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Ph.D. Dissertation

TOPORS interacts with RAD51 and mediate DNA double strand Break Repair by Homologous Recombination

Graduate School of Chosun University

Department of Bio-Materials

Gurusamy Hariharasudhan

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RAD51 결합단백질 TOPORS의 DNA이중나선절단 복구활성 조절연구

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Advisor: Prof. Ho-Jin You

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LIST OF ABBREVIATIONS

DSB	double strand break
HR	Homologous recombination
IR	irradiation
Si RNA	small interfering RNA
Sh RNA	short hairpin RNA
cDNA	complementary DNA
HA	hemagglutinin
EDTA	Ethylenediaminetetraacetic acid
PVDF	polyvinylidene Fluoride
PBS	phosphate buffer saline
DAPI	4', 6-diamidino-2-phenylindole
NaOH	sodium hydroxide
NaCl	sodium chloride
GFP	green fluorescent protein
NTR	N terminal region
RT-PCR	Reverse transcription polymerase Antigen
LZ	leucine zipper
PEST	proline, glutamine, serine and threonine
RS	arginine/serine
NLS	nuclear localization signal
SUMO	small ubiquitin-like modifier

STUbLs	SUMO-targeted ubiquitin ligases
PML	promyelocytic leukemia protein
SDS-PAGE	sodium dodecyl sulfate-polyacrylamide gel electrophoresis
RIPA	Radio immunoprecipitation assay buffer
HU	Hydroxy urea
MMC	Mitomycin C

국문초록

Rad51 결합단백질 TOPORS의 DNA이중나선 절단 복구 활성 조절연구

Gurusamy Hariharasudhan

지도교수 : 유호진

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생물신소재학과

방사선 조사, UV, 복제 손상 및 각종 시약 등에 의해 유도된 DNA 이중나선 절단은 비상동 말단결합과 상동 재결합을 통해 복구되어 유전체 안정성을 유지한다. Rad51 단백질은 DNA 이중나선 절단에 의한 상동재결합에 중요한 역할을 한다. 본 연구는 yeast-two hybrid 스크리닝을 통해 Rad51에 결합하는 새로운 단백질 TOPORS를 동정하고 RAD51과의 결합을 통해 상동재결합에 미치는 영향을 조사하였다. 먼저 in vivo와 in vitro에서 Rad51과 TOPORS의 결합을 면역침강법을 통해 확인하였다. 이 결합은 방사선 조사에 의해 증가함을 보았다. TOPORS의 중요부위 돌연변이 실험을 통해 TOPORS의

854-917aa 부위가 RAD51과의 결합에 중요함을 확인하였다. 또한 Rad51과 TOPORS는 방사선 조사 후 DNA 손상부위에 colocalization됨을 면역염색법을 통해 관찰하였다. Rad51의 결합단백질로 동정된 TOPORS가 RAD51에 의한 상동재결합에 미치는 영향을 조사하기 위해 TOPORS 유전자가 결핍된 세포주를 제작하여 방사선 손상에 따른 영향을 조사하였다. TOPORS가 결핍된 세포는 정상세포에 비해 방사선 손상과 복제 손상 등에 의한 세포손상 민감도가 증가되었다. 또한 DNA손상 마커인 γ-H2AX와 53BP1의 DNA 손상 foci형성에는 차이를 보이지 않았고, 상동재결합을 마커인 RAD51 foci는 TOPORS가 결핍된 세포에서 현저히 감소함을 확인하였다. Comet assay 와 상동재결합 분석을 통해 TOPORS가 결핍된 세포에서 DNA 손상 복구가 감소됨을 증명하였다. 따라서 본 연구는 Rad51과 TOPORS의 결합을 통해 TOPORS가 RAD51의 이중나선절단을 통한 상동재결합을 조절함을 확인함으로써 TOPORS가 방사선 손상에 있어 Rad51의 새로운 중요한 조절자임을 시사한다.

I. Introduction

1. RAD51 mediates DSB Repair by Homologous Recombination

DNA double-strand breaks (DSBs) are highly toxic lesions induced by ionizing radiation (IR), free radicals, chemicals, or replication stress. Two major pathways, error-prone non-homologous end joining and error-free homologous recombination playing an important role to maintain genome stability (Figure 1). Homologous recombination maintains high fidelity DSB repair mechanism through homologous DNA as a template (Harper et al., 2007, Ciccia et al., 2010, Pardo et al., 2009, Shrivastav et al., 2008). The core complex of HR known as collective of RAD52 group of proteins which includes Rad50,Rad51,Rad52,RAD54,RDH/TID1,RAD55,RAD57, RAD59, MRE11, and XRS2 (Symington LS et al., 2002). RAD51 is known to be backbone of homologous recombination repair which is playing a major role in homologous pairing of DSB repair. RAD51 involved in cell cycle and DNA damage response for its regulatory activity which involves transcriptional and posttranslational regulations. RAD51 can bind to many DNA repair proteins for its active regulation but this type of interactions either increase or impairs its activity (Figure 3).

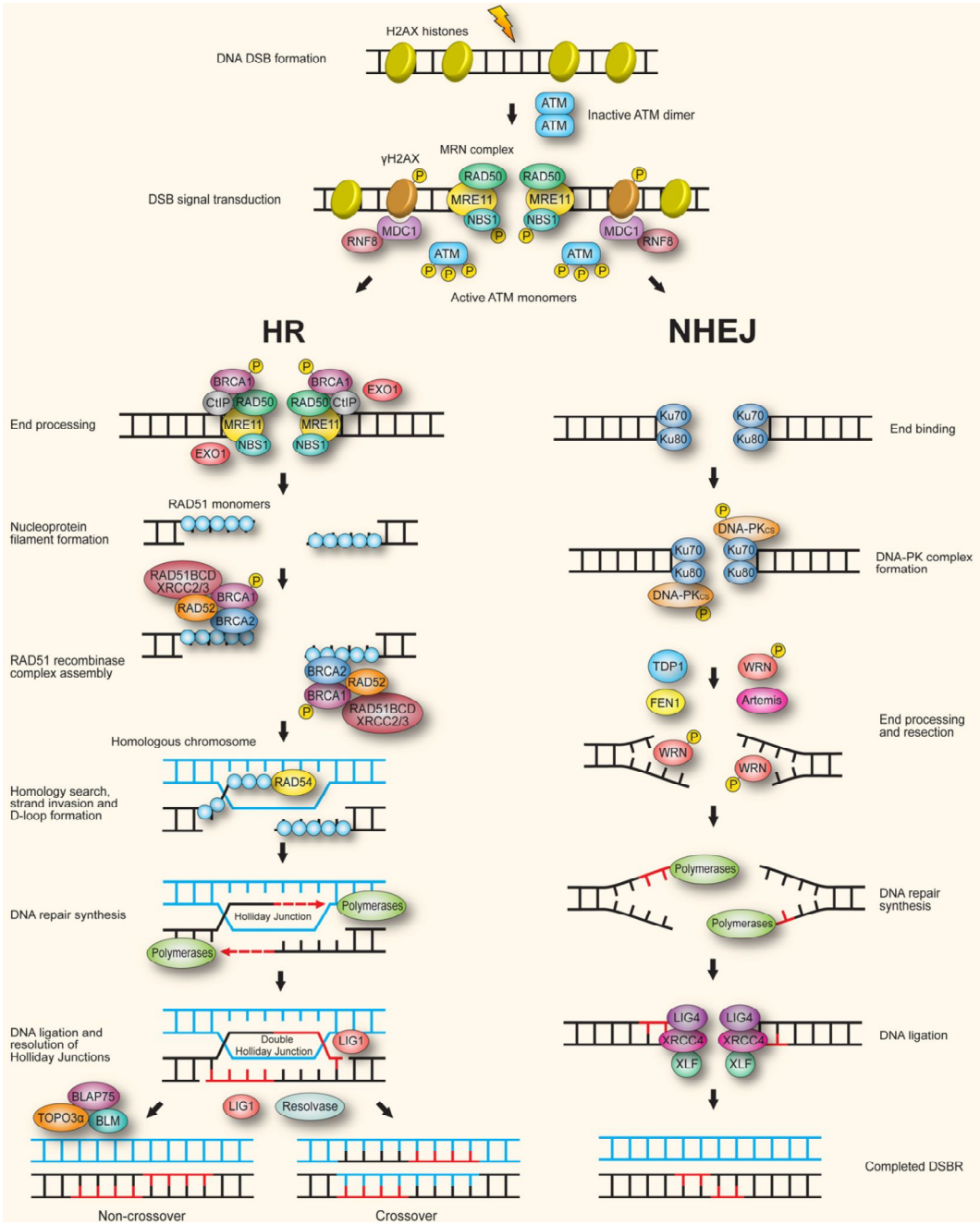


Figure 1. The two major mammalian pathways for double-strand break repair (Jeppensen et al., 2011).

2. Topors role in Cancer and DNA damage signaling

The RING finger protein TOPORS contains a RING family zinc-finger domain, a putative leucine zipper (LZ) domain, five sequences rich in proline, glutamine, serine and threonine (PEST sequences), an arginine/serine (RS) domain and a bipartite nuclear localization signal (NLS).

TOPORS was first identified as a human topoisomerase I-interacting protein by yeast two-hybrid screening (Haluska et al., 1999). TOPORS has been reported to be closely associated with the PML bodies and localized in the nucleus (Weger et al., 2003; Rasheed et al., 2002). An important role of TOPORS is its ability to interact with the tumor suppressor protein P53 (Zhou et al., 1999). Murine Topors expressed after DNA damage occurs then interacts, stabilizes p53 and enhances the p53-dependent transcriptional activities of $p21^{waf1}$, MDM2 and Bax promoters and elevates the level of endogenous $p21^{waf1}$ mRNA (Lin et al., 2005). These data explains an anti-oncogenic role for TOPORS. TOPORS protein level was undetectable in several colon cancer cell lines and the expression patterns various either it could be decreased or undetectable in colon adenocarcinomas relative to normal colon tissue (Saleem et al., 2004). Repression of TOPORS expression was also reported in progression and development of non-small cell lung cancer (Oyanagi et al., 2004). Furthermore, loss of heterozygosity in the region 9p21, the

chromosomal locus harboring TOPORS, has been frequently associated with different malignancies (Puig et al., 1995). A high-resolution genome wide mapping study identified deletion of the TOPORS genomic locus in human glial tumors, suggesting a possible role for TOPORS in gliomagenesis (Bredel et al., 2005). A missense mutation in the TOPORS gene was implicated in autosomal dominant pericentral retinal dystrophy, showing that mutations in the TOPORS gene can lead to genetic disorders (Selmer et al., 2009). Concomitant with these observations, point mutations and small insertions and deletions in the TOPORS gene was found to cause approximately 1% of autosomal dominant retinitis pigmentosa (Bowne et al., 2008). Another study reported that mutations in TOPORS cause autosomal dominant retinitis pigmentosa with perivascular retinal pigment epithelium atrophy (Chakarova et al., 2007). TOPORS role on various cellular processes has been proved by several biochemical studies. It was shown that in the nucleus TOPORS undergoes SUMO-1 modifications (Weger et al., 2003). Interestingly, TOPORS functioning as a SUMO-1 E3 ligase for many proteins for its sumoylation. For example, TOPORS can sumoylate p53 and the chromatin modifying protein Sin3A (Shinbo et al., 2005; Weger et al., 2005; Pungaliya et al., 2007). Furthermore, TOPORS induce the accumulation of polysumoylated forms of DNA topoisomerase I in vitro and in vivo (Hammer et al., 2007). Furthermore, along with sumoylation TOPORS can also function as an E3 ubiquitin ligase. TOPORS was the first example of a protein that possesses dual-roles as an E3

ligase for sumoylation and ubiquitination of other proteins. It was reported that Topors works as an E3 ubiquitin ligase with specific E2 enzymes to ubiquitinate the p53 protein and the prostrate tumor suppressor protein NKX3.1 (Rajendra et al., 2004; Guan et al., 2008). Intense investigations have been undertaken in recent years to elucidate the mechanisms of molecules that have dual E3 ligase activities for sumoylation and ubiquitination such as TOPORS. These studies have discovered a new family of proteins, designated as the small ubiquitin-related modifier (SUMO)-targeted ubiquitin ligases (STUbLs), which directly links sumoylation and ubiquitination (Perry et al., 2008). It has been suggested that similar to STUbLs, TOPORS may be recruited to its targets through SUMO-associated interactions and stimulate their ubiquitination in a RING finger-dependent manner (Perry et al., 2008). Intriguingly, TOPORS has been connected with transcriptional regulation because of its role as an E3 ubiquitin ligase. In drosophila, the homolog of human TOPORS (dTopors) ubiquitinates the Hairy transcriptional repressor, suggesting that TOPORS could be involved in regulating other transcription factors as well (Secombe et al., 2004). Indeed, it was shown that TOPORS interacts with the adeno-associated virus type 2 (AAV-2) Rep78/68 proteins and enhances the expression of a Rep78/68 dependent AAV-2 gene in the absence of the helper virus (Weger et al., 2002). Finally, it was shown that drosophila dTopors was required for the nuclear organization of a chromatin insulator, suggesting a role for TOPORS in regulation of the chromatin (Capelson et al., 2005). Topors

Widely conserved among different species. Murine Topors shows high similarity with human TOPORS (Figure 2).

