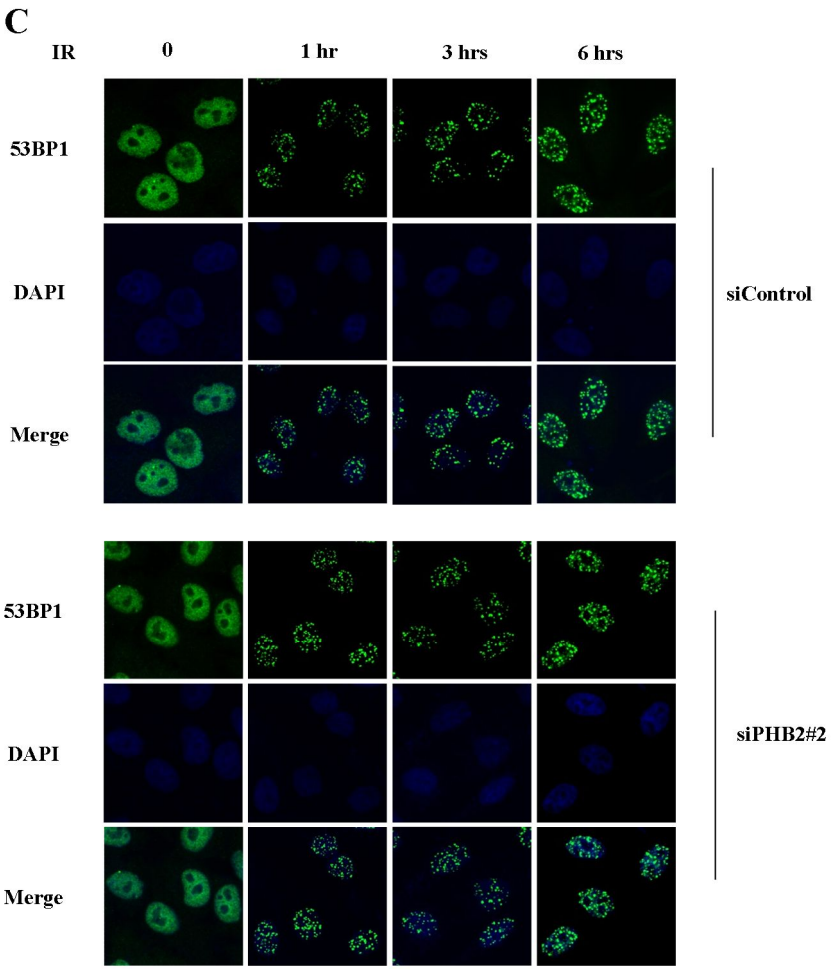


Figure 20. MDC1 and its downstream proteins nuclear foci formation in PHB2-deficient HeLa cells.

Control and PHB2-depleted HeLa cells were untreated or treated with 10Gy γ-irradiation, fixed at the indicated time points and immunostained with **(A)** MDC1, **(B)** BRCA1 and **(C)** 53BP1 antibodies. DAPI staining was performed to indicate the position of nucleus. Cells with more than 5 intense foci were counted and graphically represented.

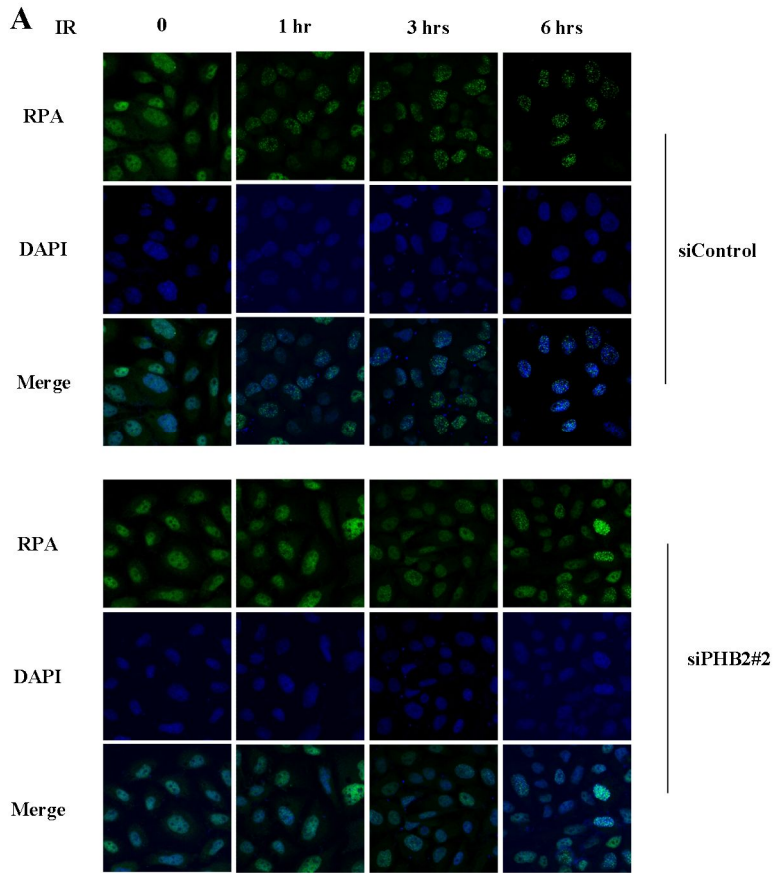
B. Down-regulation of RAD51 and RPA nuclear foci formation in PHB2 deficient cells

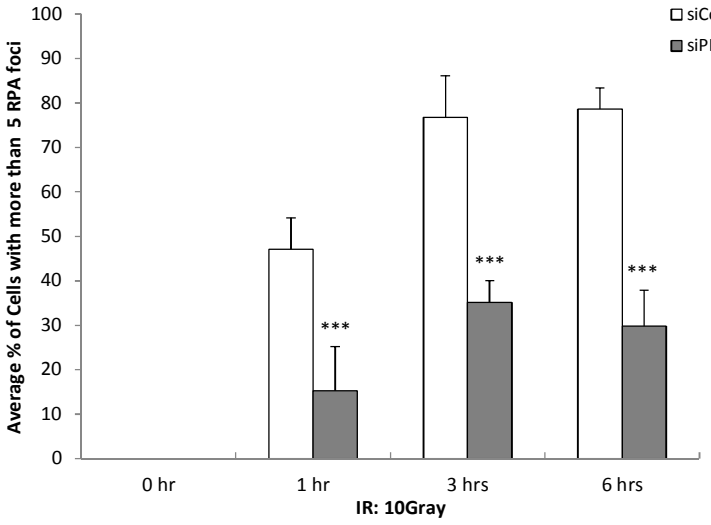
As we detected reduced number of irradiation induced nuclear foci formation for HR candidate gene BRCA1 and no difference in NHEJ gene 53BP1, we thought that PHB2 may be involve in HR. To test our hypothesis, we investigated the nuclear foci formation of two important HR pathway genes RPA and RAD51. Control and PHB2 siRNA treated cells were irradiated to induce DNA damage and stained for RPA and RAD51 nuclear foci. Foci number was found to be significantly less in siPHB2 treated cells compared to control for both these proteins (Figure 21A&B). This result showed us, PHB2 is important for DNA damage repair through HR by recruiting MDC1 and its downstream proteins.

C. PHB2 plays a role in Homologous Recombination

Since we detected a decreased number of RPA foci in siPHB2 condition, we supposed to investigate a role of PHB2 in HR. RPA binding to 3'ssDNA tail is the initiating step of HR and it prevents NHEJ (Krasner, Daley, Sung, & Niu, 2015; Nakajima et al., 2015) . The RecA/RAD51 family member binds with this 3'-ssDNA tail to initiate homologous pairing (Mimitou & Symington, 2011; Symington, 2014, 2016). Moreover DNA

damage response proteins MDC1, BRCA1 and RAD51 were found to be decreased at the site of DNA repair in PHB2 knockdown condition. Based on these results we strongly believed that PHB2 may play a role in HR. To test this hypothesis, we used DR-Gfp reporter assay. U2OS cells stably expressing DR-Gfp reporter was transfected with control and PHB2 siRNAs followed by Isce1 transfection after 6 hrs. Then cells were incubated for 48 hrs and FACS analyzed to detect HR activity through measuring Gfp expression. As we expected, Gfp expression was found to be significantly decreased in siPHB2 cells. This result confirmed the role of PHB2 in DNA damage repair though HR.





