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Understanding The Repair Mechanisms at Ionizing Radiation-induced Damage in The Human Genome

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Understanding The Repair Mechanisms at Ionizing Radiation-induced Damage in The Human Genome

by

Arijit Dutta, B.Sc., M.Sc.

Dissertation

Presented to the Faculty of the Graduate School of

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DEDICATION

To my dear parents, sister and wife, who are the core of my mind, heart and soul.

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ॐ

ज्ञानशक्तिसमारूढः तत्त्वमालाविभूषितः ।

भक्तिमुक्तिप्रदाता च तस्मै श्रीगुरवे नमः ॥

"Salutation to that noble Guru (mentor), who is established in the power of knowledge, adorned with the garland of various principles and is the bestower of prosperity and liberation," (Manah Prabodha, Shiva Madhuri).

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Understanding The Repair Mechanisms at Ionizing Radiation-induced Damage in The Human Genome

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Arijit Dutta, Ph.D.

The University of Texas Medical Branch, 2016

Supervisor: Sankar Mitra

Abstract:

Ionizing radiation (IR) such as X-rays induce damage clusters in the genome that include DNA double-strand breaks (DSB) with unligatable dirty ends, along with more frequent oxidized bases and single-strand breaks (SSB). While nonhomologous end joining and homologous recombination are major DSB repair pathways which have been extensively characterized over the past decades, contribution of error-prone alternative end joining (Alt-EJ) at X-ray-induced DNA damage is poorly characterized and underestimated. Moreover, how repair of oxidative base lesions and DSB are coordinated at damage clusters is an important unanswered question. I used recircularization of linearized plasmid reporters to monitor repair of DSBs with 3′P-blocked termini, which mimic X-ray-induced strand breaks, both *in cell* and *in vitro,* with repair complexes and measured relative efficiency of NHEJ vs. Alt-EJ based on sequence analysis of the joint site. Although NHEJ was the predominant pathway for DSB repair, Alt-EJ was significantly enhanced in pre-irradiated cells. This stimulation was dependent on XRCC1 phosphorylation by casein kinase 2 (CK2) that enhanced the interaction of XRCC1 with the end resection enzymes Mre11 and CtIP. The XRCC1 immunocomplex isolated from U2OS cells had Alt-EJ activity *in vitro;* this activity was significantly higher in the immunocomplex from pre-irradiated cells. Our studies thus suggest that activation of Alt-EJ proficient repair complexes after irradiation in surviving cells could contribute to radioresistance and could be therapeutically targeted. In a separate study, we showed that there is a hierarchy in repair of DSBs by NHEJ followed by base excision repair of oxidized bases at IR-induced damage clusters, coordinated by scaffold attachment factor-A (SAF-A), that is crucial to maintain genomic integrity.

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Alt-EJ	Alternative end joining
AP	Apurinic/Apyrimidinic
APE1	Apurinic/apyrimidinic endonuclease 1
APLF	Aprataxin and PNKP like factor
APTX	Aprataxin
ATM	Ataxia telangiectasia mutated
ATR	Ataxia telangiectasia and Rad3 related
BER	Base excision repair
BLM	Bloom Syndrome protein
BRCT	BRCA1 C Terminus
CHK1/2	Checkpoint kinase 1/2
CK2	Casein kinase 2
Co-IP	Co-immunoprecipitation
CTD	C terminal domain
CtIP	Carboxy-terminal binding protein (CtBP)-interacting protein
DBD	DNA binding domain
DG	DNA glycosylase
DNA-PK	DNA dependent protein kinase
DSB	Double strand break

FEN1	Flap Structure-Specific Endonuclease 1
FHA	Forkhead-associated
Fpg	Formamidopyrimidine [fapy]-DNA glycosylase
GST	Glutathione S-transferase
hnRNP-U	heterogeneous nuclear ribonucleoprotein U
HR	Homologous recombination
HRP	Horseradish peroxidase
IP	Immunoprecipitate
IR	Ionizing radiation
LET	Linear energy transfer
LIG3	DNA ligase 3
LIG4	DNA ligase 4
MMEJ	Microhomology mediated end joining
MRN	Mre11-Rad50-Nbs1
NEIL1	Nei Endonuclease VIII-Like 1
NHEJ	Non-homologous End Joining
NTD	N terminal domain
OGG1	8-Oxoguanine glycosylase 1
PAR	Poly(ADP)-ribose
PARP	Poly(ADP)-ribose polymerase
PCNA	Proliferating cell nuclear antigen
PCR	Polymerase chain reaction