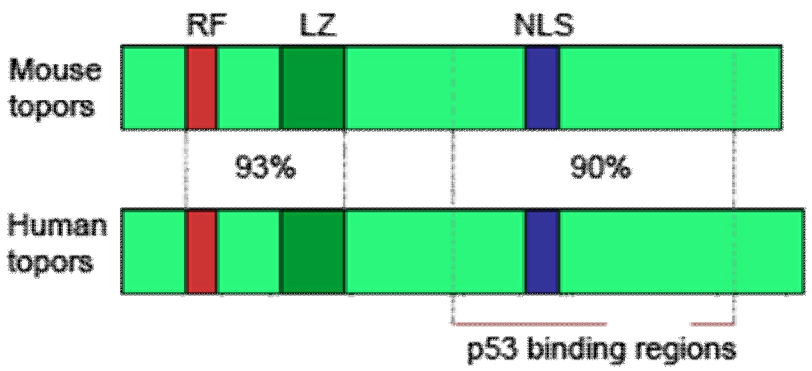


Figure 2. Schematic representation of homologous domain structure shared between mouse and human Topors (L Lin et al., 2005).

Topors colocalize and sumoylate IKKε upon DNA damage which is essential for its localization in PML-NBs (Florian Renner et al., 2010). Topors playing a major role in maintenance of genome stability and pericentric heterochromatin, topors-deficient pMEF cells showed resistant to anti-proliferative effect of histone deacetylase inhibitors (Henderson Marshall et al., 2010). The regulatory function of developing primary cilia-depentent photoreceptor was maintained by topors (Christina F. Chakarova et al., 2011). Topors function as a trans-

acting transcriptional key regulator for lung cancer-associated genes (Dong Chu et al., 2001). Moreover, Ubiquitination activity of Topors was regulated by phosphorylation at Ser-98 position without affecting its sumoylation activity (Hye-jin park et al., 2008).Topors interacts with γH2AX in cultured mammalian cells and ubiquitinates γH2AX upon DNA damage induced by various agents (ki moon seong et al 2012). Topors binds to cytoplasmic domain of sdc-1 and regulates its inhibitory effect on cell growth of platelet-derived growth factor-B induction (Kathleen R. Braun et al., 2012). The polo-like-kinase PLK1 phosphorylates Topors at Ser-718 which leads to less sumo activity as well increased ubiquitination activity and triggers Topors degradation (Xiaoming yang et al., 2009, 2010).

In this report, the results indicate, Topors deficient cells showed impaired Homologous recombination repair activity after DSB. Topors depleted cells shown hypersensitive to IR induced less colony formation which is proved by a clonal survival assay. We show depletion of endogenous Topors accumulates increased amount of unrelieved DSBs, as observed by formation of delayed γH2AX foci and Comet assay. Our results suggest that Topors is novel binding partner of RAD51, having functional connection and playing a vital role in RAD51 mediated homologous recombination repair. Topors depletion highly leads to defect in Homologous recombination repair.

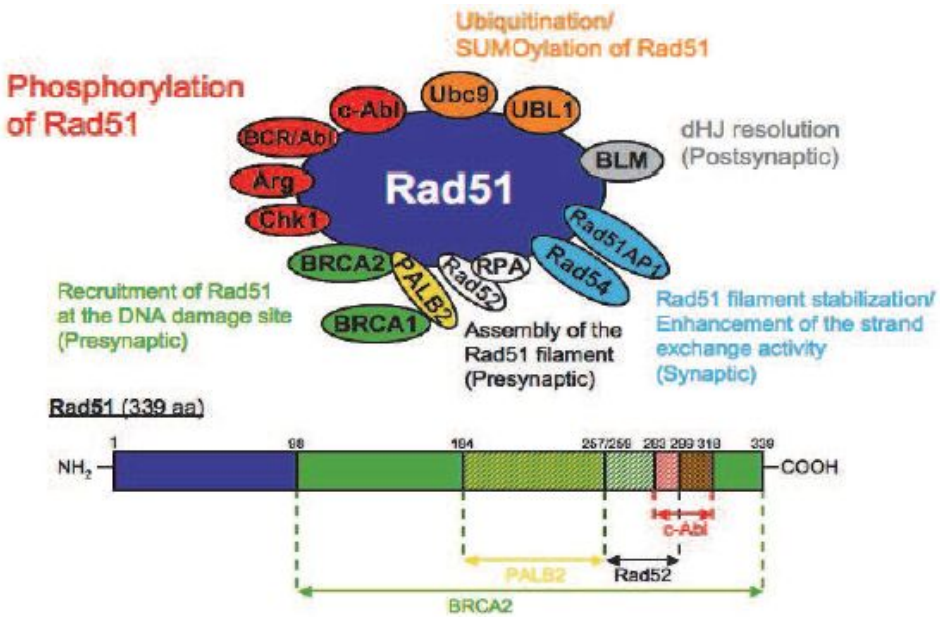


Figure 3. Schematic representation of RAD51 interaction proteins involved in HR repair and DDR (Milena Popova et al., 2011).

II. Materials and Methods

1. Cell culture and treatment

The human osteosarcoma bone morphogenetic cell line U2OS, human cervix adenocarcinoma cell line HeLa and human embryonic kidney cell line HEK293T, Topors+/+ (MEF WT) and Topors -/- Mouse embryonic fibroblasts (MEFs) 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% CO_2 at 37^{o}C. HeLa and U2OS cell lines were 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 Gray (Gy) from ^{137}Cs source (Gammacell 3000 Elan irradiator, Best Theratronics, Ottawa, Canada) and allowed to recover at 37^{o}C for various amounts of time.

2. Generation of stable Topors 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 Topors-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 Topors sequence as the reference sequence (GenBank Accession No. NM_005802). The target sequences were 5'-CCCUGCUCC UUCAUACGA A dTdT-3' (sense) and 5'-UUC GUA UGA AGG AGC AGGG dTdT-3' (antisense) for Topors siRNA #1 and 5'-GCAGUAAGGAGGCCAACUA dTdT-3' (sense) and 5'-UAGUUGGCCUCCUUACUGC dTdT-3' (antisense) for Topors siRNA #2. A non-targeting sequence, 5'-CCUACGCCACCAAUUUCGU dTdT-3' and 5'-ACGAAAUUGGUGGCGUAGG dTdT-3', was used as a negative control. To generate single knockdown clones, U2OS cells were transfected with pSilencer2.1–U6, pSilencer2.1–U6 Topors siRNA #1 or pSilencer2.1–U6 Topors siRNA #2. 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 Topors.

3. Plasmid constructs

The full-length RAD51 cDNA was amplified from GM00637 human fibroblast cells by RT-PCR using the RAD51 primers 5'-GCC TCT AGA ATGGCAATGCAGATG-3' (sense) and5'-AAT GGG CCC TCA GTC TTT GGC ATC-3'(antisense). The amplified RAD51 cDNA construct was cloned into the mammalian expression vector pcDNA3 in-frame with the hemagglutinin (HA) tag. The RAD51 sequence was confirmed by automated DNA sequencing. The human Topors full length, T1 (231-1045), T2 (539-1045) and T3 (704-1045), constructs were gifted from Prof. Eric H. Rubin laboratory and other three constructs T4 (854-1045), T5 (918-1045), T6 (854-917) prepared in our laboratory. The full-length Topors cDNA was amplified from GM00637 human fibroblast cells by RT-PCR using the Topors primers GFP_N3TOPORS_854F5'GCCGAATTCATGAAACACAAAAGGAGAAAA AGG3',GFP_N3TOPORS_918F5'GCCGAATTCATGTCTGAAGTAAAGGA GGATACAG3',GFP_N3TOPORS_1045R5'ACCGGTACCAGACATATCAC AGTCTCTACCAAG-3', GFP_N3-TOPORS_917R 5'- ACC GGT ACC ATCCTTATCACT GTCACT GTC-3'

4. Antibodies

The following antibodies were used for immunoblotting: mouse monoclonal anti Topors (1:1000 Novus Biologicals, LLC8100 SouthparkWay, A8Littleton, CO80120USA), Topors antibody (Y-30, H-127; Santa Cruz Biotechnology)

mouse monoclonal anti-RAD51 (1:1000; Calbiochem, San Diego, CA, USA), mouse monoclonal anti-RAD51 (14B4) (ab213; Abcam, Cambridge, UK) , polyclonal anti-RAD51 (1:1000; Santa Cruz Biotechnology, Santa Cruz, CA, USA), polyclonal anti-HA tag (1:1000; Santa Cruz Biotechnology), monoclonal anti-HA tag (1:1000; Santa Cruz Biotechnology), mouse monoclonal anti-HA (HA.C5) (ab18181; Abcam, Cambridge, UK) and polyclonal anti-GFP tag (1:1000; Santa Cruz Biotechnology), monoclonal anti-GFP tag (1:1000; Santa Cruz Biotechnology) monoclonal anti-α-tubulin (1:8000; Neomarkers, Fremont, CA, USA). Topors foci were detected by immunofluorescence staining using the Topors antibody (H-127; Santa Cruz Biotechnology) at a 1:200 dilution. γ- H2AX foci were detected by immunofluorescence staining using the γ-H2AX mouse monoclonal antibody (JBW301), Upstate Biotechnology, Temecula ,CA ,USA) at a 1:200 dilution. Immunoprecipitations of endogenous and exogenous proteins were performed using mouse monoclonal anti Topors (1:1000NovusBiologicals, LLC8100SouthparkWay, A8Littleton, CO80120USA), Topors antibody (Y-30, H-127; Santa Cruz Biotechnology) mouse monoclonal anti-RAD51 (1:1000; Calbiochem, San Diego, CA, USA), mouse monoclonal anti-RAD51 (14B4) (ab213; Abcam, Cambridge, UK), polyclonal anti-RAD51 (1:1000; Santa Cruz Biotechnology, Santa Cruz, CA, USA).

5. Yeast two-hybrid analysis

The two-hybrid analysis of the full length RAD51-fragment interaction was carried out using a Matchmaker two-hybrid cDNA library from human testis (Clontech, Mountain View, CA, USA). The human Topors gene was cloned from a human testis cDNA pool (purchased from Clontech) by polymerase chain reaction (PCR). The DNA fragments containing sequences derived from RAD51 and Topors 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 RAD51 and Topors sequences were introduced into the yeast strain AH109. The two-hybrid interaction between RAD51 and Topors was then tested according to the manufacturer's protocol (Clontech Matchmaker GAL4 protocol). The interaction between RAD51 and Topors 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.

6. Immunoprecipitation assay and western-blot analysis

Cells were lysed in ice-cold NP-40 lysis buffer [50mM Tris–HCl (pH 8.0), 150mM NaCl, 1% Nonidet P-40] 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), 150mM NaCl, 0.1% Tween 20] containing 5% fat-free milk at room temperature and then incubated with the indicated primary antibodies overnight at 4^{o}C After incubation for 2 h with appropriate peroxidase conjugated secondary antibodies, developed using enhanced chemiluminescence detection system. For the immunoprecipitation assay, aliquots of soluble cell lysates were precleared with protein A/G plus- agarose beads (Santa Cruz Biotechnology), G sepharose and A sepharose (GE Healthcare) as indicated and then incubated at 4^{o}C for 3 h. Next, the appropriate antibody was added, and incubated at 4^{o}C for 12 h. After the addition of fresh protein A/G plus-agarose bead, G sepharose and A sepharose, the reaction was incubated overnight at 4^{o}C with rotation. The beads were washed five times in RIPA buffer without protease inhibitors, resuspended in SDS sample buffer and boiled for 5 min. The samples were then analyzed by western blotting using the appropriate antibodies.

7. Immunostaining

To visualize γ-ray-induced foci, untreated cells or cells treated with 5Gy 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 1 h at room temperature. The cells were double-immunostained using primary antibodies directed against the indicated proteins overnight at 4^{o}C. 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). For foci quantification experiments, cells with ≥10 foci were counted as RAD51 foci-positive cells and the percentage was calculated among at least 100 cells by dividing the number of RAD51 foci-positive cells by the number of DAPI-stained cells. The error bars represent standard error in three independent experiments.

8. Cell survival assay

After treatment with IR, 5×10^2 cells were immediately seeded onto a 60-mm dish in duplicate and grown for 2–3 weeks at 37°C to allow colony formation (Shahi, A., J. H. Lee, 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.

9. Comet assay

DSB repair was assayed by alkaline single-cell agarose-gel electrophoresis as described previously (Shahi, A., J. H. Lee, et al., 2011). Briefly, control and Topors-knockdown cells were treated with 5 Gy of γ-ray followed by incubation in culture medium at 37°C for the indicated times. Cells were then harvested (~10^5 cells per pellet), mixed with low-melting temperature agarose, and layered onto agarose-coated glass slides. The slides were maintained in the dark at 4°C for all of the remaining steps. Slides were submerged in lysis solution [10mM Tris–HCl (pH 10), 2.5M NaCl, 0.1M EDTA, 1% Triton X-100, 10% dimethyl sulfoxide] for 1 h and incubated for 30 min in alkaline electrophoresis solution (300mM NaOH, 200mM EDTA at pH >13). After electrophoresis (~30 min at 1 V/cm tank length), air-dried and neutralized slides were stained with 30– 50 ml ethidium bromide (20 mg/ml). 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).