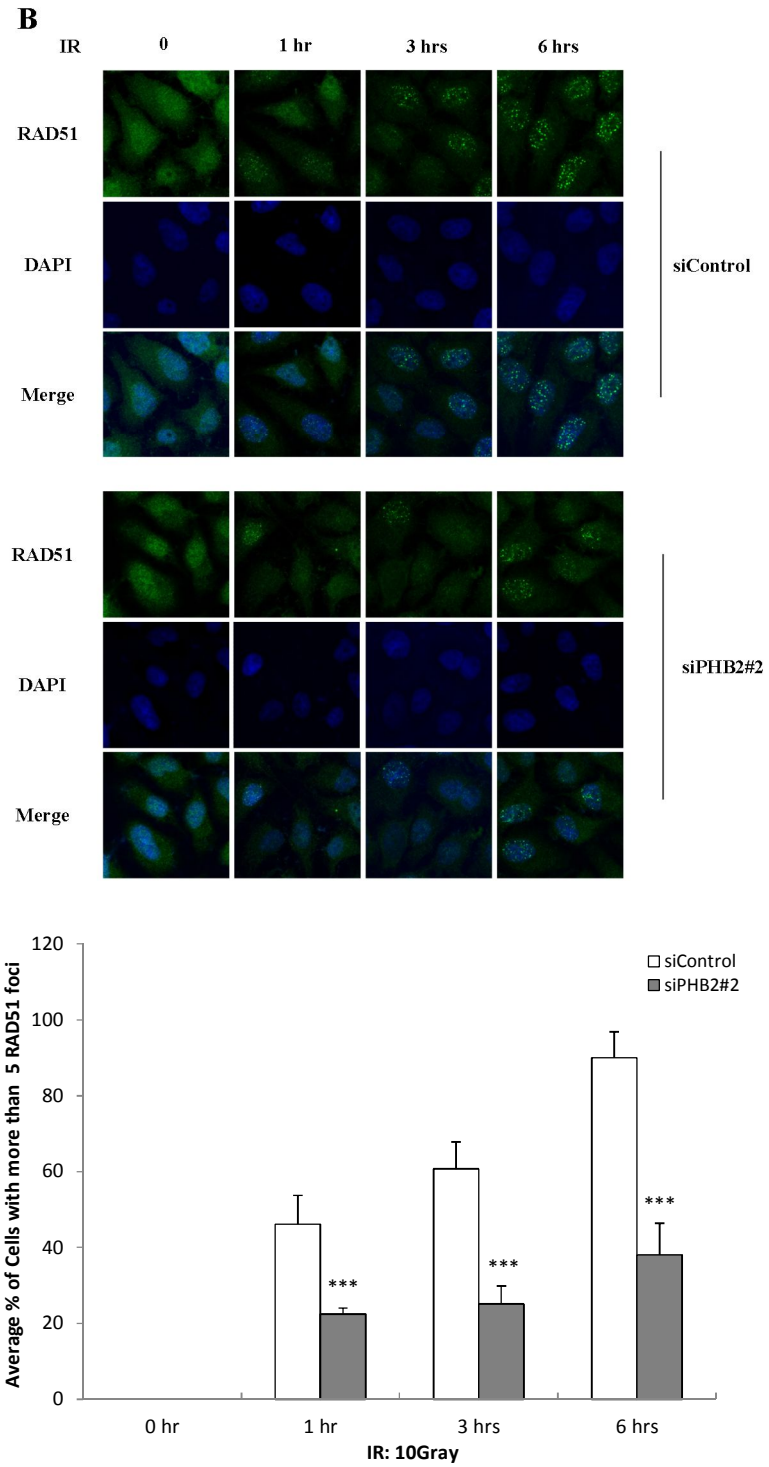


Figure 21. Down regulation of RPA and RAD51 foci formation in PHB2-deficient HeLa cells after IR treatment.

(A) Control and PHB2-depleted HeLa cells were untreated or treated with 10Gy γ-irradiation, fixed at the indicated time points and immunostained with anti-RPA antibody. DAPI staining was performed to indicate the position of nucleus. Cells with more than 5 intense foci were counted and graphically-represented as average percentage of cells with more than 5 RPA foci. **(B)** Down regulation of RAD51 foci formation in PHB2-deficient HeLa cells after IR treatment. Control and PHB2-depleted HeLa cells were untreated or treated with 10Gy γ-irradiation, fixed at the indicated time points and immunostained with anti-RAD51 antibody. DAPI staining was performed to indicate the position of nucleus. Cells with more than 5 intense foci were counted and graphically represented as average percentage of cells with more than 5 RAD51 foci.

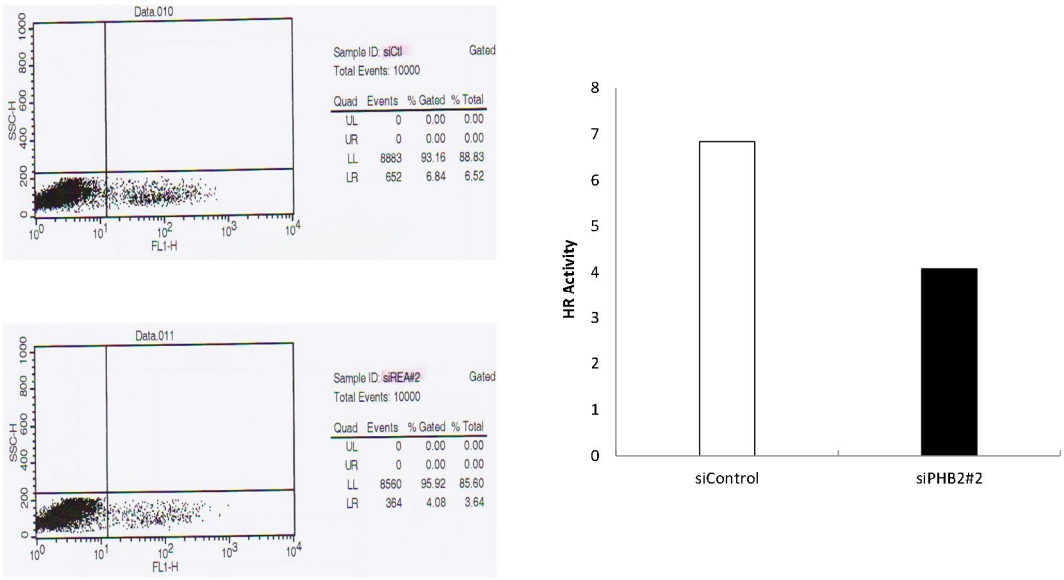


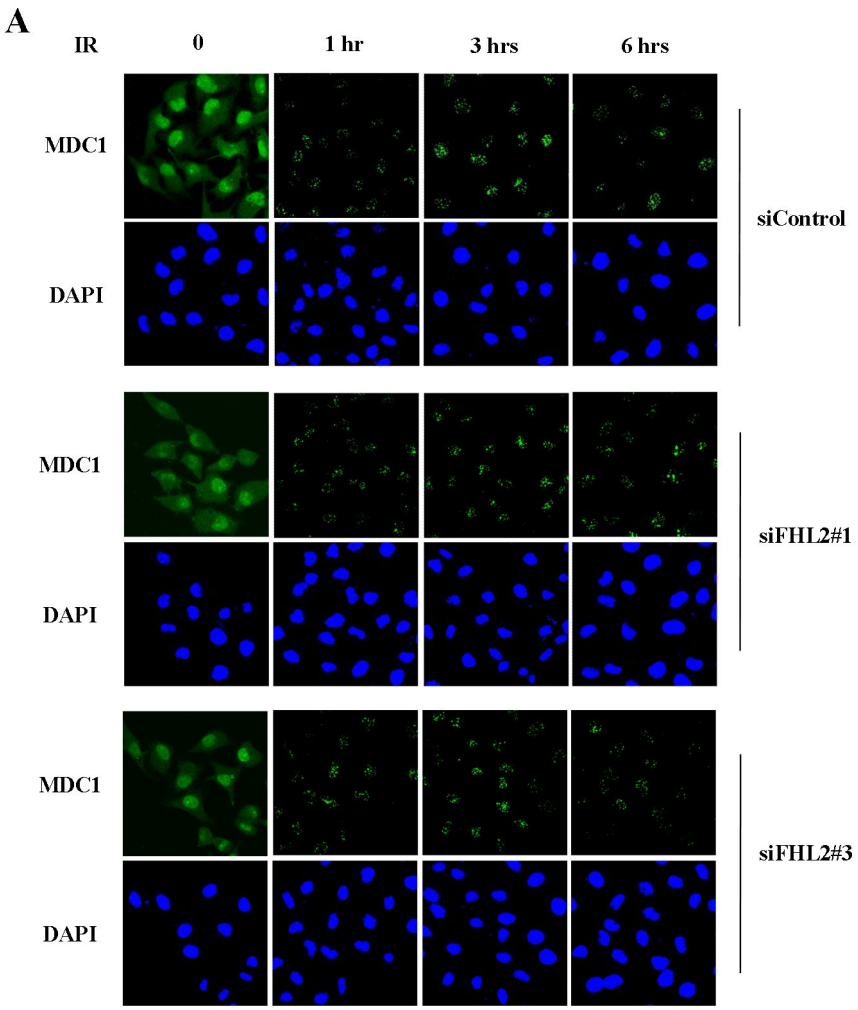
Figure 22. Impaired HR activity in PHB2 abolished cells.