PLA	Proximity ligation assay
PNKP	Polynucleotide kinase phosphatase
PONDR	Predictor of Natural Disordered Regions
RBD	RNA biding domain
RBE	Relative biological effectiveness
RFC	Replication factor C
ROS	Reactive oxygen species
RPA	Replication protein A
SAF-A	Scaffold attachment factor A
SSA	Single strand annealing
SSB	Single strand break
SSBR	Single strand break repair
UDG	Uracil-DNA glycosylase
XRCC1	X-ray repair cross-complementing protein 1
XRCC4	X-ray repair cross-complementing protein 4

CHAPTER I: BACKGROUND AND SIGNIFICANCE

1.1. IONIZING RADIATION INDUCES CLUSTERED DNA DAMAGE IN THE GENOME

Radiation therapy which involve high energy photons (x-rays or γ-rays) and charged particles (protons, neutrons, carbon ions, etc.) is used in treating about 50% of all cancer patients, either alone or in combination with surgery, chemotherapy and/or immunotherapy [1, 2]. However, growing incidences of cancer resistance towards radiation warrants revisiting our current understanding of the mechanisms involved in repair of IR-induced DNA damages [3].

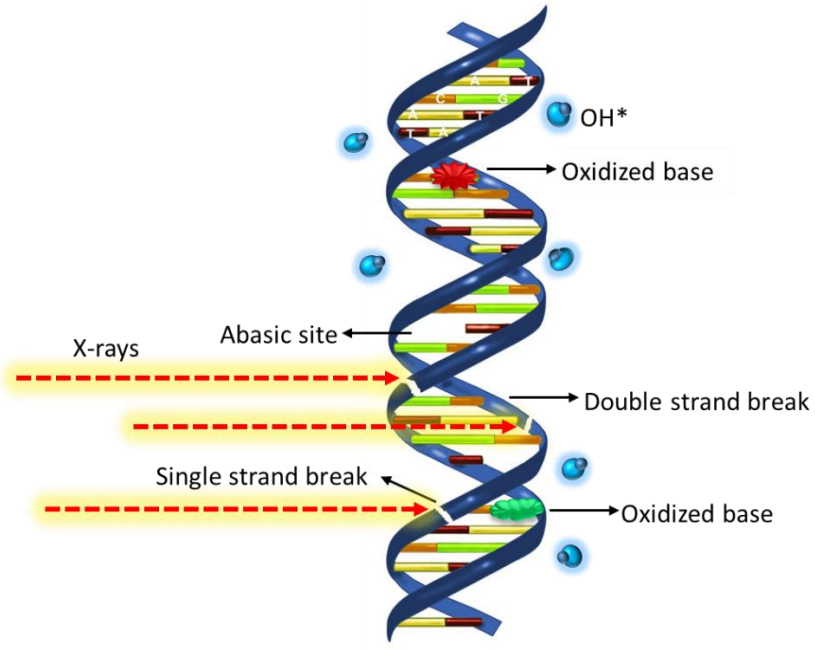


Figure 1. Ionizing radiation induces clustered DNA damage. Details are in the text. Image adapted from images.nigms.nih.gov.

Ionizing radiation (IR) liberates electrons from atoms or molecules of a substance, thereby reducing its chemical stability. Quality of ionizing radiation (IR) is evaluated in terms of linear energy transfer (LET), which is the amount of energy transferred from the radiation to a medium per unit length of the path travelled by the radiation through the medium [4]. The penetrating power of the radiation reduces with increase in LET. Charged

particles like α-particle, neutrons and carbon ions are high-LET radiation, while X-rays and γ-rays are low-LET radiation. IR kills cells by inducing DNA damage both by direct ionization of the DNA sugar-phosphate backbone or through generation of reactive hydroxyl radicals (OH•) via radio-lysis of water molecules, which induce oxidative base lesions, abasic (AP) site, single strand breaks (SSB) and double-strand breaks (DSB) (Figure 1). DSBs are the most lethal form of damage in the genome. The relative biological effectiveness (RBE) of any radiation depends upon its LET, which has been found to reach peak at 100 keV/μM and decreases beyond that value, since this density of ionization is optimum to ionize both strand at a time generating a prompt DSBs (Figure 1) [5]. While LET lower than 100 keV/μM is insufficient of producing a DSB by a single track of radiation, for those with much higher LET, the energy is wasted as the ionizing events are too close [5]. Thus, α-particle and neutrons have higher RBE than X-rays [6].