10. Analysis of Homologous Recombination activity

To analyze the role of Topors in HR in vivo, we used the pCMS-EGFP-ds RED system (Secretan et al., 2004). The plasmid was digested with EcoRI and XhoI to cut multi restriction enzyme site which is having red fluorescence and generate linearized plasmid. Supercoiled pCMS-EGFP-ds RED were used as a positive control for standardization of transfection and analysis conditions. The pCMV-SPORT 6 and pEGFP-N3 supercoiled plasmids (Clontech) used as a control for transfection efficiency. In a typical reaction, 5×10^5 cells were transfected with 0.5 µg pCMS-EGFP-ds RED or 0.5 µg supercoiled pCMS-EGFP-ds RED, together with 0.5 µg of pCMV-SPORT and pEGFP-N3 plasmid, using Lipofectamine 2000 (Invitrogen) according to the manufacturer's recommended protocol. Twenty-four hours after transfection, green (EGFP) and red (Ds Red) fluorescence was measured by flow cytometry (FACSCalibur; BD Biosciences, San Jose, CA, USA).

11. 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

1. Identification of TOPORS as a RAD51-associated protein

Topors is a serine, arginine rich nuclear protein which contains a RING-type zing finger domain and having E3 ligase activity.RAD51 is homologous recombination protein and plays a major role in homologous pairing of DNA double strand break (DSB) repair. To identify unknown RAD51 interacting proteins yeast two-hybrid screening for human RAD51 as bait was carried out. From 2×10^6 transformants, eight clones was identified which is activated both *URA3* and *ADE2* expression. One of the positive clones was identified by yeast two-hybrid screening encoded human TOPORS. Further confirmation was performed with expression of *LacZ* by filter assay (Figure 4).

2. Binding of RAD51 and Topors upon DNA Damage from intact cells

It has been reported that Topors interacts with the adeno-associated virus type 2 (AAV-2) Rep78/68 proteins and involved in transcriptional regulation (Weger et al., 2002) and also have interact with the tumor suppressor protein P53 (Zhou et al., 1999). To investigate Topors interacts with RAD51, cultured

mammalian cancer cells expressing both proteins were utilized and co-Immunoprecipitation assay were performed.

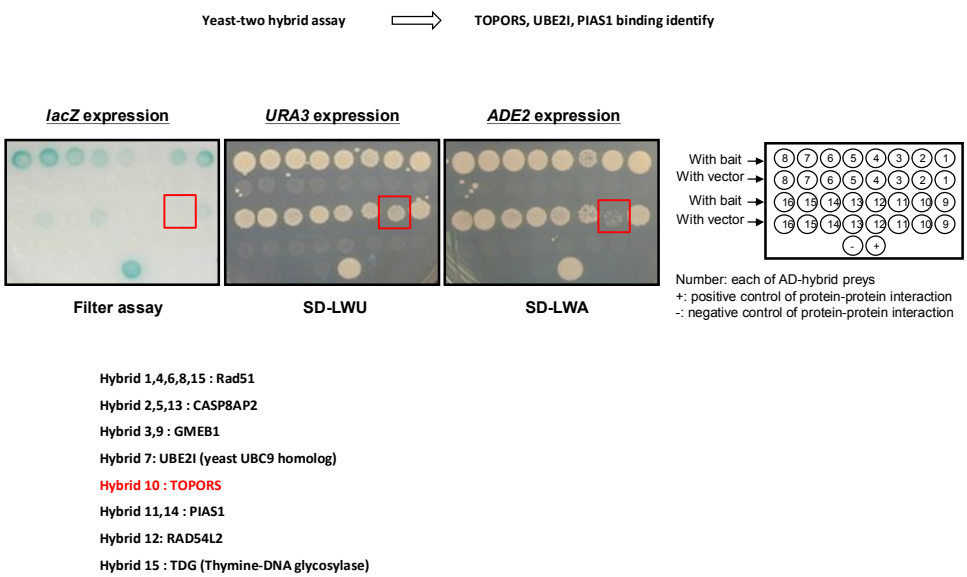


Figure 4. Protein interaction study by Yeast two-hybridization assay

U2OS cells was treated with 5Gy IR to make DSB and harvested after 3hrs, untreated cells was used as control. The cells were lysed, and endogenous RAD51 was immunoprecipitated with a RAD51 specific antibody. Immunoprecipiates were subjected to western blotting with an anti-Topors antibody. Immunoprecipitation results through western blotting showed us the RAD51 binding to Topors was increased after IR induced DSB. The reciprocal experiment was performed, Topors antibody was coimmunoprecipitated with RAD51 (Figure 5A). These results showed us interaction between RAD51 and Topors might be direct or indirect endogenously. To identify specific binding rabbit or mouse IgG coimmunoprecipitated with RAD51 or Topors, our results showed us binding was specific and it's not due to antibody non-specific interaction. Immunoprecipitation for both RAD51 and Topors antibody was also performed.

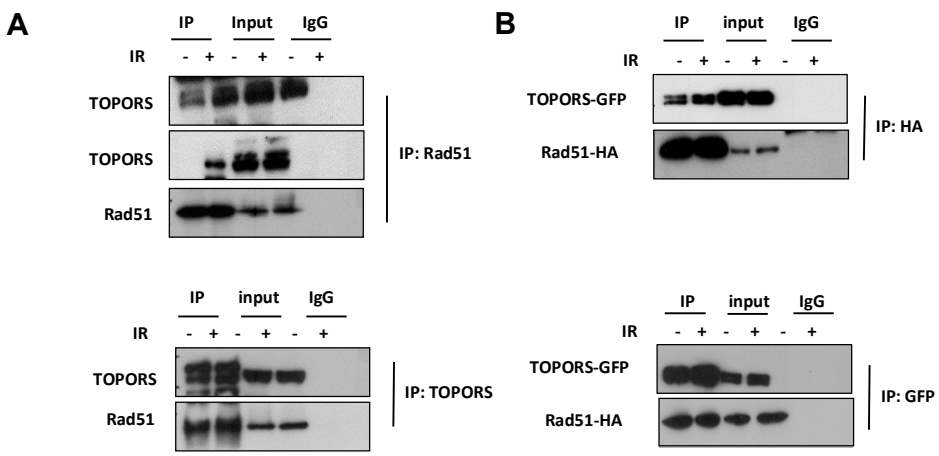


Figure 5. Interaction between Rad51 and TOPORS.

A. Rad51 interacts with TOPORS after IR induced DNA damage (endogenous). U2OS cells were untreated or treated with 5Gy γ irradiation for 3hrs (5A top figure). Proteins were immunoprecipitated from the lysates using an RAD51 antibody. Immunoprecipitates were then subjected to western-blot analysis using antibodies specific for Topors or RAD51. The third or fourth lane contains 5% input for RAD51 and 20% input for Topors. Normal rabbit IgG was used for negative control immunoprecipitations. Topors also interacts with RAD51 after DNA damage. U2OS cells were untreated or treated with 5Gy γ irradiation for 3hrs (5A bottom figure). Proteins were immunoprecipitated from

the lysates using a Topors antibody. **B.** HA-Rad51 interacts with GFP-TOPORS after IR induced DNA damage (exogenous). HEK293T cells were transfected with full-length GFP-Topors and HA-RAD51 expression vectors. After 24 or 48 hrs, cells were untreated or treated with 5Gy γ irradiation for 3hrs (5B top figure).Proteins were immunoprecipitated from the lysates using an anti-HA RAD51 antibody. Immunoprecipitates were then subjected to western-blot analysis using antibodies specific for GFP-Topors or HA-RAD51. The third or fourth lane contains 5% input for HA-RAD51 and 20% input for GFP-Topors. Normal rabbit IgG was used for negative control immunoprecipitations. GFP-Topors also interacts with HA-RAD51 after DNA damage. HEK293T cells were transfected with full-length GFP-Topors and HA-RAD51 expression vectors. After 24 or 48 hrs, cells were untreated or treated with 5Gy γ irradiation for 3hrs (5B bottom figure). Proteins were immunoprecipitated from the lysates using a Topors antibody.

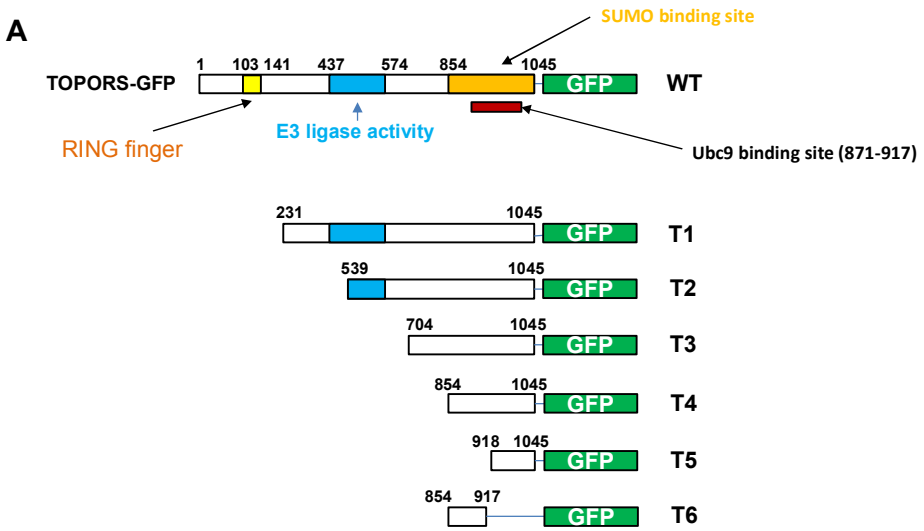
Our endogenous binding result was further confirmed by exogenous co-Immunoprecipitation using full length RAD51 and Topors. HEK 293T cells were transiently transfected with full-length RAD51 tagged with HA and EGFP tagged Topors. Co-Immunoprecipitation assay were performed by using an anti-HA antibody and immunoblotting was done with anti-GFP antibody. Immunoprecipitation results through western blotting showed us the HA-RAD51 binding to GFP-Topors was increased after IR induced DSB, results similar to endogenous Co-Immunoprecipitation. The reciprocal experiment was performed, Topors antibody was Coimmunoprecipitated with RAD51 (Figure 5B). These results showed us interaction between HA-RAD51 and GFP-Topors might be direct or indirect exogenously. To identify specific binding rabbit or mouse IgG coimmunoprecipitated with HA-RAD51 or GFP-Topors, our results showed us binding was specific and it's not due to antibody non-specific interaction. Immunoprecipitation for both HA-RAD51 and GFP-Topors antibody was also performed.

3. Identification of a RAD51 Binding site/domain in Topors

It was previously reported that, C-terminal region of Topors is required for punctate nuclear localization (Rasheed et al., 2002) and residues 437 to 574 was sufficient for both sumoylation and localization to nuclear speckles (Weger et al., 2003).Topors N-terminal RING finger domain function as E3 ligases for

P53 ubiquitination (Weger et al., 2005). These previous studies, our endo and exo co-immunoprecipitaion assays were insisted us to identify specific/unique RAD51 binding domain of Topors.

To identify RAD51 binding domain, we used six Topors fragments consisting of residues T1-T6 (Figure 6A) (231-1045), (539-1045), (704-1045), (854-1045), (918-1045), (854-917). Topors full length, T1, T2 and T3 constructs were gifted from Prof. Eric H. Rubin laboratory and other three constructs T4, T5, T6 prepared in our laboratory. HEK 293 T cells were transfected with Topors constructs T1-T6, N-Terminal T1 and T2 constructs was showed nuclear body localization (Figure 6C) and others were not shown nuclear body localization.


A
SUMO binding site
TOPORS-GFP
1
103 141
437
574
854
1045
GFP
WT
RING finger
E3 ligase activity
Ubc9 binding site (871-917)
231
1045
T1
539
T2
704
T3
854
T4
918
T5
917
T6

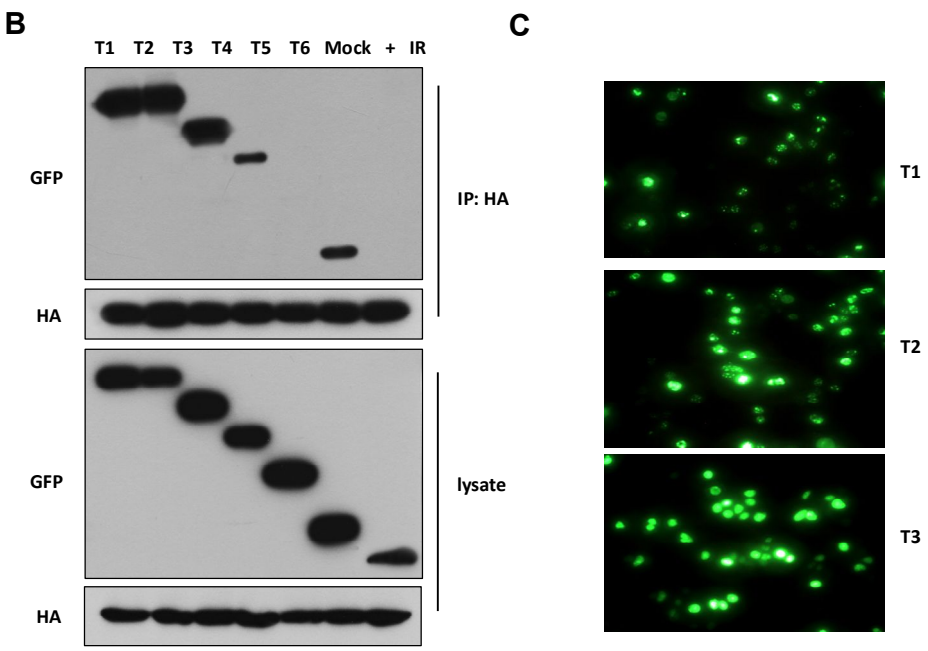

B
T1 T2 T3 T4 T5 T6 Mock + IR
GFP
HA
IP: HA
lysate
C
T1
T2
T3

Figure 6. Identification of Rad51 binding site in TOPORS.