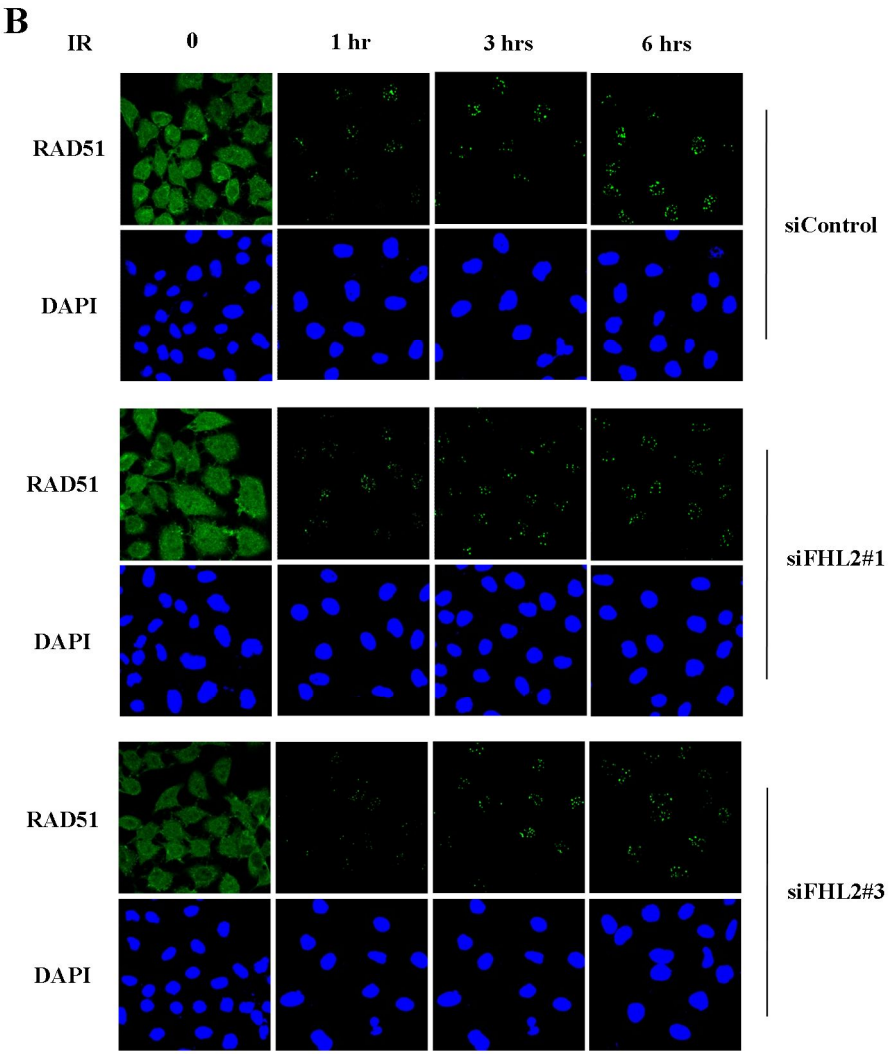
DR-GFP-U2OS cells were transfected with siControl or siPHB2, at 6 h after incubation cells were again transfected with Isce-1 endonuclease expression vector. After 48 h cells were extracted and FACS analyzed for GFP expression. FACS analyzed results were graphically represented as HR activity.

Chapter 4 – FHL2

A. FHL2 is not involved in irradiation induced DNA DSB repair

As an initial stage to screen FHL2, we investigated its possible role in γ-irradiation induced DNA double strand break repair. For this purpose we induced DNA DSBs by exposing siControl and siFHL2 treated cells to 10Gy γ-irradiation and stained to detect two important DNA damage response proteins MDC1 and RAD51. There is no difference between siControl and siFHL2 treated cells in number of irradiation induced nuclear foci for both these proteins. So it showed us FHL2 may not play a role in DSB repair. We are not able to come to a conclusion based on this one experiment, so we next investigated the accumulation of DNA DSBs between siControl and siFHL2 treated cells through measuring phosphorylated H2AX (γH2AX) foci in late hours. We detected no significant difference in γH2AX foci number between control and FHL2 siRNA treated cells. So we are clear that FHL2 is not involved in DNA DSB repair. The same fact was clarified though clonal survival assay. We detected no significant difference between siControl and siFHL2 treated cells in terms of survival rate after γ-irradiation induced DNA DSBs. Based on all these experiments we come to a conclusion that FHL2 is not involved in irradiation induced DNA DSB repair.





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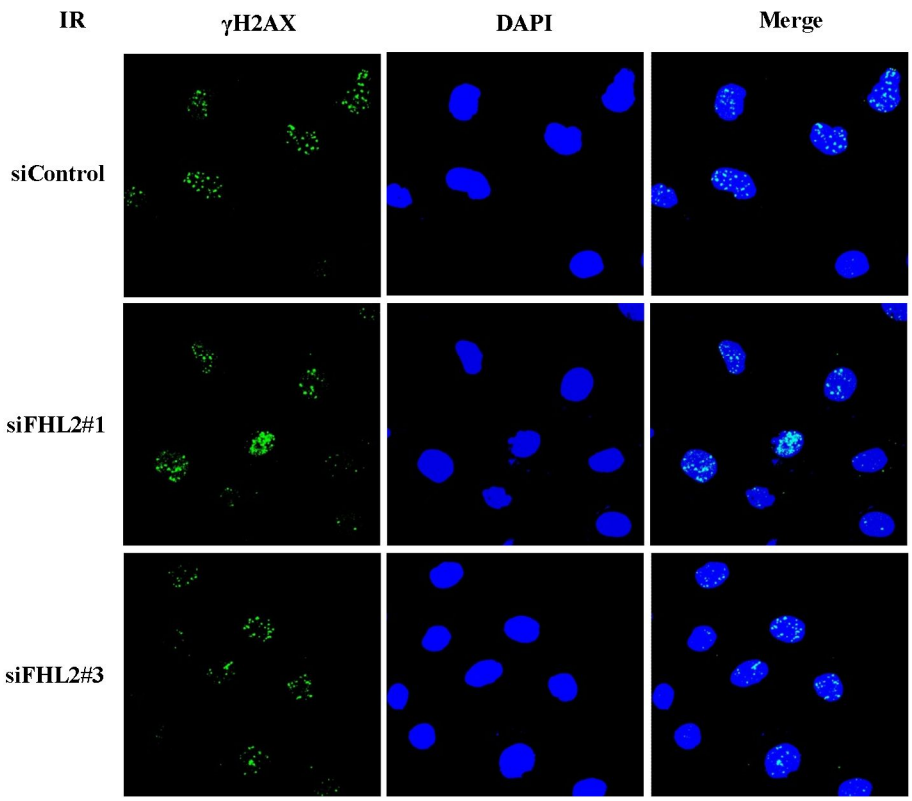