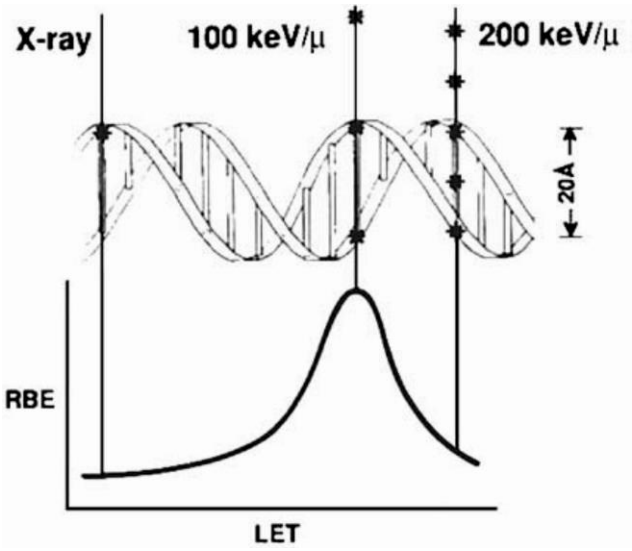


Figure 2. Schematic diagram showing ionizing radiation with LET of at 100 keV/μM has greatest RBE. This is because at this density of ionization the average separation between ionizing events is equal to the diameter of the DNA double helix (~20 Å), thus has highest probability of generating DSBs with single track of absorbed dose of radiation. Reprinted by permission from Macmillan Publishers Ltd: Oncogene [5], copyright 2003.

Radiation	Energy	Relative LET value (keV/µm)
X-Rays	250 kV	3
	3 MV	0.3
Cobalt 60	1.17–133 MV	0.3
Beta	10 kV	2.3
	1 MV	0.25
Neutron	2.5 MV	20
	19 MV	7
Proton	2 MV	16
Alpha	5 MV	100

Table 1: LET values of different types of ionizing radiation. (adpated from [6])

Although, a typical therapeutic dose of 2 Gy/fraction of sparsely ionizing radiation generates only 3000 DNA lesions per cell, compared to 50,000 lesions per cell produced daily by endogenous ROS, it shows significant tumor cell killing capacity [7]. This is due to spatial distribution of the lesions induced by the radiation track. Sutherland et. al. first reported that high energy γ-rays as well as X-rays induce clustered DNA damage which consist closely placed (10-20 bp) DSBs and 4-8 times more non-DSB lesions like oxidized bases, abasic sites and SSBs [8, 9]. Lomax et. al. reviewed that around 450 purine lesions, 850 pyrimidine lesions, 1000 SSB and 20-40 DSB/cell/Gy could be generated by low LET γ-rays (Table. 1) [7]. Common oxidative base lesions include 8-oxo-7, 8 dihydroguanine, thymine glycol, formamidopyrimidine, 5-formyluracil, 5-hydroxymethyluracil, and 5-methylcytosine [10]. Apart from prompt DSBs, secondary DSBs with long single strand DNA (ssDNA) overhangs could be generated at closely spaced bistranded SSBs or oxidative base lesions at the damage clusters [11]. The strand breaks mostly contain blocked termini such as phosphate, phosphoglycerate, and phosphoglycolaldehyde at the 3′ end, and hydroxyl and phosphodeoxyribose derivatives at 5′ end [12]. Clustered DNA damage accompanied with complex DSBs appears to have reduced reparability compared

to single lesions which contributes to effective killing of tumor cells [13]. Moreover, closely spaced base lesions exhibited generation of DSBs which were repaired with errors, and thus could confer genomic instability [14, 15]. Although repair of single lesions and DSBs via distinct repair pathways have been characterized well, repair mechanisms at the clustered DNA damage is poorly understood, whose characterization could lead to identification of new molecular targets for effective sensitization of resistant fraction of tumor cells.

Radiation-induced lesions in cellular DNA	Number/Gy/cell	Number/Gy/cell
	γ-radiation	$^{12}C^{6+}$ ions (31.5 keV/μm)
5,6-thymine glycol (Tg)	582	372
5-(hydroxymethyl)-2′-deoxyuridine	174	72
5-formyl-2′-deoxyuridine	132	66
FapyG	234	132
8-oxo-7,8-dihydro-2′-deoxyguanosine	120	60
Single-strand breaks	1000	

Table 1: Approximate quantity of major lesions generated at the genome by ionizing radiation (adapted from [7]).