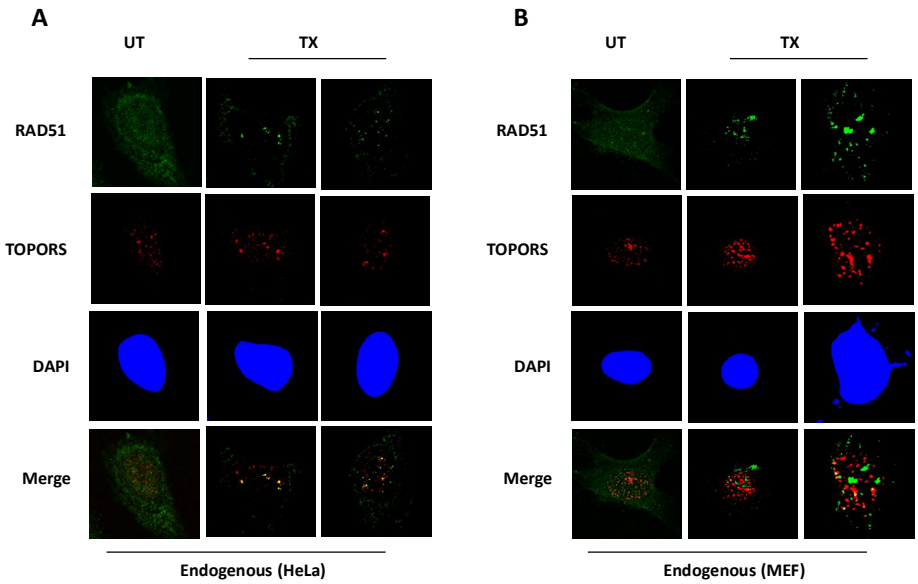
A. Schematic representation of Topors full length and other six constructs. **B and C**. Co-Immunoprecipitation of RAD51-HA and Topors Constructs T1-T6 and TOPORS nuclear body localization T1 and T2, T3~T6: no localization.HEK293T cells were co-transfected with HA-RAD51 and various GFP-Topors constructs named T1-T6 and mock as control GFP expression vectors. After 48 hrs, cells were treated with 5Gy γ irradiation for 3hrs. Proteins were immunoprecipitated from the lysates using an anti-HA RAD51 antibody. Immunoprecipitates were then subjected to western-blot analysis using antibodies specific for GFP-Topors antibody. The second or third western blotting contains 20% input for HA-RAD51 and GFP-Topors constructs T1-T6 and control GFP vector.

HEK 293T cells were transiently transfected with full-length RAD51 tagged with HA and EGFP tagged Topors constructs T1-T6.After 48 hrs of Co-transfection cells were IR with 5Gy and harvested after 3hrs. The lysed cells, Co-Immunoprecipitated by using an anti-HA antibody and immunoblotting was done with anti-GFP antibody. Co-Immunoprecipitation results through western blotting showed us Topors constructs T1, T2, T3, T4 and T6 binding to HA-RAD51 but construct T5 did not binding so immunoblotting was not shown any band (Figure 6B).

These results indicate/confirmed us binding site of RAD51 is C-terminal region 854-917 amino acid of Topors. Immunoprecipitation for HA-RAD51 antibody was also performed.

4. TOPORS localizes to the sites of DNA damage and colocalizes with Rad51

The DNA damage induced by IR or other damaging agent's localize/recruit/interact many DNA repair proteins in the nucleus and form distinct structures called as foci. It was previously shown that RAD51 can form discrete foci in nucleus after DNA damage by IR or other damaging agents (Haff et al., 1995, Raderschall et al., 1999, Tarsounas et al., 2003). Topors is localized in punctate nuclear regions or it forms a speckled pattern in nucleus (Rasheed et al., 2002, Weger et al., 2003). It was insisted us to find out whether Topors and RAD51 can be colocalize after DSB. We performed RAD 51, Topors foci formation in time and dose dependent manner (data not shown) and choose particular time and dose to do other experiments including Colocalization. In 5Gy γ-irradiation after 3 hrs was choosed, in untreated Hela cells forms diffuse nuclear staining and treated cells forms discrete RAD51 nuclear foci colocalize with Topors foci endogenously (Figure 7A). Similar to Hela cells, MEF Topors +/+ or MEF WT cells also forms IR induced discrete RAD51 nuclear foci colocalize with Topors foci endogenously after treatment of 5Gy γ-irradiation in 3hrs (Figure 7B).


A
UT
TX
RAD51
TOPORS
DAPI
Merge
Endogenous (HeLa)
B
UT
TX
RAD51
TOPORS
DAPI
Merge
Endogenous (MEF)

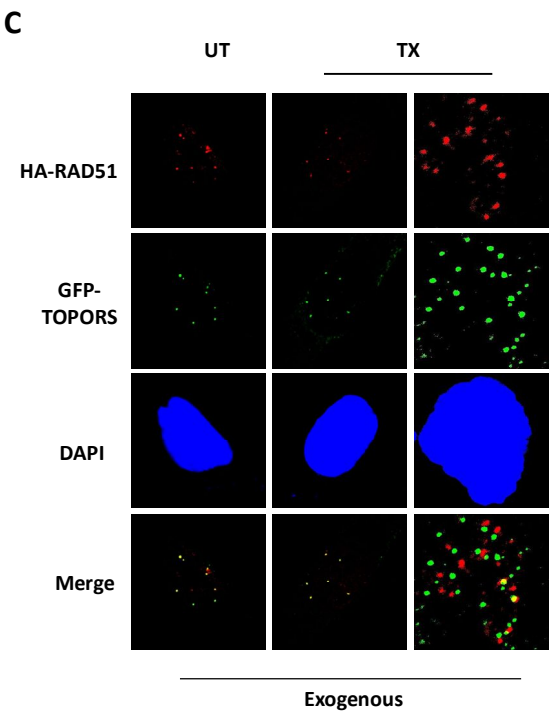

C
UT
TX
HA-RAD51
GFP-
TOPORS
DAPI
Merge
Exogenous

Figure 7. Colocalization of TOPORS and RAD51 in nucleus after DSB by IR. A. HeLa cells were untreated or treated with 5Gy γ irradiation for 3hrs. After 3 hrs cells were fixed and immunostained using anti-Topors and anti-RAD51 antibody. **B**. MEF WT cells were untreated or treated with 5Gy γ irradiation for 3hrs. After 3 hrs cells were fixed and immunostained using anti-Topors and anti-RAD51 antibody. **C**. Hek293T cells were co-transfected with full length HA-RAD51 and GFP-Topors expression vectors. After 48 hrs of Co-transfection cells were treated with 5Gy γ-irradiation and fixed, immunostained after 3hrs using anti-GFP and anti-HA antibody. Representative cells containing foci are shown. Nuclei were stained with DAPI. Topors forms nuclear foci and colocalize with RAD51 by 5Gy γ-irradiation after 3 hrs. At least 100 cells were analyzed for each treatment. Topors and RAD51 or GFP and HA antibodies were visualized with Alexa Fluor 488-or Alexa Fluor 594 conjugated secondary antibodies, respectively, followed by confocal microscopy. Colocalization of Topors (red) and RAD51 (green) or GFP (green) and HA (red) in cells appears yellow in merged images.

Our endogenous colocalization result was further confirmed by using full length RAD51 and Topors. HEK 293T cells were transiently transfected with full-length RAD51 tagged with HA and EGFP tagged Topors. After 48 transfection cells were harvested and fixed. Immunofluorescence assay was carried out by using an anti-HA, anti-GFP antibody. 40-6-Diamidino-2-phenylindole (DAPI) staining was performed to indicate the position of nuclei (Figure 7C).

5. TOPORS is required for the recruitment of Rad51 to DSBs

Our previous experiments results from co-immunoprecipitation and colocalization of Topors with RAD51 reasoned that Topors might be playing a vital role in the functional regulation of RAD51 mediate DSB repair by homologous recombination. To further confirm this hypothesis, Topors was silenced in U2OS cells using plasmid-mediated sh RNA expression technology to create stable Topors knockdown cells lines (Topors shRNA#1 and Topors shRNA#2). Immunoblotting confirmed that expression of endogenous Topors was reduced by more than 90% in U2OS cell lines stably transfected with the two different Topors shRNAs in comparison to control shRNA-transfected cells (Figure 8A). Topors +/+ and Topors -/- knockout MEF cell lines were prepared and immunoblotting confirmed to check the endogenous expression of Topors.

Similar to U2OS stable cells Topors was reduced more than 90% in Topors -/- MEF cells compare to Topors +/+ MEF cells (Figure 8B).

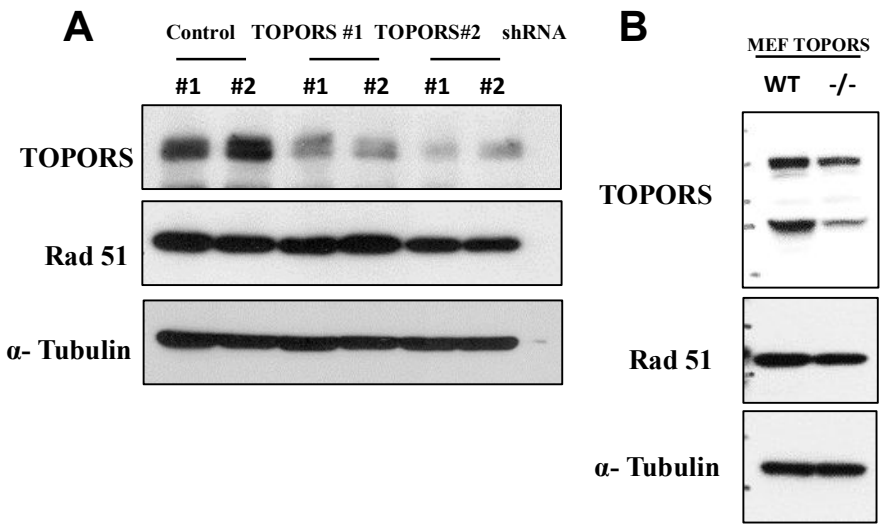


Figure 8. Preparation of TOPORS knockdown cells. A. U2OS cells were transfected with control or one of two different Topors shRNA expression vectors (Topors shRNA #1 and #2) and selected with 400 µg/ml G418. Four weeks later, the expression level of the Topors protein was determined by immunoblotting analysis using an anti-Topors antibody. **B.** MEF cells were transfected with control or one of two different Topors shRNA expression vectors (Topors shRNA #1 and #2) and selected with 400 µg/ml G418. Four

weeks later, the expression level of the Topors protein was determined by immunoblotting analysis using an anti-Topors antibody.

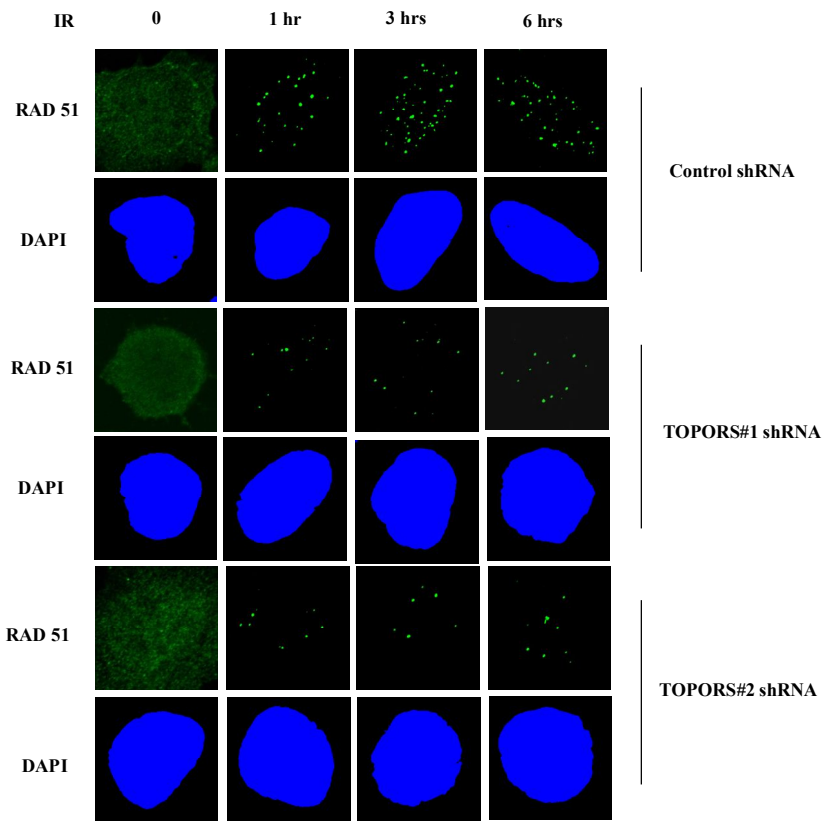
We further investigated RAD51 foci formation after DSB in control and both Topors stable knockdown U2OS stable cells in time dependent manner. Control and Topors knockdown U2OS cells were treated with 5Gy γ-irradiation as well as MMC and HU harvested in different time intervals. Cells were fixed with 4%paraformaldehyde and immunofluorescence assay performed. It was previously reported that knockdown of DNA damage response proteins impact on down regulation of RAD51 foci formation (Zhang et al., 2005 Boichuk et al., 2011) as well RNF4 ubiquitin E3 ligase showed RAD51 recruitment to DSB site was severely impaired in RNF4 depleted cells whereas γH2AX foci was normal (Galanty et al., 2012, Yin et al., 2012).After observing our results, RAD51 foci formation was approximately 25% at early time points (1hr) but it has increased double the amount (approximately 45%) and reached peak at 3hrs then reduced to 35% in 6hrs in case of control ShRNA U2OS cells. In contrast to control ShRNA cells both Topors knockdown U2OS cells showed decreased percentage of RAD51 foci formation. Since from early to late time points RAD51foci formation didn't increased and it was below 15% whereas control cells showed up to 45 % (Figure 9). Topors +/+ and Topors -/- knockout MEF cell lines were harvested, fixed and immunofluorescence confirmed to check the RAD51 foci formation in MEF cell lines. Almost Similar to U2OS stable cells RAD51 foci formation was approximately 25% at early time points (1hr)

but it has not increased like U2OS stable cells. The amount of foci number in Topors +/+ MEF cell has been little decreased in late times 3hrs and 6hrs. Compare to Topors +/+ MEF cells in case of Topors -/- MEF cells RAD51 foci formation has been decreased approximately 40% at increased time course (Figure 10).