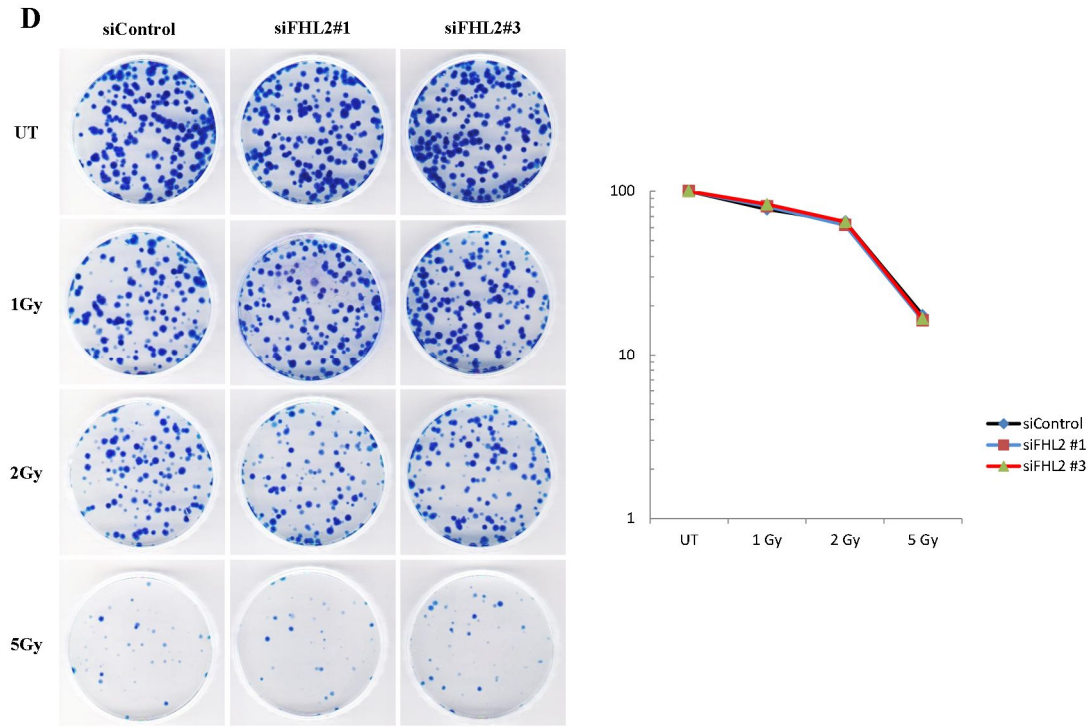


Figure 23. No difference in MDC1, RAD51 & late γ–H2AX foci, and IR sensitivity

No difference in IR induced MDC1, RAD51 and late γ-H2AX foci formation between FHL2-deficient and control HeLa cells. Control and FHL2-depleted HeLa cells were untreated or treated with 10Gy γ-irradiation, fixed at the indicated time points and immunostained with **(A)** anti-MDC1, **(B)** anti-RAD51 and **(C)** anti-γH2AX antibodies. **(D)** FHL2 knockdown does not sensitized cells to IR treatment. FHL2 depletion not affects survival of cells following exposure to γ-irradiation. Control and FHL2- depleted HeLa cells were untreated or treated with 1, 2 and 5 Gy ionizing radiation, after 3 weeks colonies were stained with methylene blue and counted.

B. FHL2 and DNA replication stress

The negative results of FHL2 role in DSB repair makes us to investigate the involvement of FHL2 in replication stress. Hydroxyurea (HU) is a chemotherapeutic agent that causes DNA replication stress by upsetting the process of DNA replication. Hydroxyurea reduces the availability of deoxynucleotide (dNTP) pools at DNA replication folks, so DNA polymerases don't have enough dNTPs to carry out the normal process of DNA replication (Koc, Wheeler, Mathews, & Merrill, 2004; Yarbro, 1992). We used HU to induce DNA replication stress for study the role of FHL2.

C. FHL2 abolishment leads to increased HU sensitivity of cells due to inefficient replication stress induced DNA damage repair

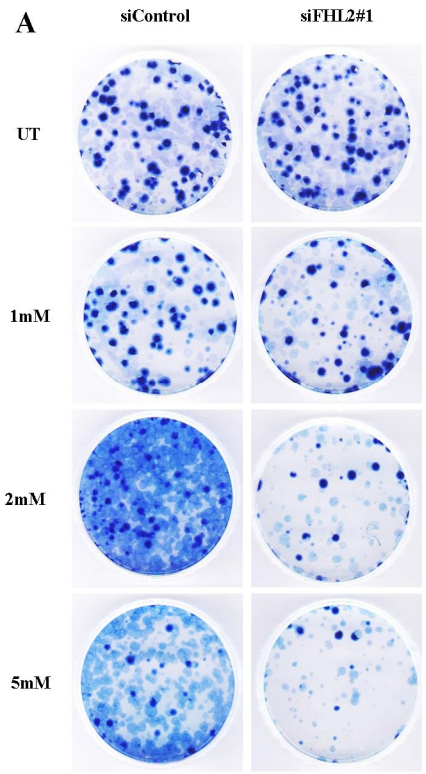
First of all we investigated HU sensitivity in control and FHL2 knockdown cells though colony survival assay. Sensitivity of cells towards HU is measured by colony formation ability after treatment. The colonies were observed in control and FHL2 knockdown cells after 0, 1, 2 and 5 mM HU treatments for 2 and 3 weeks of incubation. The observed results shown us, FHL2 knockdown cells showed decreased number of colonies compared to control cells after 2 and 3 weeks of HU (Figure 24A). This result showed that FHL2 depletion leads to increased HU sensitivity.

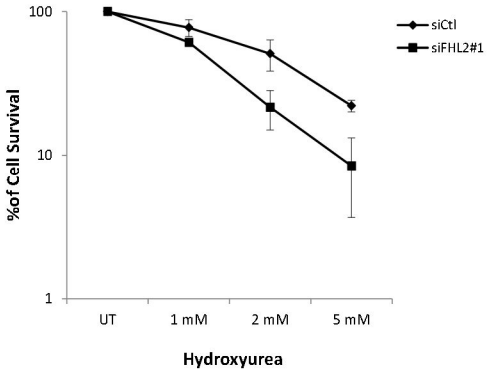
Since FHL2 knockdown resulted in increased HU sensitivity in cells, we intended to investigate the effect of FHL2 knockdown in DNA DSB repair. For this we assessed the late time focus formation of marker protein γH2AX after HU treatment. Control and FHL2 knockdown cells were IR treated and incubated for 24hrs. After incubation cells were fixed and immunofluorescent stained for γH2AX foci detection. Control cells were found to have less number of γH2AX foci, whereas FHL2 knockdown cells were counted with high number of foci (Figure 24B).

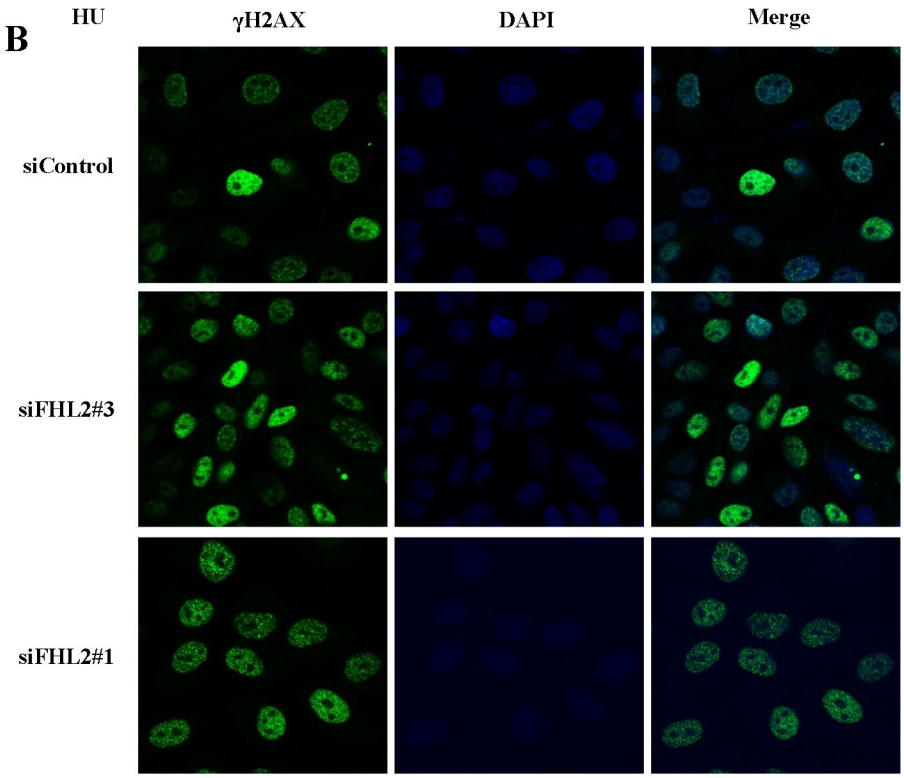
D. FHL2 plays a role in replication stress induced DNA damage repair through Homologous Recombination (HR)

Previous results provided enough evidences that FHL2 is involved in the process of replication stress induced DNA damage repair. We wanted to determine thought which pathway it regulates DSB repair. For this we examined the effect of FHL2 over nuclear foci formation of HR candidate gene RAD51 in HU treated cells. Control and FHL2 siRNA transfected HeLa cells were treated with HU (10mM) to induce replication stress mediated DNA damage. Then cells were incubated for different time intervals followed by staining with anti-RAD51 antibody. FHL2 depleted cells were found to form significantly less number of RAD51 nuclear foci compared to controls (Figure 25A).