1.2. PATHWAYS WHICH REPAIR IONIZING RADIATION INDUCED DNA DAMAGE

Oxidative base lesion, SSBs and DSBs induced by IR are repaired by base excision repair (BER), SSB repair (SSBR), and DSB repair pathways of homologous recombination (HR), and nonhomologous end joining (NHEJ), respectively. Removal of oxidative base lesions by BER leads to generation of SSBs which are repaired similar to prompt SSBs though shared repertoire of SSBR factors. HR repairs DSB utilizing the sister chromatid as template and hence is restricted to S/G2 phase of the cell cycle. HR is error-free but could take 7h or longer to complete [16]. NHEJ, on the other hand is the predominant and

fast DSB repair pathway throughout the cell cycle, where the DSBs could be repaired approximately within 30 mins, however it can induce insertions or deletions of few nucleotides depending upon the complexity of the broken termini [16]. More recently a highly error-prone DSB repair pathway called alternative end joining (Alt-EJ) has emerged, that requires stabilization of the DSB ends through annealing at short homology sequences [17]. Although several DNA repair factors have been implicated in Alt-EJ, there is poor understanding of its regulation and contribution towards repair of IR-induced DNA damage. This section gives a brief synopsis of the basic mechanisms and role of key proteins involved in each pathway.

1.2.1 BASE EXCISION REPAIR

All bases lesions are repaired via the evolutionarily conserved pathway of BER, first demonstrated by Tomas Lindahl, for which he was one among the 2015 Nobel laureates in Chemistry [18]. Mechanistically, BER is a four step process initiated by removal of the damaged base, followed by strand scission at the apurinic/apyrimidinic (AP) site, cleaning of the chemically blocked termini, gap filling with nucleotides and finally ligation of the nicked DNA strand. Damaged bases (oxidized and alkylated) are recognized and excised by a family of protein called DNA glycosylase (DG), which are of functionally two types – monofunctional DGs which have only base excision activity and bifunctional DGs which have both base excision and AP lyase activity to cut the strand at the AP site. Oxidative base lesions are mostly excised by bifunctional DGs like 8-oxoguanine glycosylase (OGG1) and NTH1 via β elimination reaction, or Nei endonuclease VIII-Like (NEIL) proteins (NEIL1-3) via β, δ-elimination. Strand scission with β-elimination leads to generation of 3' α,β-unsaturated aldehyde (PUA) and 5' phosphate (P) termini while β, δ-elimination causes 3' P and 5' P termini [12]. Next, blocked 3' termini are cleaned by end processing activities of AP endonuclease 1 (APE1) and polynucleotide kinase/ phosphatase

(PNKP) which remove 3' PUA and 3' P, respectively, only in mammalian cells, to generate DNA polymerase compatible 3' OH termini. This is followed by filling of the single nucleotide gap by DNA polymerase β (Polβ) and ligation of the nick by DNA ligase III-α (LIG3) which exist as a heteromeric complex with X-ray cross complementing protein 1 (XRCC1) in mammalian cells [19]. XRCC1 not only stabilizes LIG3 but interacts with several BER proteins through direct protein-protein interaction, thus promoting formation of distinct BER complexes through its scaffolding action (Figure 20) [20, 21]. This mode of BER involving repair of the damaged base via removal of a single nucleotide is called single nucleotide or short patch BER or (SN/SP-BER) (Figure 3) [22].

The second sub-pathway is long-patch BER (LP-BER), where a 5' blocking group such as 5' deoxyribose phosphate (dRP) which when fails to get cleaned by 5'dRP-lyase activity of Polβ, the 5' strand could be displaced by extension of the 3' strand by Polβ or replication DNA polymerase Polδ/ε upto 8 nts long, followed by removal of the 5'-flap by 5'-flap endonuclease 1 (FEN-1). Proliferating cell nuclear antigen (PCNA) loaded at the lesion by replication factor-C (RFC) recruits Polδ/ε [23, 24]. In LP-BER the nick is sealed by DNA ligase 1 (LIG1). How the choice between SN-BER and LP-BER is made is poorly understood, the latter has been suggested to be essential for repair of stalled replication forks [25] and could be regulated by local ATP concentration [26].

More recent studies from our lab and other groups have found association of BER with other physiological processes involving DNA transaction such as replication and transcription which are facilitated through unique protein-protein interactions. We found DNA glycosylase NEIL1 is co-opted at the replication machinery, which acts as a 'cow-catcher' to detect any damaged base ahead at the track of DNA replication [27]. NEIL1's interaction with replication proteins PCNA, RFC, Polδ and LIG1 is facilitated through its disordered C-terminal domain (CTD) that allows formation of replication associated 'BERosome' [28]. Moreover, NEIL1's recruitment at the chromatin is regulated by its acetylation at the CTD *(Sengupta, S., unpublished*); thus several studies including ours

showed that BER is regulated through post-translational modifications such as acetylation, phosphorytlation, ubiquitylation and SUMOylation [29]. NEIL2 which acts as a backup DG for NEIL1 has been found to be associated with transcription coupled BER [30]. Recently we reviewed role of scaffold factor XRCC1 and non-canonical proteins such as heterogeneous ribonucleoprotein-U (hnRNP-U), high mobility group B1 (HMGB1), and YB-1 in BER which could facilitate protein-protein interactions through extended intrinsically disordered regions in the proteins [21].