In case of MMC induced RAD51 foci formation, Control U2OS cells shown increased number of foci formation when time is increasing. The RAD51 foci formation approximately 25% at early time points (3hr) but it has increased 35% in 6hr and reached peak at 12hrs in case of control ShRNA U2OS cells. In contrast to control ShRNA cells both Topors knockdown U2OS cells showed decreased percentage of RAD51 foci formation. Since from early to late time points RAD51foci formation didn't increased and it was below 15% whereas control cells showed up to 50 % (Figure 11A). In case of HU induced RAD51 foci formation, the RAD51 foci formation approximately 25% at early time points (3hr) but it has not increased like MMC induced RAD51 foci formation. The number of foci was almost similar in three different timings (3, 6,12hrs) or it has been slightly decreased similar to MEF IR induced RAD51 foci (figure 10). In contrast to control ShRNA cells both Topors knockdown U2OS cells showed decreased percentage of RAD51 foci formation. Since from early to late time points RAD51foci formation didn't increased and it was decreased up to 50 % (Figure 11B).

Our observation showed Topors knockdown or knockout in U2OS and MEF cells impairs DSB induced (IR/MMC/HU) RAD51 foci formation in early to late time points. But both Topors knockdown stable cells show no effect on well-known DSB markers γH2AX or 53BP1 foci formation (Figure 12).

A


IR
0
1 hr
3 hrs
6 hrs
RAD 51
DAPI
Control shRNA
RAD 51
DAPI
TOPORS#1 shRNA
RAD 51
DAPI
TOPORS#2 shRNA

B

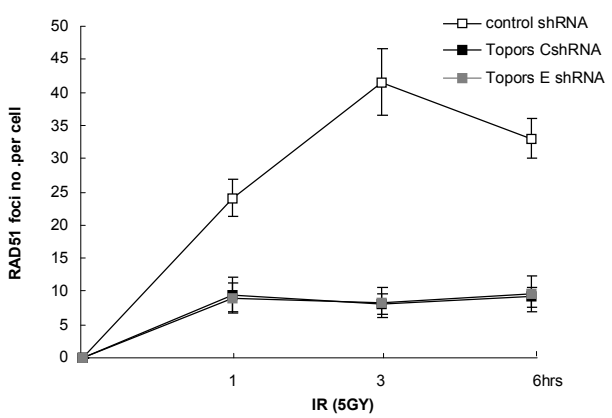
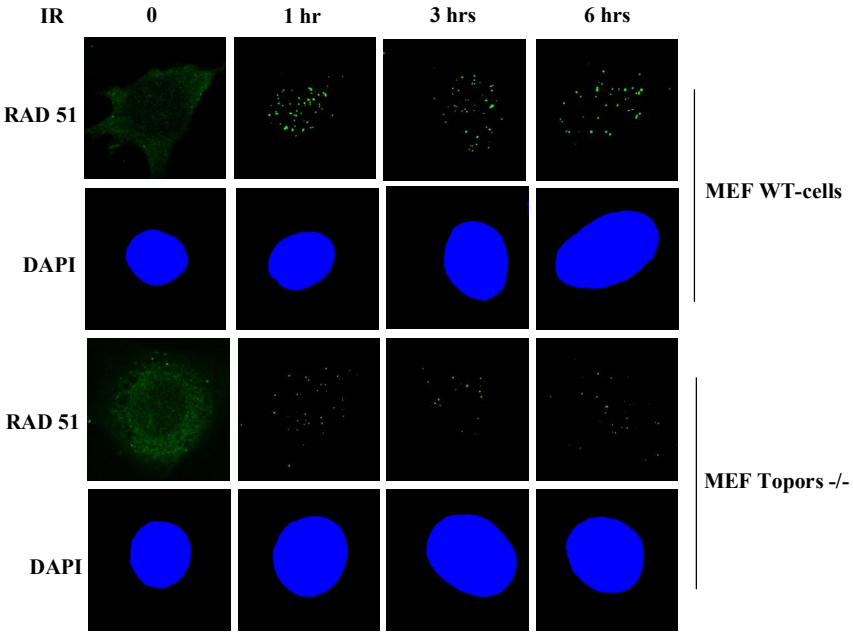

control shRNA
Topors CshRNA
Topors E shRNA
RAD51 foci no .per cell
50
45
40
35
30
25
20
15
10
5
0
1
3
6hrs
IR (5GY)

Figure 9. TOPORS knockdown impairs Rad51 foci in IR treatment.

A. Down regulation of RAD51 foci formation in Topors-deficient U2OS cells after IR treatment. Control and Topors-depleted U2OS cells were untreated or treated with 5Gy γ-irradiation and were then fixed at the indicated times. Cells were stained with an anti-Rad51 antibody and the DNA was counterstained using DAPI. **B.** Quantification of cells with RAD51 foci in control or Topors sh RNA cells with or without IR induced DNA damage in time dependent manner. The number of Rad51 foci positive cells determined by confocal microscopy (*N*=3). Error bars represents the mean ± SD. Asterisk denotes $P<0.01$.

A


IR
0
1 hr
3 hrs
6 hrs
RAD 51
DAPI
MEF WT-cells
RAD 51
DAPI
MEF Topors -/-

B

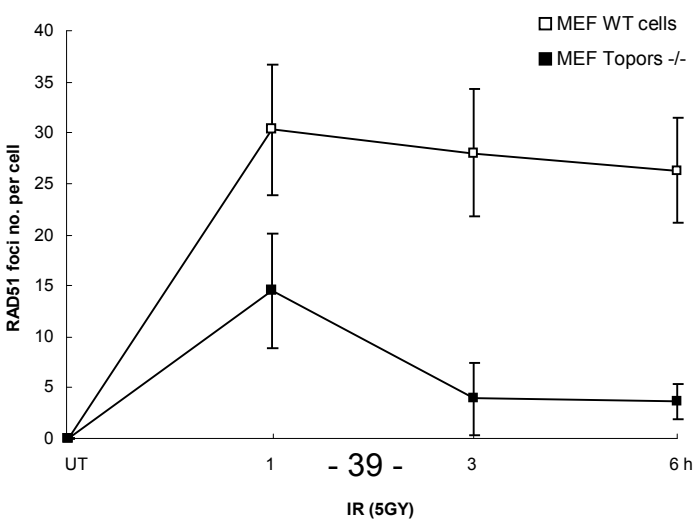
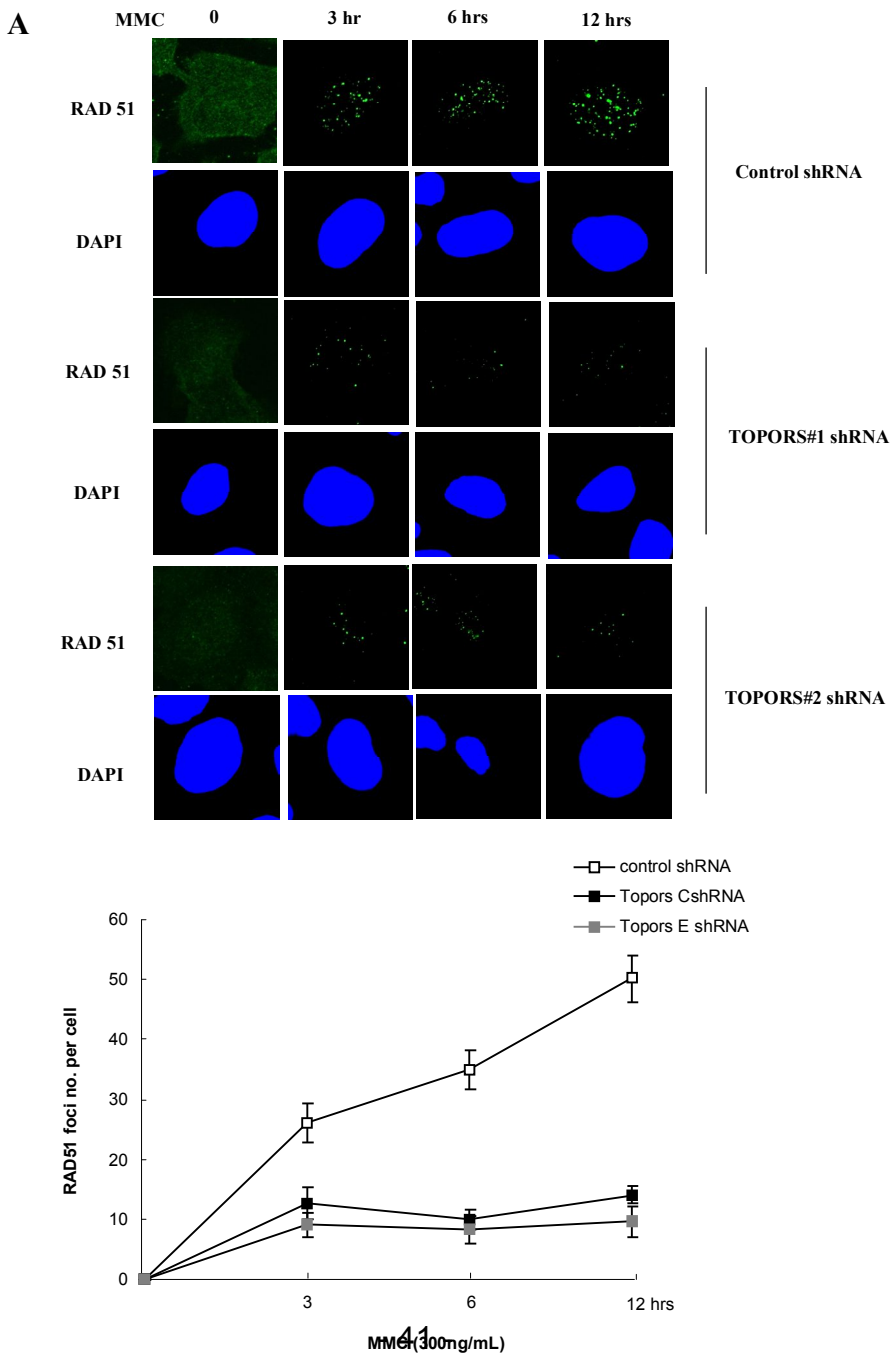
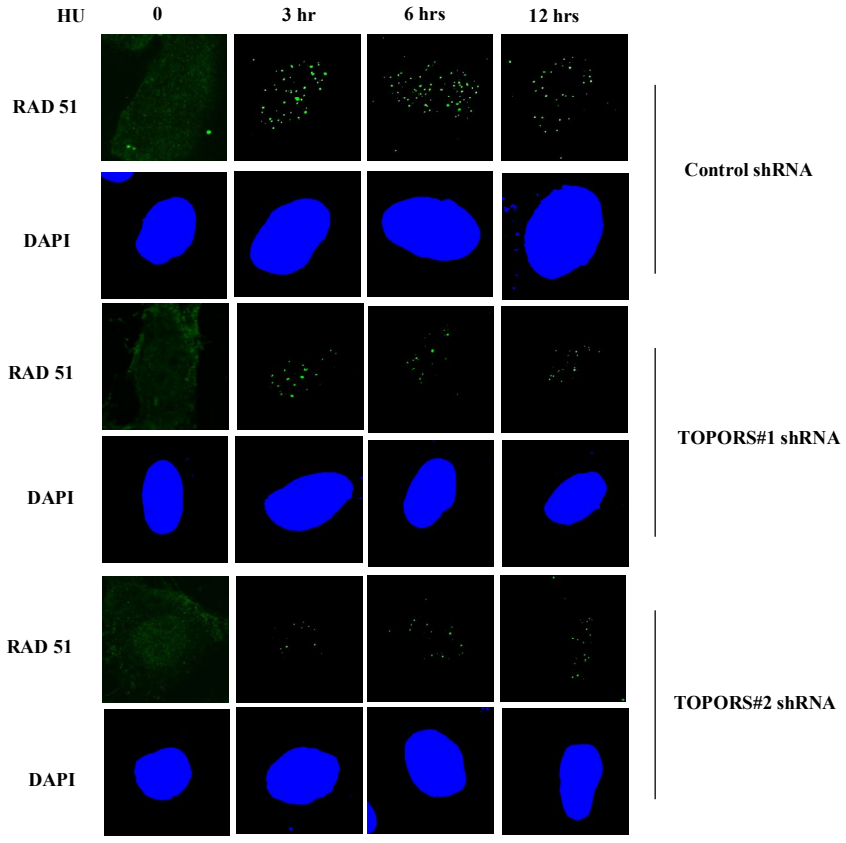

MEF WT cells
MEF Topors -/-
RAD51 foci no. per cell
40
35
30
25
20
15
10
5
0
UT
1
3
6 h
IR (5GY)

Figure 10. TOPORS knockout impairs Rad51 foci in IR treatment.