Next we intended to investigate the effect of FHL2 knockdown over HR repair activity through DR-Gfp reporter assay. DR-Gfp U_2OS cells were seeded and transfected with control and FHL2 siRNAs followed by Isce1 plasmid transfection after 6 hrs. Then cells were incubated for recovery. After 48hrs cells were analyzed through FACS (fluorescence activated cell sorting) machine to measure Gfp fluorescence as a measure of HR activity. Positive Gfp expressions in FHL2 siRNA treated cells were found to be significantly decreased compared to control (Figure 25B). These results clearly showed that FHL2 plays a role in replication stress induced DNA damage repair though HR pathway.







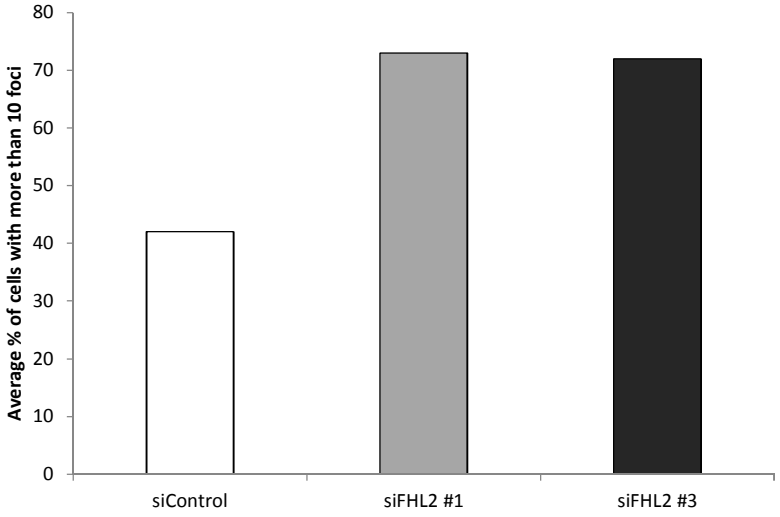
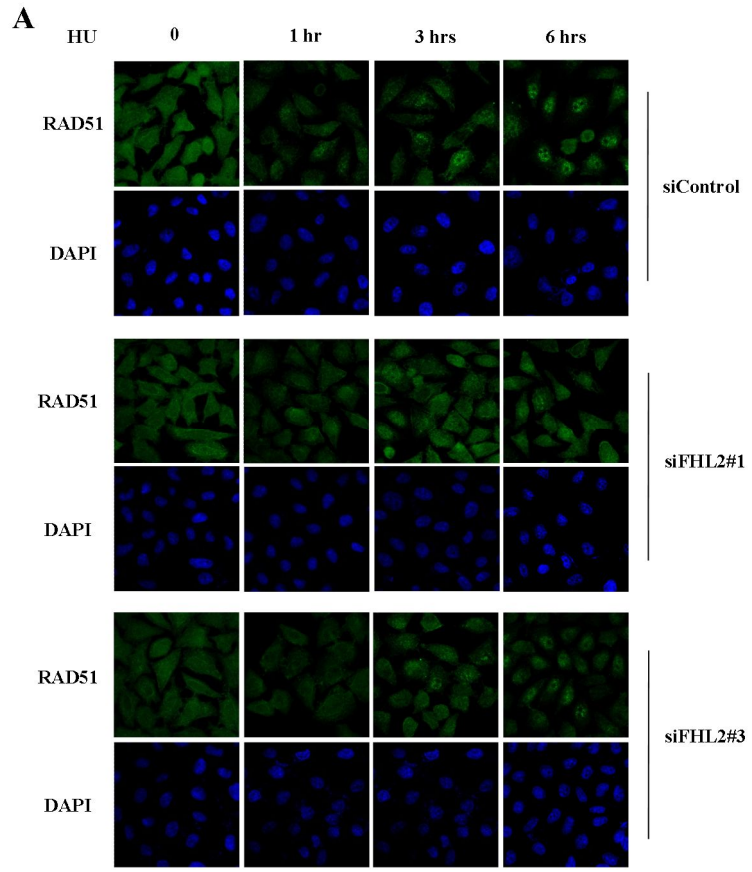
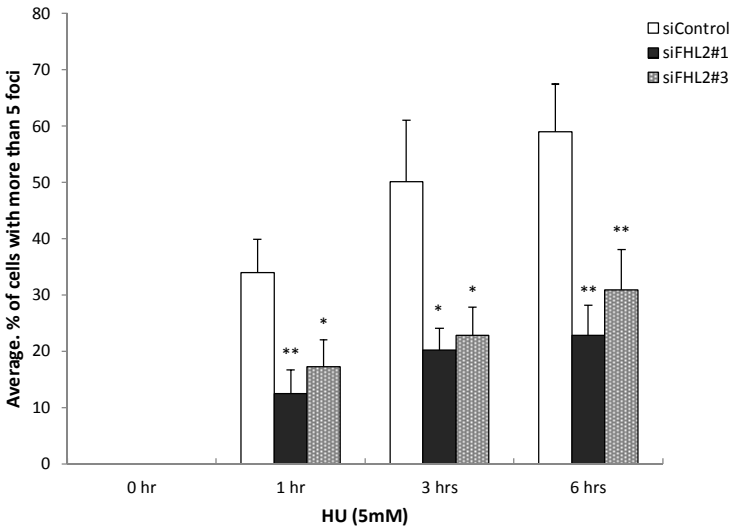


Figure 24. FHL2 knockdown sensitized cells to HU treatment and exhibits prolonged γ-H2AX foci after HU treatment

(A) FHL2 depletion affects survival of cells following exposure to HU. Control and FHL2- depleted HeLa cells were untreated or treated with 1, 2 and 5 mM HU, after 3 weeks colonies were stained with methylene blue and counted. **(B)** Control and FHL2-depleted HeLa cells were treated with HU (10mM) and incubated for recovery. Cells were fixed and analyzed by immunofluorescence staining with γ-H2AX antibody. Cells with more than 10 intense foci were counted and graphically represented as average percentage of cells with prolonged γ-H2AX foci.