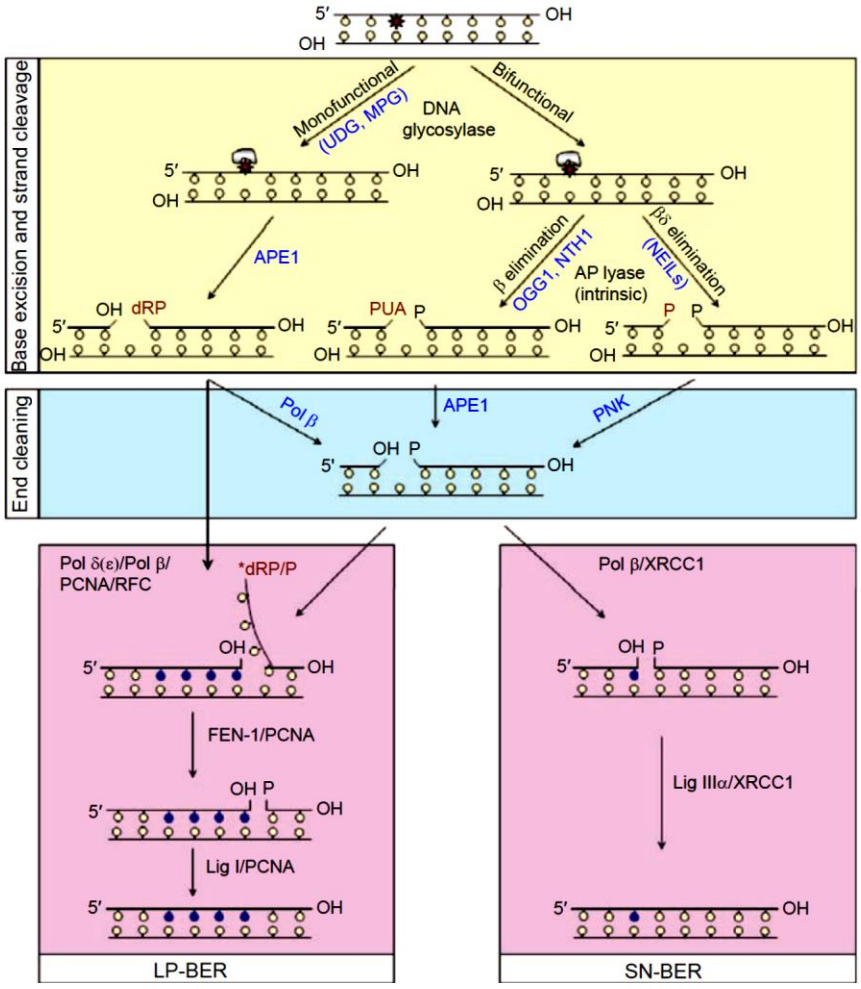


Figure 3. Base excision repair pathways – long patch repair and single nucleotide repair (details in the text). Reprinted by permission from Macmillan Publishers Ltd: Cell Research [12], copyright 2008.

1.2.2. SINGLE STRAND BREAK REPAIR

SSBs are nicks or one nucleotide gaps in one of the strand of DNA duplex that are often accompanied with blocks at 3' and 5' termini. SSBs can be generated during strand scission step of BER or directly by oxidative cleavage of the sugar backbone (Figure 4). DNA topoisomerase 1 (TOP1) also create transient SSBs to relieve topological stress during transcription and DNA replication, followed by religation; however an error in this process due to collision with RNA or DNA polymerases or interaction with a closely spaced DNA lesion could lead to generation TOP1 covalently-linked SSBs/DSBs [20]. Unrepaired SSBS can lead to generation of DSBs in dividing cells due to encountering a replication fork, which are mostly corrected by homologous recombination. In non-dividing cells like post-mitotic neurons accumulated SSBs can lead to cell death by affecting transcription or over-activation of SSB sensor poly(ADP-ribose) polymerase 1 (PARP1) and depletion of cellular NAD+ and ATP [31].