A. Down regulation of RAD51 foci formation in Topors -/- MEF cells after IR treatment. Topors +/+ and Topors -/- MEF cells were untreated or treated with 5Gy γ-irradiation and were then fixed at the indicated times. Cells were stained with an anti-Rad51 antibody and the DNA was counterstained using DAPI. **B.** Quantification of cells with RAD51 foci in Topors +/+ and Topors -/- MEF cells with or without IR induced DNA damage in time dependent manner. The number of Rad51 foci positive cells determined by confocal microscopy (N=3). Error bars represents the mean ± SD. Asterisk denotes $P<0.01$.


A
MMC
0
3 hr
6 hrs
12 hrs
RAD 51
DAPI
RAD 51
DAPI
RAD 51
DAPI
Control shRNA
TOPORS#1 shRNA
TOPORS#2 shRNA
control shRNA
Topors CshRNA
Topors E shRNA
RAD51 foci no. per cell
60
50
40
30
20
10
0
3
6
12 hrs
MMC(300ng/mL)

B


HU
0
3 hr
6 hrs
12 hrs
RAD 51
DAPI
Control shRNA
RAD 51
DAPI
TOPORS#1 shRNA
RAD 51
DAPI
TOPORS#2 shRNA

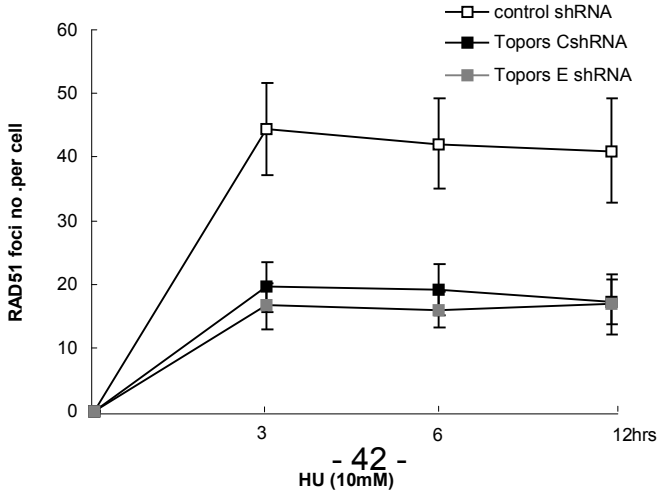

control shRNA
Topors CshRNA
Topors E shRNA
RAD51 foci no .per cell
60
50
40
30
20
10
0
3
6
12hrs
HU (10mM)

Figure 11. TOPORS knockdown impairs in response to replication stress. **A and B** Down regulation of RAD51 foci formation in Topors-deficient U2OS cells after MMC and HU treatment. Control and Topors-depleted U2OS cells were untreated or treated with 300ng/mL concentration of MMC and 10mM HU were then fixed at the indicated times. Cells were stained with an anti-Rad51 antibody and the DNA was counterstained using DAPI. Quantification of cells with RAD51 foci in control or Topors sh RNA cells with or without MMC and HU induced DNA damage in time dependent manner. The number of Rad51 foci positive cells determined by confocal microscopy (*N*=3). Error bars represents the mean ± SD. Asterisk denotes $P<0.01$.

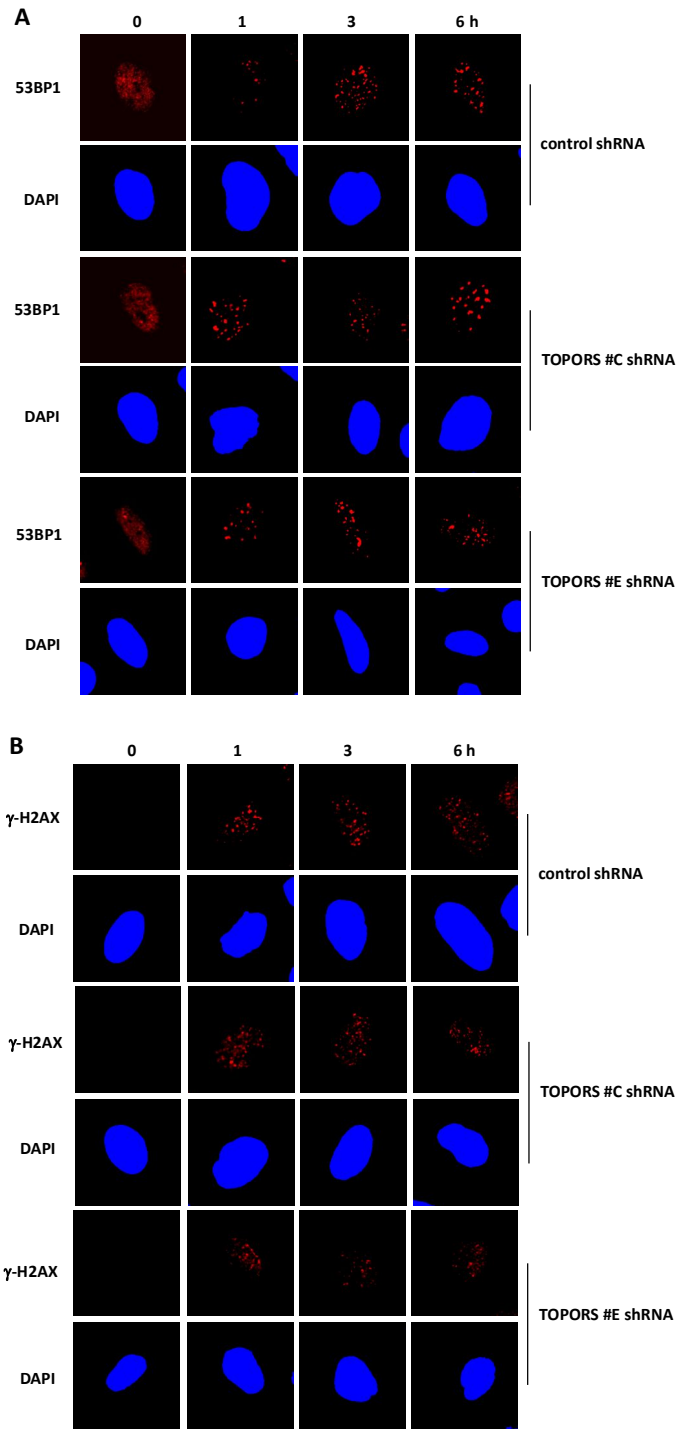

A
0
1
3
6 h
53BP1
DAPI
control shRNA
53BP1
DAPI
TOPORS #C shRNA
53BP1
DAPI
TOPORS #E shRNA
B
0
1
3
6 h
γ-H2AX
DAPI
control shRNA
γ-H2AX
DAPI
TOPORS #C shRNA
γ-H2AX
DAPI
TOPORS #E shRNA

Figure 12. TOPORS is not affected 53BP1 and γH2AX foci recruitment after IR treatment. A. Control and Topors-depleted U2OS cells were untreated or treated with 5Gy γ-irradiation and were then fixed at the indicated times. Cells were stained with an anti-53BP1 antibody and the DNA was counterstained using DAPI. **B.** Control and Topors-depleted U2OS cells were untreated or treated with 5Gy γ-irradiation and were then fixed at the indicated times. Cells were stained with an anti-γH2AX antibody and the DNA was counterstained using DAPI.

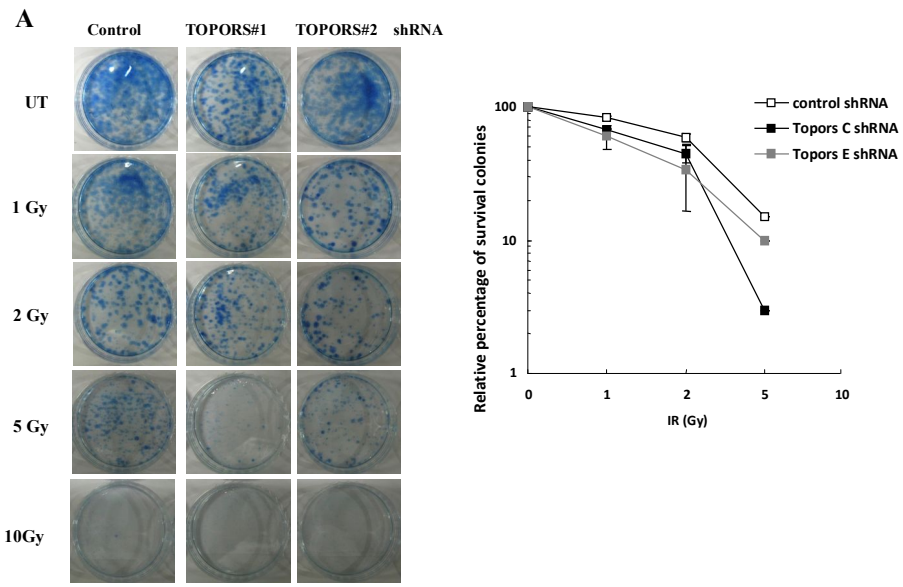


Figure 13. Knockdown of TOPORS sensitized cells to IR treatment.

A. Topors depletion affects survival U2OS cells following exposure to γ-irradiation. Control and Topors- depleted U2OS cells were untreated or treated with 1, 2, 5 and 10 Gy ionizing radiations, after 2 weeks stained with methylene blue.

B. Control and Topors-knockdown U2OS stable cells were untreated or treated with the indicated doses of ionizing radiation and cell survival was assessed using a clonogenic assay. Data represent the mean±SD of three independent experiments. Asterisk denotes $P<0.01$.

6. Deletion of TOPORS leads to decreased homologous recombination repair

To address biological consequences of defective Rad51 foci formation following DNA damage in TOPORS-depleted cells, we analyzed the sensitivity of cells to IR treatment and DSB repair.TOPORS- depleted cells showed significantly compromised clonogenic cell survival after exposure to IR (Figure 13).

Next, to investigate Homologous recombination repair activity we used the pCMS-EGFP-ds RED system (Secretan et al., 2004). The plasmid was digested with EcoRI and XhoI to cut multi restriction enzyme site which is having red fluorescence and generate linearized plasmid. Supercoiled pCMS-EGFP-ds RED (Figure 14B and 14C) was used as a positive control for standardization of transfection and analysis conditions. The pCMV-SPORT 6 and pEGFP-N3 supercoiled plasmids (Clontech) used as a control for transfection efficiency. Control and Topors knockdown U2OS cells were transfected with 0.5 µg pCMS-EGFP-ds RED or 0.5 µg supercoiled pCMS-EGFP-ds RED, together with 0.5 µg of pCMV-SPORT and pEGFP-N3 plasmid, using Lipofectamine 2000 (Invitrogen) according to the manufacturer's recommended protocol. Twenty-four hours after transfection, green (EGFP) and red (Ds Red) fluorescence was measured by flow cytometry (FACSCalibur; BD Biosciences,

San Jose, CA, USA). In Control cells HR activity was nearly 80% whereas both Topors knockdown cells shown only 40-50% of HR activity (Figure 14).In control cell showed effective HR repair due to presence of Topors ,this results indicated Topors playing an effective role in HR regulation and its involvement in DNA damage repair. The HR activity was where EcoRI digested 37.81±7.73%, 56.31±10.66 %in the Topors shRNA #1 cells and XhoI digested was 27.44±5.61% and 45.37±8.59% in the Topors shRNA #2 cells relative to control shRNA transfected cells.