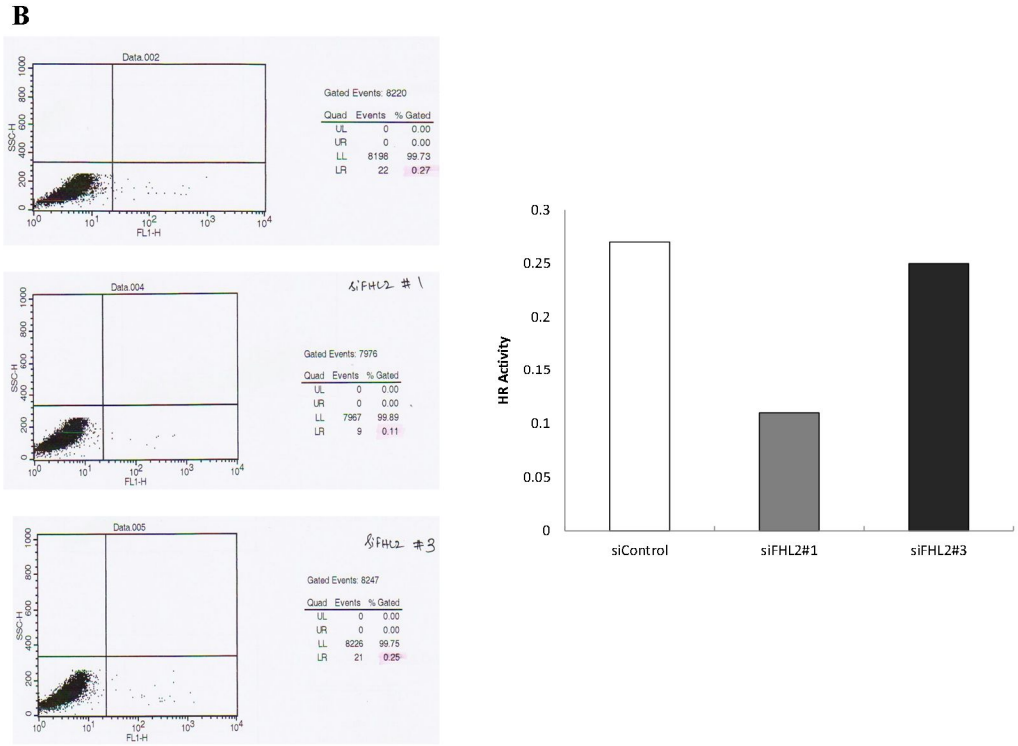
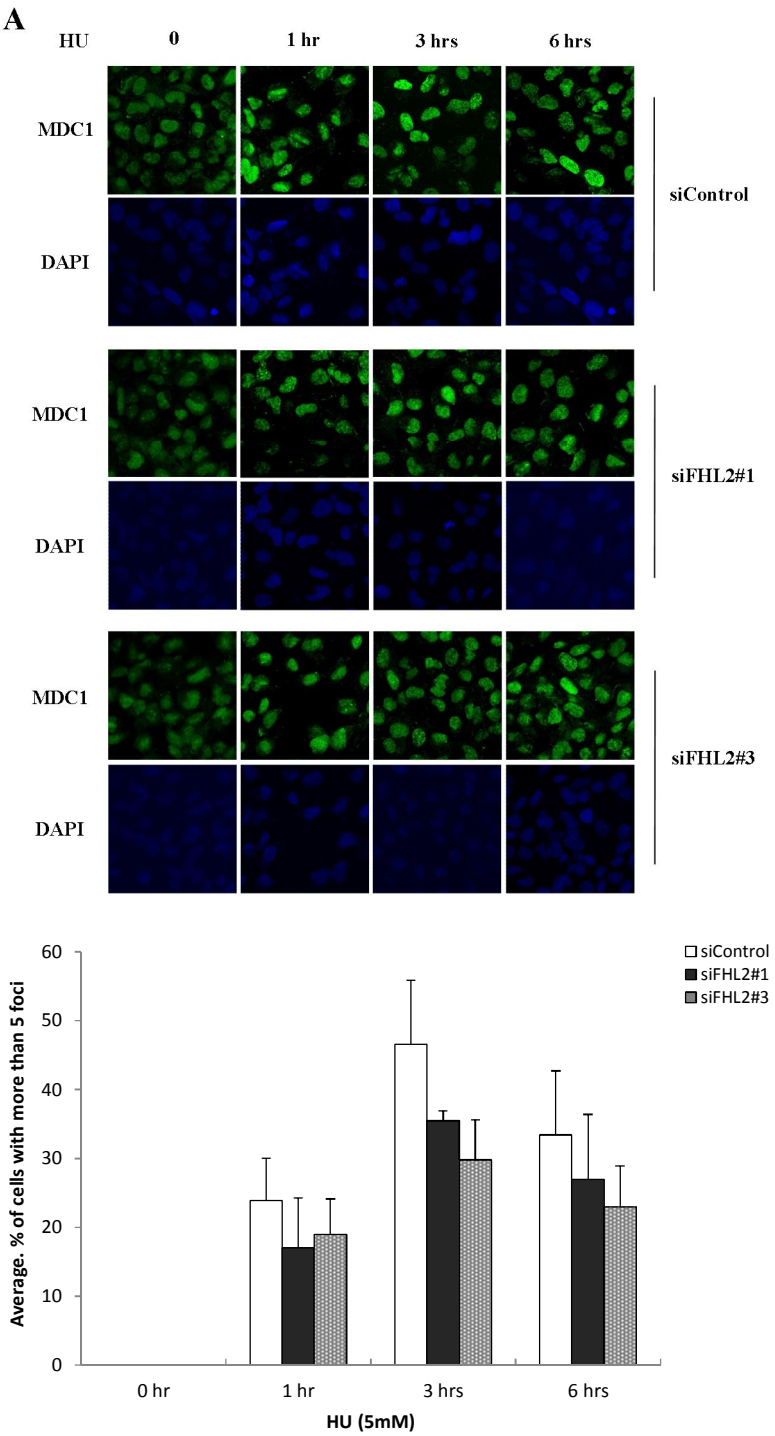


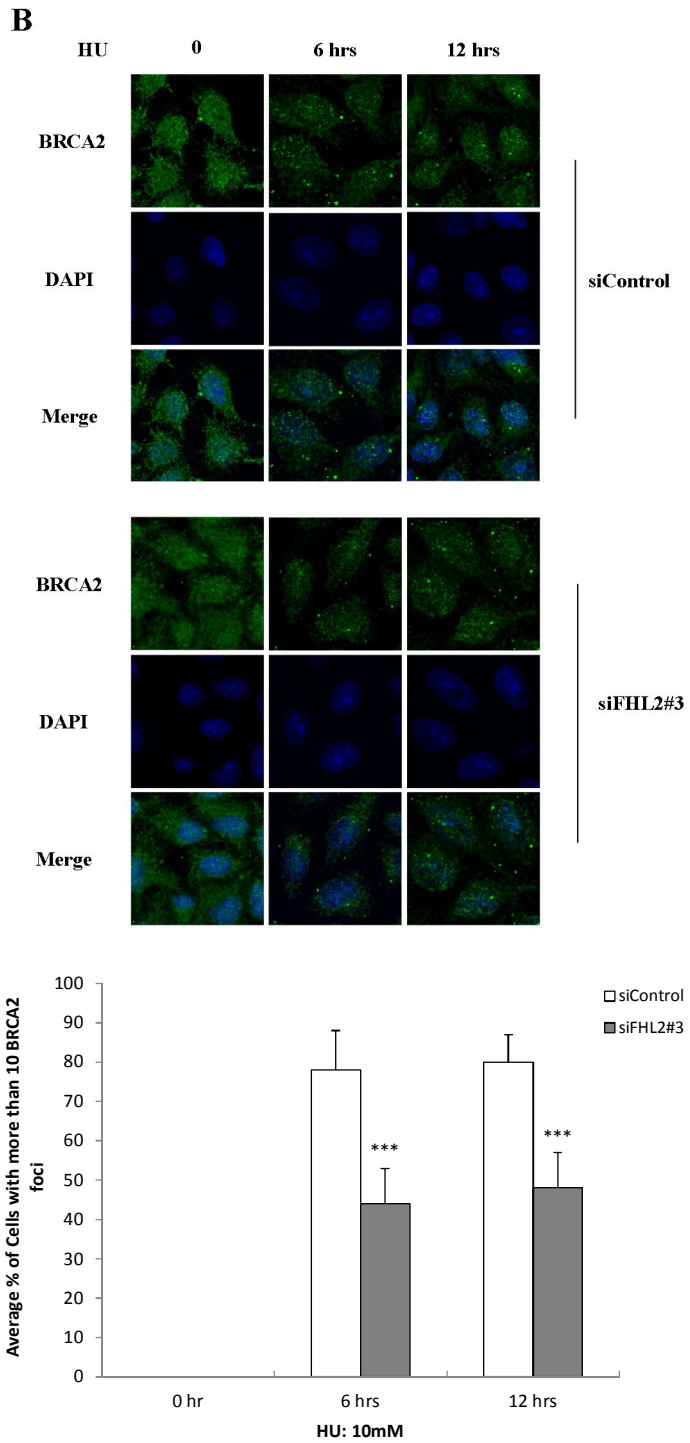
Figure 25. Down regulation of RAD51 foci formation and reduced HR activity in FHL2-deficient cells after HU treatment.

(A) Control and FHL2-depleted HeLa cells were untreated or treated with HU (10mM), fixed at the indicated time points and immunostained with anti-RAD51 antibody. Cells with more than 5 intense foci were counted and graphically- represented. **(B)** DR-GFP-U2OS cells were transfected with siControl or siFHL2, at 6 h after incubation cells were again transfected with Isce-1 endonuclease expression vector. After 48 h cells were extracted and FACS analyzed for GFP expression. FACS analyzed results were graphically represented as HR activity.

E. Effect of FHL2 knockdown over the foci formation ability of DNA damage repair proteins in replication stress induced cells

Since we detected a role for FHL2 in replication stress induced DNA damage repair though HR pathway, we next examined the effect of FHL2 over the ability of DDR proteins nuclear foci formation. Control and FHL2 siRNA transfected HeLa cells were treated with HU (10mM) to induce replication stress mediated DNA damage. Then cells were incubated for different time intervals followed by staining for DNA damage response proteins MDC1, BRCA2 and 53BP1. Among these three proteins, MDC1 and BRCA2 were found to form significantly less number of nuclear foci in siFHL2 treated cells compared to controls (Figure 26A&B). Whereas, there is no significant difference between siFHL2 and siControl cells were detected in terms of NHEJ candidate gene 53BP1 foci number (Figure 26C). These results showed that FHL2 plays a role in stress induced DNA damage repair specifically through HR pathway.