SSBs are detected by PARP1 which catalyzes formation of branched chains of poly(ADP-ribose) or PAR that leads to recruitment of XRCC1/LIG3. PARP1 auto-PARylates itself, XRCC1 and several chromatin associated factors [32]. PARylation of XRCC1 prevents its polyubiquitylation to increase its retention until the SSB is repaired [33], while PARylation of histones results in chromatin decondensation that facilitates repair and other DNA transactions like transcription [34]. PAR chains are degraded by PAR glycohydrolase (PARG) which releases PARP1 from the breaks [35]. Steps in SSB repair is identical to those in late BER, where XRCC1 has a major role in recruiting several downstream proteins like PNKP, aprataxin (APTX), Polβ and LIG1/LIG3 for end cleaning, gap filling and ligation, either through short patch or long patch repair. While blocked termini generated by oxidative damage such as 3′ PUA and 3′ P are processed by APE1 and PNKP, TOP1-SSBs generated due to abortive TOP1 activity are processed by tyrosyl-DNA phosphodiesterase 1 (TDP1) resulting in 5′ AMP-SSBs which are processed by

APTX [36]. XRCC1's phosphorylation by CK2 has been shown crucial for SSBR through stabilization XRCC1/LIG3 complex and facilitating multiple interactions with PNKP and APTX [37-39].