We further investigated DSB marker γH2AX foci formation in late hours after IR to identify effective DNA repair. Phosphorylation of C-terminal ser-139 histone variant H2AX is one of the early events after DNA damage and it's a landmark for DNA damage site where as other repair factors assemble (Rogakou et al., 1999). For this control and both Topors knockdown cells were treated with IR and kept for 16 hrs incubation. After incubation cells were fixed and immunofluorescence assay was performed. Our results showed us, Control cells having less number of γH2AX foci formation which is due to effective DSB repair. In contrast both Topors knockdown cells having abundant number of γH2AX foci, because of delayed DSB repair. This data showed Topors involvement effective in DSB repair (Figure 15)

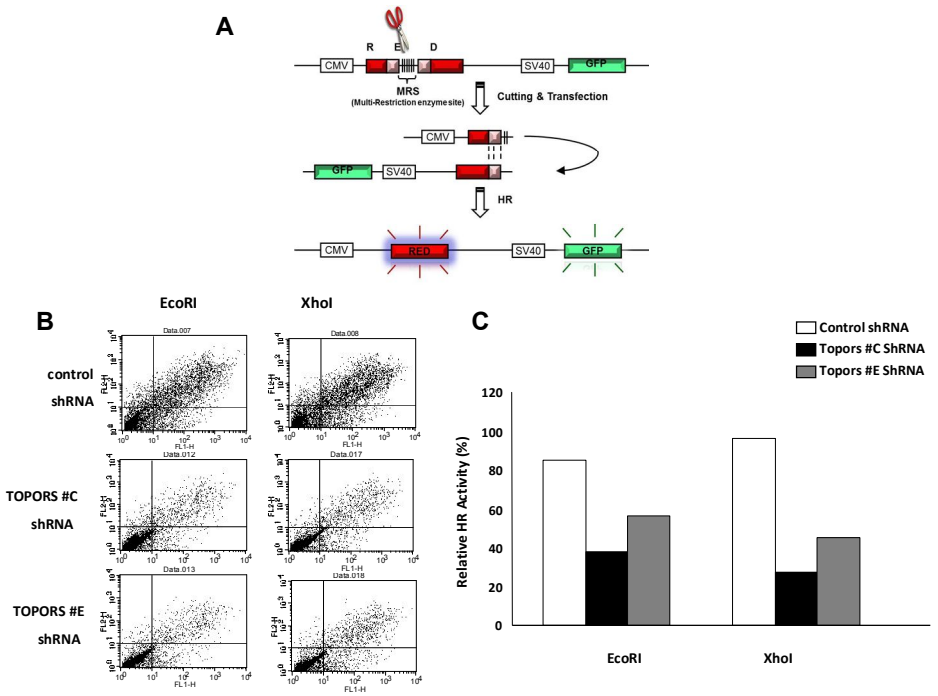


Figure 14. HR activity in TOPORS depleted cells. A. Schematic diagram of pCMS-homologous DNA for HR assay. Repair efficiency after treatment with restriction enzymes EcoRI and XhoI was measured.Down regulation of Homologous recombination activity in Topors deficient U2OS cells. **B.** U2OS stable cells were transfected with full length and restriction (EcoRI and XhoI) digested pCMS-HR expression vector. To quantify HR events, the cells were analyzed by flow cytometry (FACS) 24 hrs after transfection. Ds Red expression was used to normalize transfection efficiency. The ratio of GFP-

positive (GFP+) to Ds Red-positive (Ds Red+) cells was used as a measure of HR activity. Relative levels of plasmid joining compared to control shRNA-transfected cells were calculated by dividing the GFP+/Ds Red+ ratios of the samples and plotted.

C. Quantification of cells with Homologous recombination repair activity was approximately 50% impaired in Topors depleted cells compare to control cells. Means are representative of at least three independent experiments. Error bars indicate the SD. Asterisk denotes P<0.

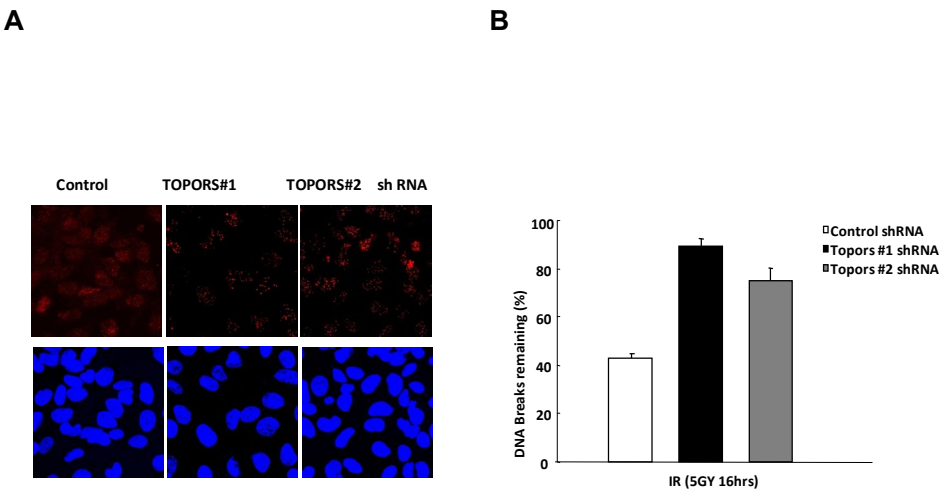
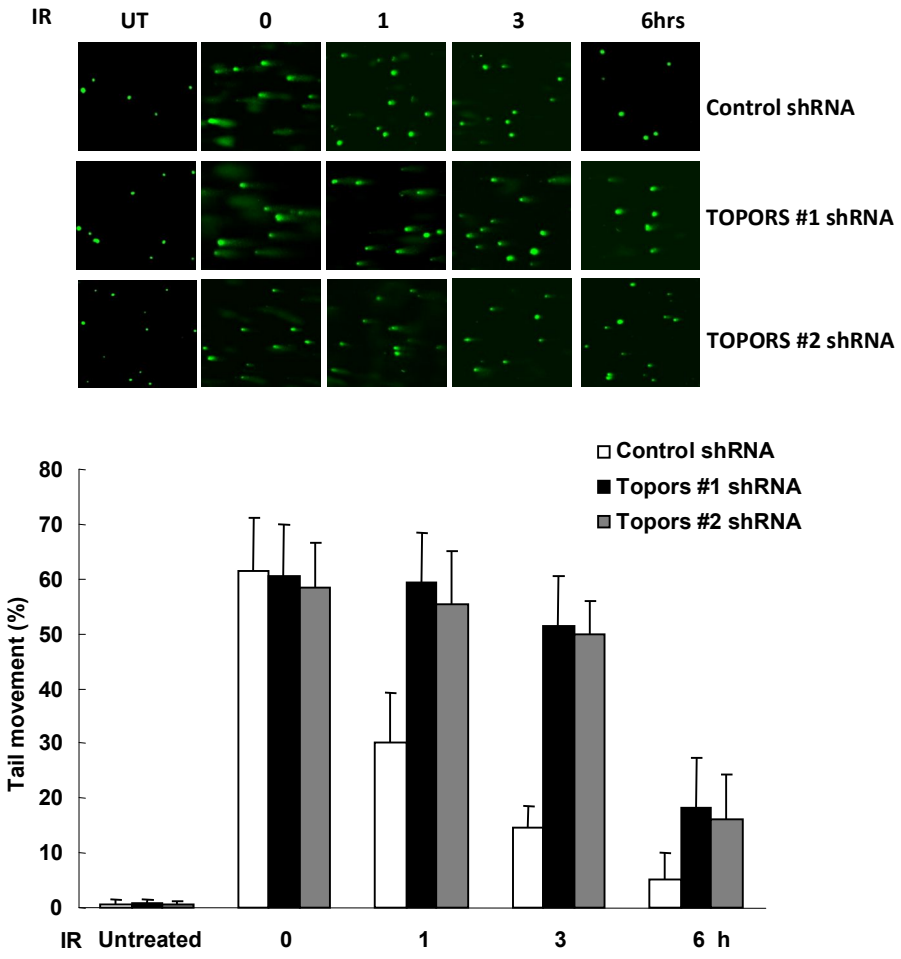


Figure 15. Topors knockdown cells exhibit prolonged γ-H2AX foci formation after IR A. Control and Topors-depleted U2OS stable cells were untreated or treated with 5Gy γ-irradiation and were then fixed after 16hrs cells were fixed, immunostained with an anti- γ-H2AX antibody and the DNA was counterstained using DAPI. **B.** Topors deficient cells shown increased percentage of γ-H2AX foci positive cells compare to control cells. The number of γ-H2AX foci positive cells divide by DAPI stained cells determined by confocal microscopy (*N*=3). Error bars represents the mean ± SD. Asterisk denotes P<0.01.

We used single-cell gel electrophoresis (comet assay) to measure IR induced DSB repair in Topors knockdown cells. For this, Control and both Topors knockdown shRNA U2OS cells were treated 5Gy IR to make DSB and harvested in different time points. Topors +/+ and Topors -/- MEF cells also were treated 10Gy IR to make DSB and harvested in different time points. This method is very sensitive, versatile and relatively simple to perform even it can measure very low level of DNA breaks (Collins 2004, Olive et al., 2006). Based on DNA mobility or comet tail movement DSB repair will be measured. In the initial time point's tail movement will be similar between control and Topors knockdown or knockout U2OS/MEF cells. But time persist, the DSB repair was measured based on tail movement. The unrepaired DNA strand breaks percentage of control cells decreased in different time (0-6hrs) whereas Topors knockdown cells showed long comet tails (Figure 16 A). Similar to Topors depleted U2OS cells, MEF Topors -/-also showed long comet tails (Figure 16 B) compare to MEF Topors +/+ cells in different time points (0-6hrs). The observation of long comet tails in Topors deficient cells indicates impaired DNA DSB repair. These results proved that Topors knockdown cells unable to repair IR induced DSBs.

A


IR
UT
0
1
3
6hrs
Control shRNA
TOPORS #1 shRNA
TOPORS #2 shRNA
Control shRNA
Topors #1 shRNA
Topors #2 shRNA
Tail movement (%)
80
70
60
50
40
30
20
10
0
IR
Untreated
0
1
3
6 h

B

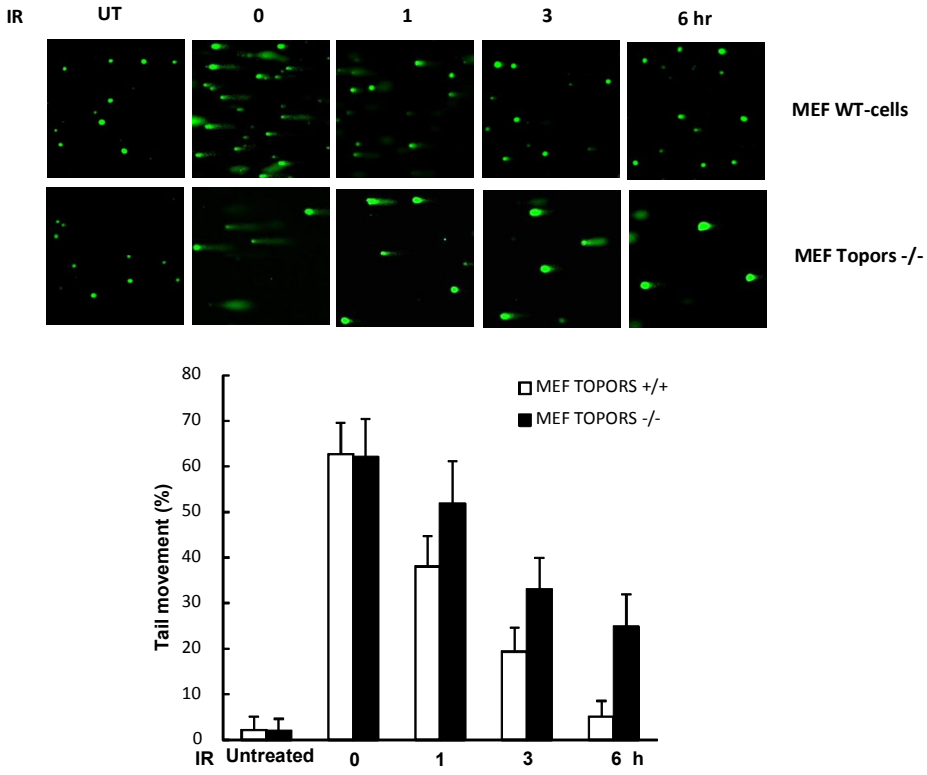

IR
UT
0
1
3
6 hr
MEF WT-cells
MEF Topors -/-
MEF TOPORS +/+
MEF TOPORS -/-
Tail movement (%)
80
70
60
50
40
30
20
10
0
IR
Untreated
0
1
3
6 h

Figure 16. Topors knockdown results in decreased DSB repair.

A. Control and Topors- depleted U2OS cells were untreated or treated with 5Gy ionizing radiation. At the indicated time points, cells were harvested for comet tail formation assay under alkaline conditions. Comet images were captured using fluorescence microscopy, and tail movement was analyzed in 75-80 randomly chosen comets using Komet 5.5 analysis software. **B.** Topors +/+ and Topors -/- MEF cells were untreated or treated with 10Gy ionizing radiation. At the indicated time points, cells were harvested for comet tail formation assay under alkaline conditions. Comet images were captured using fluorescence microscopy, and tail movement was analyzed in 75-80 randomly chosen comets using Komet 5.5 analysis software. Representative comet images observed in U2OS stable and Topors knockout MEF cells at different time points after IR treatment are shown. Changes in relative percentage of comet tail moments between control and Topors knockdown U2OS /MEF cells after treatment of ionizing radiation. Histograms represent the average of three independent experiments. Error bars represent the mean±SD. Asterisk denotes $P<0.01$.

IV. Discussion

Here, we report that Topors is a novel RAD51-interacting protein identified by yeast two-hybrid screening. We showed Topors interacts with RAD51 in endogenous and exogenous co-immunoprecipitation assay and binding was increased after IR induced DNA damage. Different Topors constructs co-immunoprecipitation assay showed us binding site of RAD51 is C-terminal region 854-917 amino acid of Topors.RAD51 and Topors forms a discrete IR induced nuclear foci and colocalize at DSB site. We report that Topors knockdown cells shows depletion of RAD51 foci but no change in γH2AX or 53BP1 foci after γ-irradiation. Moreover, Topors deficient cells also showed impaired Homologous recombination repair activity after DSB. Topors depleted cells shown hypersensitive to IR induced less colony formation which is proved by a clonal survival assay. We showed depletion of endogenous Topors accumulates increased amount of unrelieved DSBs, as observed by formation of delayed γH2AX foci and Comet assay. These observations confirmed us Topors is novel interactor of RAD51 and having critical role in homologous recombination.