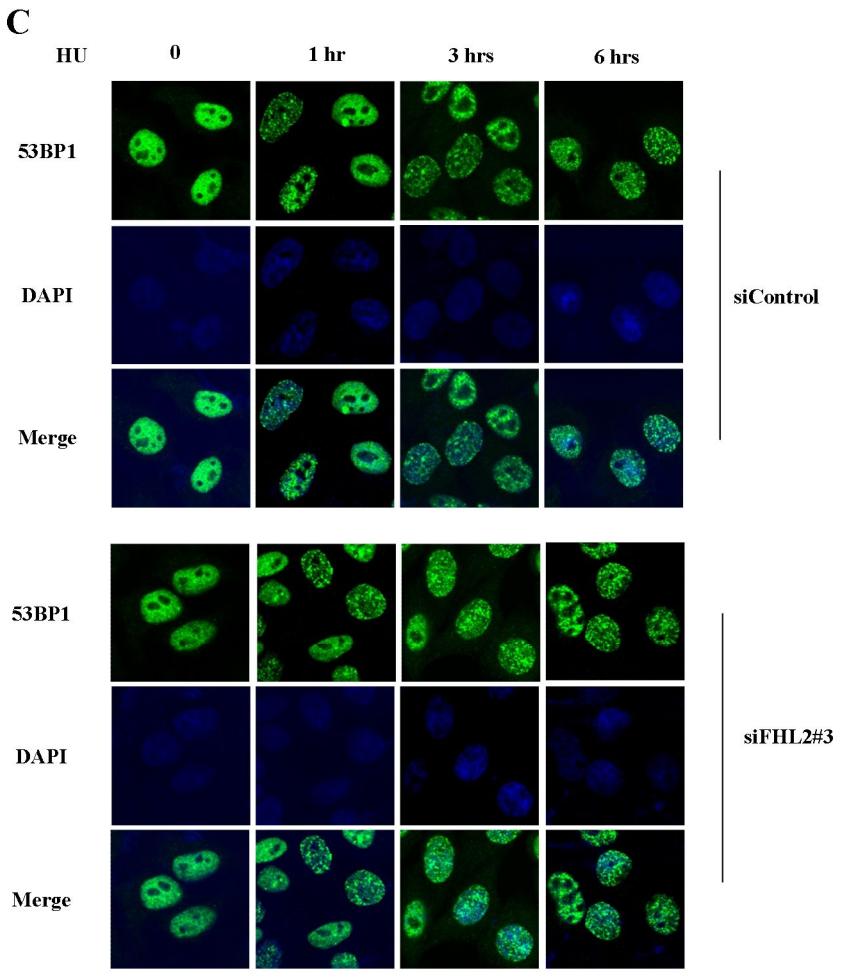


Figure 26. Down regulation of MDC1 and BRCA2 foci formation in FHL2-deficient HeLa cells after HU treatment.

Control and FHL2-depleted HeLa cells were untreated or treated with HU (10mM), fixed at the indicated time points and immunostained with (A) MDC1, (B) BRCA2 and (C) 53BP1 antibodies. DAPI staining was performed to indicate the position of nucleus.

IV. Discussion

Chapter 1 – KPNA2

A. KPNA2 mediated nuclear translocation of MDC1 in response to γ-irradiation induced DNA DSB

We initially found that KPNA2 is binding with MDC1 in yeast-two hybrid screening. Then we confirmed this binding through endogenous and exogenous co-immunoprecipitation of mammalian cell lysates. This binding insists us to find a reason behind this. KPNA2 belongs to a transporter family of proteins which translocate NLS containing cargo proteins from the cytoplasm to the nucleus (Chook & Blobel, 2001; Huang et al., 2013; Teng et al., 2006; Zannini et al., 2003) and MDC1 as NLS domain containing well-known DNA damage repair protein (Lou, Minter-Dykhouse, Wu, & Chen, 2003; Stucki & Jackson, 2004; Wu, Luo, Lou, & Chen, 2008), we hypothesized that KPNA2 may be involved in the transport of MDC1 after DNA DSB. So, we induced DSB by treating the cells with γ-irradiation and then nuclear portion was extracted and examined. In control cells, MDC1 expression was increased in nuclear portion after irradiation whereas in KPNA2 stably knock-down cells MDC1 expression was found to be the same. It indicates that MDC1 was unable to translocate to the nucleus in response to irradiation in absence of KPNA2. This result was validated by transfecting

HEK293T cells with HA-tagged full length MDC1 and KPNA2 siRNA. The HA-MDC1 expression in nuclear portion of irradiated cell lysate was found to be less in KPNA2i cells compared to control siRNA treated cells. These results showed that KPNA2 mediates the transport of MDC1 from cytosol to the nucleus after irradiation.

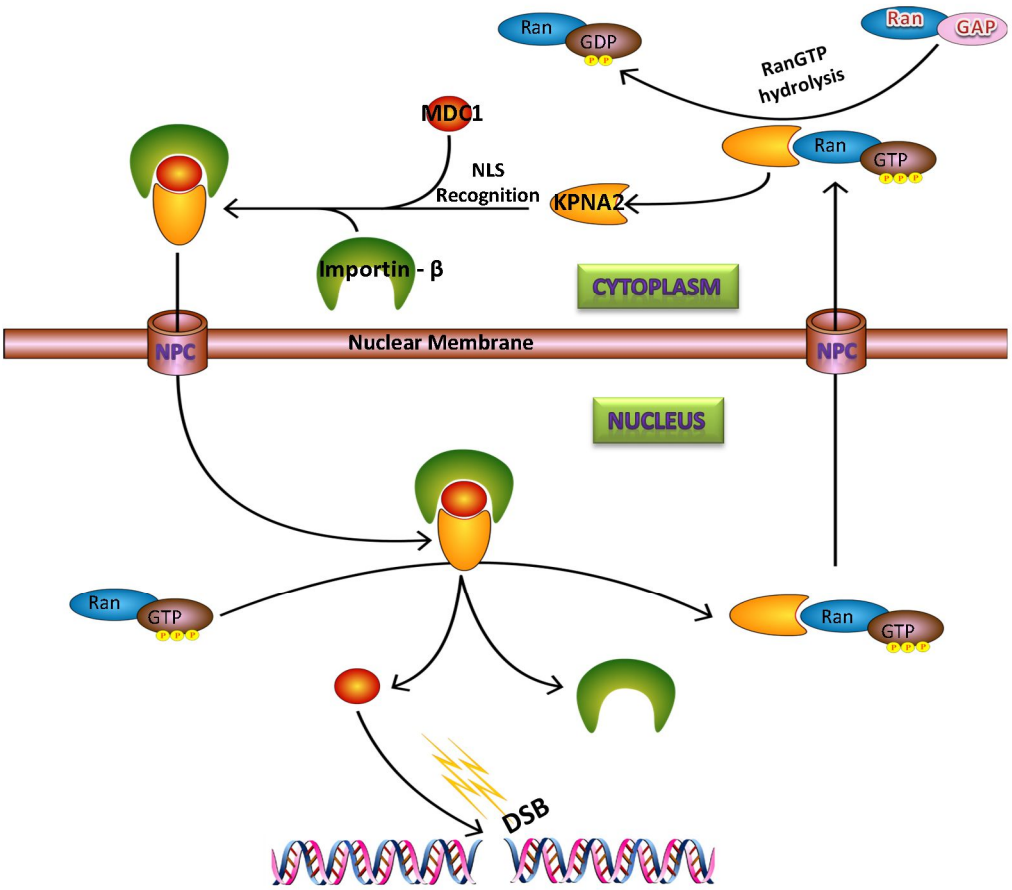


Figure 27. Schematic representation of KPNA2 mediated MDC1 nuclear transport.

B. KPNA2 plays a role in DNA damage repair through recruiting MDC1 to the site of action

It was previously reported that MDC1 forms irradiation-induced foci (IRIF) in response to DNA damage (Lou et al., 2003; G. S. Stewart et al., 2003; Stucki et al., 2005). As our results indicated KPNA2 is responsible for MDC1 translocation in response to DNA damage, we thought of examine the effect of KPNA2i over MDC1-IRIF formation. For this we induced DNA damage by treating control and KPNA2 knock-down cells with γ-irradiation and incubated for different time intervals to recover. Then cells were fixed with 4% paraformaldehyde and immuno-stained with MDC1 antibody. Interestingly, MDC1 nuclear foci formation was found to be decreased in KPNA2 abolished condition compared to control. Previous reports have indicated that MDC1 is required for amplification of DNA damage repair signal by recruiting DNA repair proteins to the site of DNA damage repair (Lou et al., 2006; G. S. Stewart et al., 2003). So we also examined MDC1 downstream repair proteins 53BP1 and BRCA1 nuclear foci formation. As we expected, these MDC1 downstream repair proteins also formed reduced IRIF in KPNA2 stably knock-down cells. But, the MDC1 upstream target γH2AX IRIF formation was not affected by KPNA2 knock-down. These findings clearly showed us KPNA2 is involved in DNA damage repair though recruiting MDC1 to the site of action. If MDC1 is not recruited to the site of

action, the repair pathway downstream of MDC1 is affected which leads to improper DNA damage repair and accumulation of DSBs.