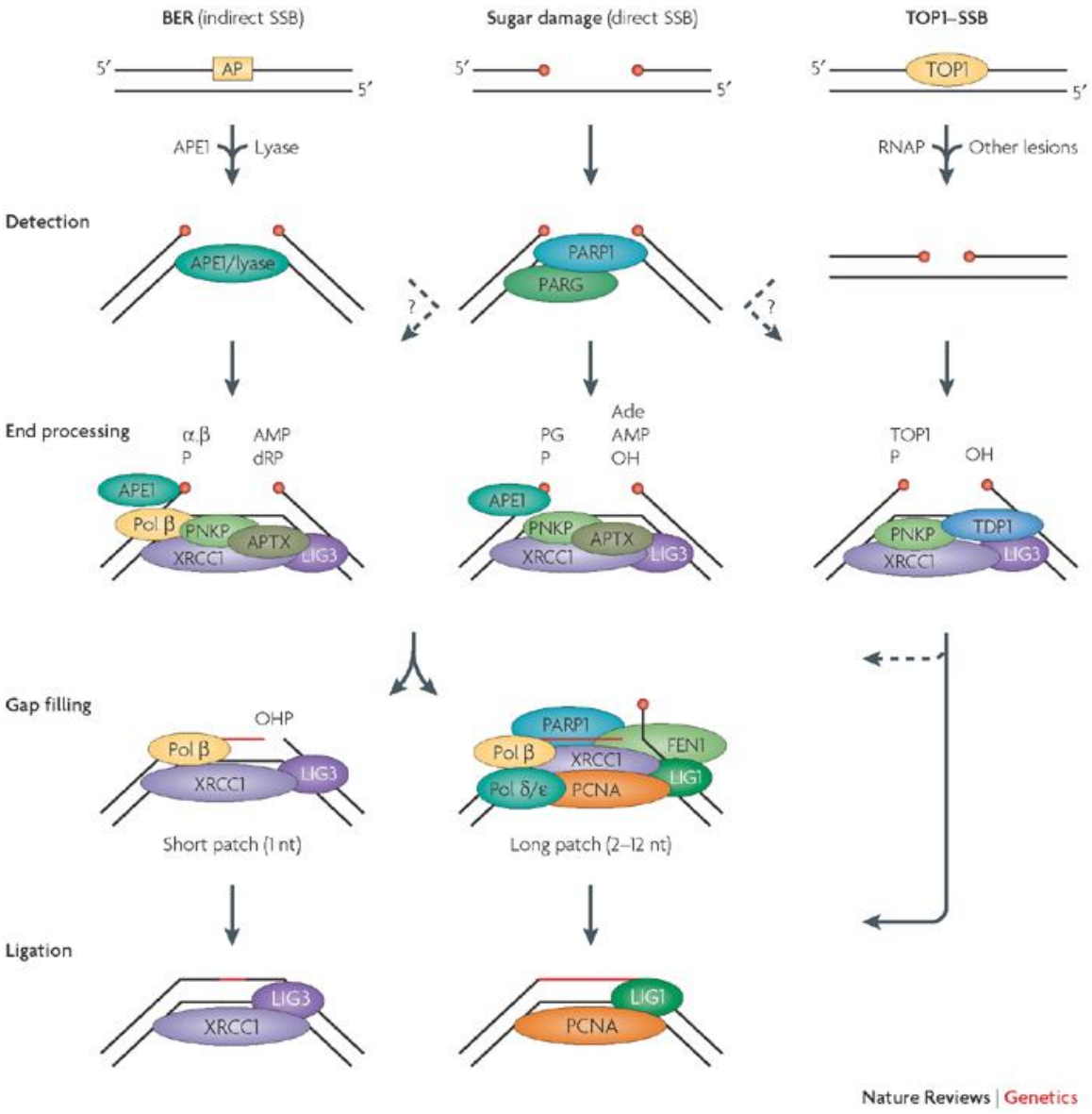


Figure 4. Modes of single strand break repair (details in the text). Reprinted by permission from Macmillan Publishers Ltd: Nature Reviews Genetics [20], copyright 2008.

1.2.3. NON-HOMOLOGOUS END JOINING REPAIR

DSBs are most lethal DNA lesions which if not repaired could lead to cell death by mitotic catastrophe, apoptosis or senescence. NHEJ is the predominant mode of DSB repair which unlike homologous recombination (HR) is not dependent upon the cell cycle phase. NHEJ directly joins the DSB ends without requirement of a homologous template. The key steps of NHEJ are detection of the DSB and bridging the broken ends by protein complexes to provide stability, making the ends compatible for ligation through end processing enzymes that could introduce minor deletions, gap filling by DNA polymerases, and finally end-joining followed by disengagement of the NHEJ complex (Figure 5) [40]. Activation of NHEJ is also accompanied with a cascade of DNA damage response signaling through Ataxia telangiectasia mutated (ATM)/ ATM and Rad3 related (ATR) kinases [41].

The DSB ends are recognized by Ku heterodimer Ku70/Ku80, followed by recruitment of DNA-dependent protein kinase catalytic subunit (DNA-PKcs) to form the DNA-PK holoenzyme that holds the two ends of the broken DNA as a synaptic complex [42]. DNA-PK phosphorylates itself and several downstream proteins of NHEJ complex and histone H2A.X [43]. Many studies have shown that Ku80 but not other early DSB biding proteins such as Mre11-Rad50-Nbs1 (MRN) or structural maintenance of chromosomes protein 1 (SMC1) is absolutely required for providing positional stability to the DSB ends [44]. Depletion of Ku results in severe chromosomal instability in irradiated S phase cells, suggesting NHEJ's preference for DSB repair over HR [45]. Ku also recruits XRCC4-DNA ligase IV (LIG4) and XRCC4 like factor (XLF) by direct protein-protein interactions [46, 47]. Recent studies have shown that XRCC4-XLF complex forms filament like structure that complement Ku70/Ku80-DNA-PKcs in bridging DNA ends [48, 49].

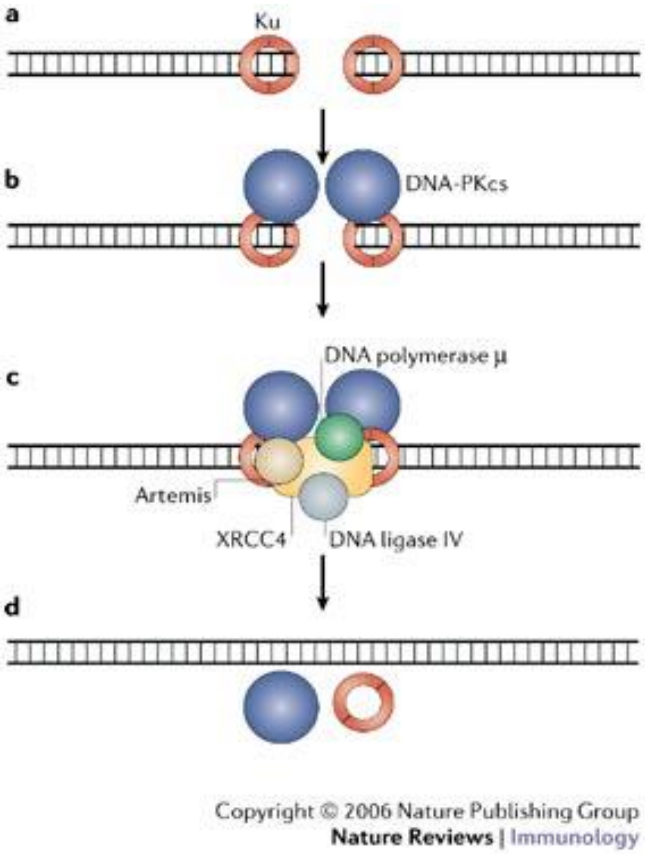


Figure 5. Steps in nonhomologous end joining (details in the text). Reprinted by permission from Macmillan Publishers Ltd: Nature Reviews Immunology [50], copyright 2006.