1. Topors plays critical role in HR Repair by interacting with RAD51 in the site of Double strand break

It was previously reported, Topors interacts with the adeno-associated virus type 2 (AAV-2) Rep78/68 proteins and involved in transcriptional regulation (Weger et al., 2002) and also have interaction with the tumor suppressor protein P53 (Zhou et al., 1999).Topors also having interaction with N-terminus of topoisomerase I (Haluska et al., 1999). Recent reports showed us Topors interacts with a well-known DNA DSB marker protein γH2AX act as E3ligase for its ubiquitination (Seong Ki moon et al., 2012). In past one decade many researchers investigated the Topors involvement in various transcriptional regulation, tumor suppressor and E3 ligase activity in different species. Here we have identified Topors as a novel interacting partner of RAD51 and involved in functional regulation of HR. We performed yeast two-hybrid screening for preliminary confirmation of Topors interaction with RAD51 (Figure 4). Our further investigation was found that Topors, RAD51 interaction increased after IR induced DNA damage via endo and exogenous co-immunoprecipitation assay (Figure 5A and 5B).This observation was insist us to found C-terminal region 854-917 amino acid of Topors is RAD51 binding domain/site (Figure 6). Interestingly previously reported that, C-terminal region of Topors is required for punctate nuclear localization (Rasheed et al., 2002)

and C-terminal sequence of Topors shown interaction with ubc9 and Sumo-1 by yeast two-hybrid screening (Weger et al., 2003). So our further investigations are targeting and experimenting towards Sumoylation of RAD51 by Topors and how Topors is mediating or involved in that mechanism of regulation. Furthermore, our endo and exogenous immunofluorescence assay found Topors was colocalizing with RAD51 after IR induced DNA damage (Figure 7).

2. Knockdown of Topors down regulates RAD51 foci formation and Homologous Recombination Repair activity

Topors interaction and colocalization with RAD51 data from our studies insisted us to find further functional role of Topors in knockdown conditions. It has been proved that, knockout of Topors in mouse increased rate of malignancy and genetic instability (Marshall et al., 2010). Topors functioning as a tumor suppressor in different malignancies as well reports have proven Topors suppression role in non-small cell lung cancer (Oyanagi et al., 2004, saleem et al., 2004 and Lin et al., 2005). Murine Topors has shown high similarity with human Topors (Lin et al., 2005). Recent years many researchers have found the various roles of many E3 ligases on DNA damage responses.

Interestingly, mammalian E3 ligases PIAS1 and PIAS4 recruited at DSB site and involved in DNA damage dependent Sumoylation, PIAS4 along with SUMO-1 mediated the accumulation of RAD51 at DSB site (Galanty et al.,

2009).Furthermore, at the time of our thesis preparation data was published that PIAS1 mainly involved in RAD51 foci formation and recruiting at DSB site (shima et al., 2013). Human Hela cancer cells shown defect in mitotic chromosome segregation whereas PIASγ gene was knockdown (Martinez et al., 2006).PIAS1 and PIAS4 knockout mouse shown significant effect on embryonic lethality and an inability to drive viable cells (Tahk et al., 2007).Taken together, E3 ligase PIAS 1 depletion shown various effect on DSB response and RAD51 foci regulation and homologous recombination repair comparatively our E3 ligase Topors stably knockdown U2OS or Topors knockout MEF cells shown similar effect on impaired IR/MMC/HU induced RAD51foci formation and decreased homologous repair activity (Figure 9, 10, 11 and Figure 14).

On the other hand, other E3 ligases knockdown showed various effects on impaired RAD51 foci and HR activity. RNF4 depleted cells inhibits accumulation of RAD51 on DSB sites (Galanty et al., 2012 Yin et al., 2012).RNF8 or RNF 168 were required for RAD51 assembly at DSB site in 53BP1 expressing cells and BRCA1 is regulating RNF8 independent RAD51 assembly (Nakada et al., 2012). NBS1 recruitment was decreased in RNF8 mutant cells which leads to impaired HR repair activity (Chi-sheng Lu et al., 2012).RAD51 foci formation is widely abundant in tumor cell lines compare to non-malignant control cell line (Raderschall et al., 2002).Impaired formation of

RAD51 foci has been reported in mammalian BRCA1 or BRCA2 defective cells and hamster or chicken cells defective in RAD51 paralogs XRCC2,XRCC3,RAD51B,RAD51C (chen et al.,1999, Takata et al.,2001, Yuan et al.,1999).

Furthermore apart from E3 ligases, RAD51 interacts with many other DNA repair proteins such as BRCA1, BRCA2, and MDC1 for its dynamic regulation (Yu et al., 2003, Liu et al., 2010, Thorslund et al., 2010, Holloman et al., 2011). MDC1 depleted cells showed decreased formation of RAD51 foci and HR repair activity but the RAD51 mechanism of regulation is unknown (Zhang et al., 2005). It was previously investigated; after DNA damage PML colocalized with RAD51 in SV40 moreover PML depletion impaired RAD51 foci and HR repair activity (Boichuk et al., 2011). RAD51 might have shown non-covalent interaction with SUMO1 and hydroxy urea induced RAD51 foci were decreased, HR repair activity also down regulated in SUMO mutant BLM cells (Ouyang et al., 2009). So it's understood from our Topors knockdown results down regulation of RAD51 will leads to the severe effect of entire DNA damage repair system and its dynamic regulations (Figure 17). Furthermore, Topors have been showed us the dual functional role in homologous recombination repair activity and recruitment of RAD51 foci at DSB site.

Currently our experiments target on RAD51 sumoylation by Topors. Because Topors E3 ligase activity might be require for sumoylating RAD51 in DSB site. It has been proved that RAD51 and Topors shown interacting SUMO1 (Shen et al., 1996 and Weger et al., 2005).PML might be having functional connection with this studies whereas PML shown interaction with RAD51, Topors and SUMO1 (Boichuk et al., 2011, Rasheed et al., 2002, Duprez et al., 1999).It has been hypothesized by us Polo like kinase1 might be regulating this whole function and finally degradating Topors. It has been proved that Polo like kinase 1 phosphorylates RAD51 and Topors (Yata et al., 2012 and Yang et al., 2009).

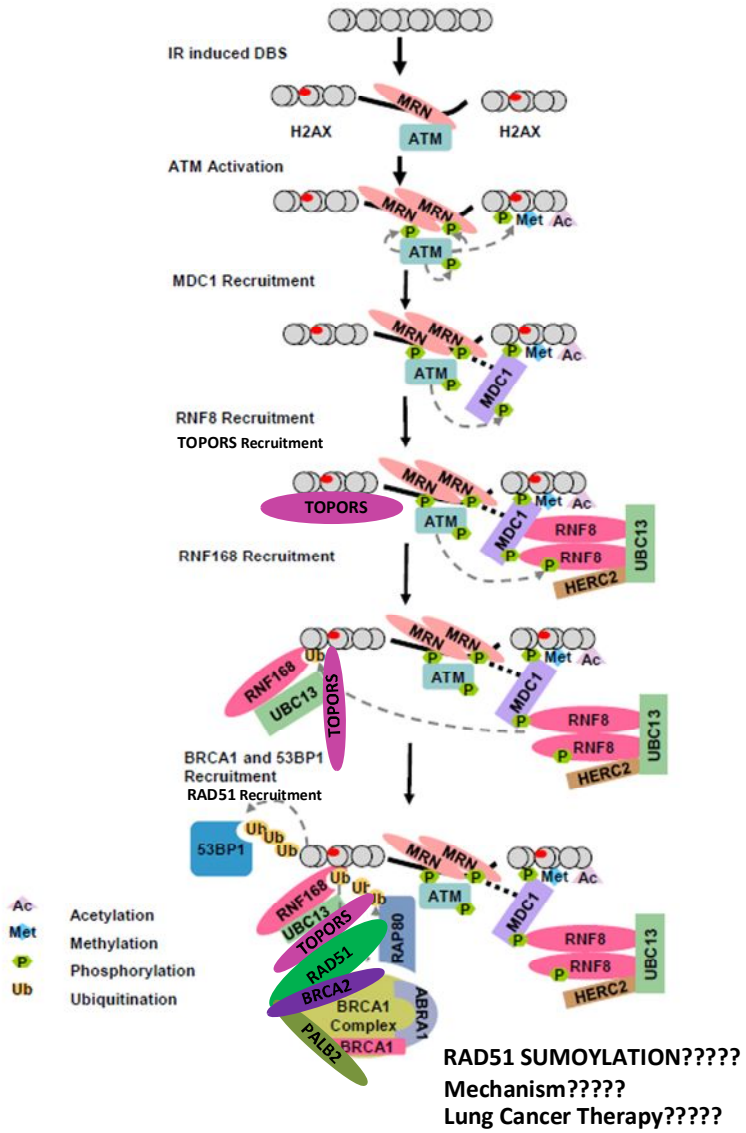


Figure 17. A model for TOPORS/RAD51 interactions after IR induced DSB response and other DNA repair proteins recruitment (Nur,Yucer et al., 2012).

3. Knockdown of Topors affects cell survival and delays repair activity, delays γH2ax foci

The present studies provide us direct evidence that Topors depleted cells shown hypersensitive to IR. The data obtained from clonal survival assay suggesting us defect in HR repair activity was linked to development of IR sensitivity. Topors knockdown U2OS stable cells formed less colony numbers compare to negative control cells (Figure 13).Loss of any DNA repair protein shows sensitivity to IR. It has been revealed down regulation of FIGNL1 by shRNAs resulted in sensitivity to camptothecin and IR (Yuan et al., 2013).Anchorage-independence prostate cancer cell line shown sensitivity to CPT and IR which associated with impaired homologous recombination repair (Wang et al., 2005). The SiRNA mediated silencing of E3 ubiquitin ligase RBX1 (RING BOX Protein 1) shown sensitivity to IR which was proved by clonal survival assay (Jia et al., 2010).

The results obtained from comet assay and delayed γH2AX foci formation assay results further supports clonal survival assay data and confirmed those Topors deficient cells hypersensitive to IR because endogenous Topors depletion accumulates increased amount of unrelieved DSB's. Comet assay was performed in Topors knockdown U2OS stable cells or Topors knockout MEF cells, showed Topors depleted cells tail length was longer than control U2OS cells or Topors +/+ MEF cells (Figure 16A and B). The damage was more in

Topors knockdown conditions which was clearly demonstrated us absence of Topors cells hypersensitive to IR. The similar observation was obtained from delayed γH2AX foci formation assay. Topors down regulation results in the persistent foci of DNA damage marker γH2AX (Figure 15). Interestingly, chromo domain helicase DNA-binding protein 4 depleted cells were showed long tail length compare to control cells (Smeenk et al., 2010).It was previously reported that E3 ubiquitin ligase Iduna shown long tail movement in shRNA Iduna cells (Kang et al.,2011). The SiRNA mediated silencing of E3 ubiquitin ligase RBX1 (RING BOX Protein 1) shown sensitivity to IR which was proved by comet assay (Jia et al., 2010). Loss of RING finger and WD repeat domain3 (RFWD3) showed delayed γH2AX foci formation due to hypersensitivity to IR and it's associated with impaired DNA damage repair (Liu et al., 2011).

In this study we concluded, Topors is a novel interacting partner of RAD51 and functionally involved in RAD51 foci formation at DSBs, HR repair activity in mammalian cells. The previous studies revealed that Topors act as tumor suppressor for non-small lung cancer (Oyanagi et al., 2004, and Lin et al., 2005).RAD51-dependent HR repair was studied/targeted in non-small-cell lung cancer cell line which was shown radiation sensitivity (Sak et al., 2005).Taken together further investigations may give a direction of developmental tools for lung cancer diagnosis and therapy.

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ABSTRACT

TOPORS interacts with RAD51 and mediate DNA double strand Break Repair by Homologous Recombination

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DNA double-strand breaks (DSBs) are highly toxic lesions induced by ionizing radiation (IR), free radicals, chemicals, or replication stress. Two major pathways, error-prone non-homologous end joining and error-free homologous recombination playing an important role to maintain genome stability. Homologous recombination maintains high fidelity DSB repair mechanism through homologous DNA as a template. RAD51 is known to be backbone of homologous recombination repair which is playing a major role in homologous pairing of DSB repair. Here, we report that Topors is a novel RAD51-interacting protein identified by yeast two-hybrid screening. We showed Topors interacts with RAD51 in endogenous and exogenous co-

immunoprecipitation assay and binding was increased after IR induced DNA damage.

Different Topors constructs co-immunoprecipitation assay showed us binding site of RAD51 is C-terminal region 854-917 amino acid of Topors.RAD51 and Topors forms a discrete IR induced nuclear foci and colocalize at DSB site.

We report that Topors knockdown cells shows depletion of RAD51 foci but no change in γH2AX or 53BP1 foci after γ-irradiation. Moreover, Topors deficient cells also showed impaired Homologous recombination repair activity after DSB. Topors depleted cells shown hypersensitive to IR induced less colony formation which is proved by a clonal survival assay. We show depletion of endogenous Topors accumulates increased amount of unrelieved DSBs, as observed by formation of delayed γH2AX foci and Comet assay.

Taken together, our results suggest that, TOPORS is a critical regulator of the Rad51-mediated homologous recombination repair and has a role in activation of Rad51 activity and maintenance of genetic stability.

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