C. Role of KPNA2 in DDR via HR pathway

Non-Homologous End Joining (NHEJ) and Homologous Recombination (HR) are two extensively studied repair pathways in which the mammalian cells repair DNA double-strand breaks (Aparicio, Baer, & Gautier, 2014; Chapman, Taylor, & Boulton, 2012; Jackson, 2002; Mao, Bozzella, Seluanov, & Gorbunova, 2008). Among these two repair systems, HR repair pathway is considered as error-free process because it uses damage free sister chromatid as a template. On the other hand, NHEJ is normally error-prone as it repairs DNA DSBs by directly ligating broken ends (Betermier, Bertrand, & Lopez, 2014; Burma, Chen, & Chen, 2006; Guirouilh-Barbat, Lambert, Bertrand, & Lopez, 2014). As our results showed us KPNA2 is involved in DSB repair, we thought of investigating through which pathway it functions. For this we studied an important HR pathway protein, Rad51 (Baumann & West, 1998; Pohl & Nickoloff, 2008). In absence of KPNA2, the IRIF formation of Rad51 was found to be reduced. So we next examined the HR repair efficiency in absence of KPNA2 via DR-GFP-U2OS cells. In which the I-Sce1 endonuclease induced DSBs were inefficiently repaired in KPNA2i cells compared to control. NHEJ efficiency was studied using the same principle as DR-GFP, but with HeLa cells. In which we could

not found any difference in NHEJ activity in repairing I-Sce1 induced DSBs between control and KPNA2 eliminated cells. Through these results we established a role of KPNA2 in DDR via HR repair pathway.

D. Knockdown of KPNA2 affects cell survival rate and delays repair activity

As we found that abolished KPNA2 resulted in inefficient HR activity, we thought of examine this effect over the accumulation of DSBs in IR treated cells. In response to DNA DSBs, the histone variant H2AX is phosphorylated to γH2AX as initial step in DDR pathway. The γH2AX nuclear foci are considered to be a bio-marker of DSB and reduction in γH2AX foci indicates the efficient DSB repair (Ivashkevich et al., 2011; Lobrich et al., 2010; Olive, 2011). So we analyzed γH2AX foci formation to monitor DSB repair efficiency. As a result of DSB accumulation, prolonged γH2AX foci were detected in KPNA2 knock-down cells compared to control. To further confirm this inefficient DSB repair in KPNA2 knock-down cells, we employed neutral comet assay. It is a sensitive and efficient method to detect DSBs (Collins, 2004; Lorenzo, Costa, Collins, & Azqueta, 2013; Olive & Banath, 2006; Olive, Wlodek, & Banath, 1991) because it measure DNA damage in individual cells, hence the name single cell gel electrophoresis. We observed increased movement of comet tails in KPNA2 vague cells compared

to control which indicates the abundance of DSBs. As a result of these observations we confirmed the accumulation of DSBs in absence of KPNA2. Accumulation of DSBs leads to genome instability and ultimately cell death (Eastman & Barry, 1992; Khanna & Jackson, 2001; Rich, Allen, & Wyllie, 2000; Roos & Kaina, 2013). We thought of confirm KPNA2i mediated DDR inefficiency through clonal survival assay. The survival rate of KPNA2i cells were reduced in compared to control cells which indicate the inefficient DNA damage repair in KPNA2 knock-down cells.

In conclusion, KPNA2 is critical for nuclear transportation and nuclear focus formation of MDC1. Any delay or absence of MDC1 translocation leads to impaired DNA repair and accumulation of DNA lesions with in the cells. The KPNA2-MDC1 functional interaction clarifies the molecular mechanism of MDC1 drive to DNA damaged site.

Chapter 2 - 4 (ZNF114, PHB2 & FHL2)

Here we took a first step in disclosing the novel functions of three proteins namely ZNF1144, PHB2 and FHL2 in DNA damage response. We have done an initial screening of these proteins to confirm their role in DDR. All these proteins are found to have a role in DNA damage repair. Among these three proteins, ZNF114 is found to be involved in ionizing radiation induced DNA DSB repair through HR. FHL2 is involved in hydroxyurea (HU) mediated replication stress induced DNA damage repair. There is a huge possibility for PHB2 involvement in the process of end-resection.

We induced DNA DSBs by treating cells with γ-irradiation and examined the effect of ZNF114 in DSB repair. ZNF114 involvement in DNA damage repair is confirmed through detection of reduced IR induced nuclear foci formation of DDR candidate genes. Surprisingly we detected no effect of ZNF114 knockdown over MDC1 foci formation, but positive effect for other DDR proteins RAD51, BRCA1 and RPA. So, ZNF114 involvement in DDR may be downstream of MDC1. This might be the reason why ZNF114 knockdown not affected MDC1 but have a positive effect on MDC1 downstream targets. Since we detected a positive effect of ZNF114 over DDR proteins, next we thought to investigate the repair efficiency in absence of ZNF114 though late time γH2AX foci (Mah et al., 2010; Siddiqui, Francois,

Fenech, & Leifert, 2015) and neutral comet assay (Anderson & Laubenthal, 2013; Speit & Hartmann, 2005). Bothe these experiments confirmed a role for ZNF114 in DDR. We also confirmed that it specifically involved in HR by using DR-GRP reporter assay. This reporter assay showed a reduced HR activity for cells devoid of ZNF114 compared to controls. Finally we investigated the effect of ZNF114 abolishment in survival rate of cells after γ-irradiation though clonal survival assay. ZNF114 knockdown cells showed reduced survival rate due to inefficient repairing of DNA damaged caused by ionizing radiation. Taken together, all these results showed a novel fucntion of ZNF114 in DNA damage repair.

We initially treated cells with γ-irradiation to induce DSBs and studied the effect of FHL2 in terms of DNA damage repair, but we got all negative results. So we thought that FHL2 may be involved in repairing other forms of DNA damages. To test this argument, we used HU to induce replication stress and then examined the role of FHL2. In HU mediated replication stress induced DNA damage repair we detected a positive role of FHL2. We done confocal microscopy detection of DDR protein's nuclear foci formation, late time γH2AX foci detection, DR-GFP reporter assay and clonal survival assay to determine FHL2 function in DDR.

PHB2 is involved in DNA DSB repair though HR. We detected a significant decrease in nuclear foci formation of end-resection protein RPA in absence of PHB2. So we think that PHB2 may be involved in end-resection,

the process very important for selecting HR pathway over NHEJ to repair DNA damages (Liu & Huang, 2016; Shrivastav et al., 2008; Symington, 2016).

In conclusion, we did an initial screening to elucidate the novel function of ZNF114, PHB2 and FHL2 in DNA damage repair. Basic experiments using transiently knockdown cells confirmed the involvement of these three proteins in DDR. Further detailed research on these proteins in terms of DDR warranted.

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ABSTRACT

The Regulatory Mechanism of DNA Damage Checkpoint Protein MDC1

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Mediator of DNA damage checkpoint protein 1(MDC1) plays a vital in DNA damage response (DDR) to repair DNA damages, especially DNA double strand breaks (DSBs). In response to DSBs, MDC1 relocates to the damaged site to mediate recruitment of DNA repair proteins. Any delay or impairment in DSB induced translocation of MDC1 to the nucleus leads to disorganized DNA repair results in accumulation of DNA damages, carcinogenesis, chromatin instability and ultimately cell death. Here, we found Karyopherin α-2 (KPNA2, a member of importin-α family responsible for nuclear transport of proteins bearing Nuclear Localization Sequence) as MDC1 interacting protein in yeast two-hybrid screening and further confirmed by

immunoprecipitation assay. In absence of KPNA2, DSB induced nuclear transport and nuclear foci formation of MDC1 was reduced which affected the recruitment of downstream repair proteins RAD51, 53BP1 and BRCA1. We also showed that KPNA2 depleted cells accumulate DSBs which is exposed by detecting increased number of late γH2AX foci after DSB and hyper tail movement in neutral comet assay. Furthermore, DR-GFP reporter assay revealed a lesser amount of Homologous Recombination (HR) activity in KPNA2i condition. Cells showed hypersensitivity to IR-induced cell death in absence of KPNA2, as disclosed by clonogenic cell survival assay. These results suggest that KPNA2 is important for DSB induced nuclear translocation and proper functioning of MDC1 in DDR pathway, especially in HR. We also did an initial screening to elucidate the novel functions of ZNF114, PHB2 and FHL2 in DNA damage repair.

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"I dedicate this Doctorate to my Parents"

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"By what great penance did his father be get him" is the benefit which a son should render to his father.

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The mother who hears her son called "a wise man" will rejoice more than she did at his birth.