Several end processing and end resection enzymes such as PNKP, aprataxin, aprataxin and PNKP like factor (APLF), artemis, Werner (WRN), could engage at the NHEJ complex depending upon the complexity at the ends. PNKP phosphorylates 5′ OH and removes 3′ P [51], while aprataxin removes adenylate groups from 5′ P termini [52]. Ku also has been shown to have 5′-dRP/AP lyase activity [53]. Artemis is phosphorylated by DNA-PKcs and has several end processing functions at the DSB ends which are 5′ endonuclease activity at 5′ overhang to generate a blunt end, 5′ to 3′ exonuclease activity on ssDNA and removal of 3′-phosphoglycolate (PG) blocks from DSB ends [54]. WRN, a RECQ-like helicase and APLF have 3′ to 5′ exonuclease activity [55, 56]. Apart from end cleaning, gap filling could also be required at DSB ends, which may be carried out by family-X DNA polymerases Polμ, Polλ, and terminal deoxynucleotidyl transferase (TdT).

While Polμ carries out template-dependent synthesis, Polλ and TdT add nucleotides at the DSB termini in a template-independent manner; however TdT expresses only in B and T lymphocytes and their precursor cells [57].

The final step of NHEJ is ligation of the DSB ends by LIG4. XRCC4 stabilizes LIG4 and stimulates its activity through BRCA1 C Terminus (BRCT) domain mediated interaction [58]. XLF and APLF also stimulates LIG4 activity [59, 60]. Phosphorylation of DNA-PK at Ser2056 and Thr2609 is required for its dissociation from DSBs and proper completion of NHEJ; while DNA-PK autophosphorylates itself at Ser2056 and Thr2609, ATM and ATR phosphorylates at Thr2069 [61-63]. E3 ubiquitin ligase, RING finger protein 8 (RNF8) has also been shown to promote release of Ku from DNA ends [64].

1.2.4. HOMOLOGOUS RECOMBINATION

HR is the key DSB repair pathway during late S phase to late G2 phase in proliferating mammalian cells and particularly in cancer cells, that precisely repairs the damaged DNA utilizing the sister chromatid as the repair template. It is carried out in a well-orchestrated manner that preserve the genomic stability, and which when mutated lead to developmental abnormalities and tumorigenesis. HR involves DNA strand exchange which is invasion of the broken DSB termini at the homologous sequence of the intact sister chromatid (Figure 6). This requires extended 3′ DNA overhangs which is generated by resection of the 5′ termini by a two-step process. Several studies in both yeast and mammalian system have suggested that the initial resection is carried out by Mre11/Rad50/Nbs1 (MRN) which has both endonuclease and 3′→5′ exonuclease activity and carboxy-terminal binding protein (CtBP)-interacting protein (CtIP), which stimulates Mre11 and possesses independent 5′ flap endonuclease activity to remove DNA adducts at DSBs [65-67]. Based on *in vitro* experiments it has been suggested that EXO1 or DNA2, stimulated by Bloom's syndrome protein (BLM), extends Mre11 resected ends to generate

long 3′ overhangs which are stabilized by ssDNA-binding protein replication protein A (RPA) [68, 69]. This is followed by formation of Rad51 nucleofilament through replacement of RPA by RAD51 at the ssDNA that requires RAD51 loader BRCA2 and Rad51 paralogues, RAD51A, RAD51B, RAD51D, XRCC2 and XRCC3 [70]. The Rad51 nucleofilament ensures strand invasion and formation of heteroduplex D-loop [71], where templated extension of the broken DNA strand is carried out preferentially by Polδ, or other DNA tranlesion polymerases REV1 and Polζ [72, 73]. At this point the newly synthesized strand can displace and anneal with the other end of the DSB resulting in a non-crossover DSB repair, a process known as synthesis-dependent strand annealing (SDSA), which is preferred during mitosis [74].

Alternatively, the other DSB termini could be captured to form the double-Holliday junction which could be resolved to generate non-crossover via SDSA mechanism or cross-over products via double-strand break repair (DSBR) model during meiosis [75]. In certain situations, the D-loop can also be undergo another fate called break-induced replication (BIR), where the homologous duplex DNA undergo replication till the chromosome end, that could lead extended loss of heterozygosity [76]. The detailed molecular mechanisms of different subpathways of HR, which include role of cell cycle factors, are beyond the scope of my current studies.

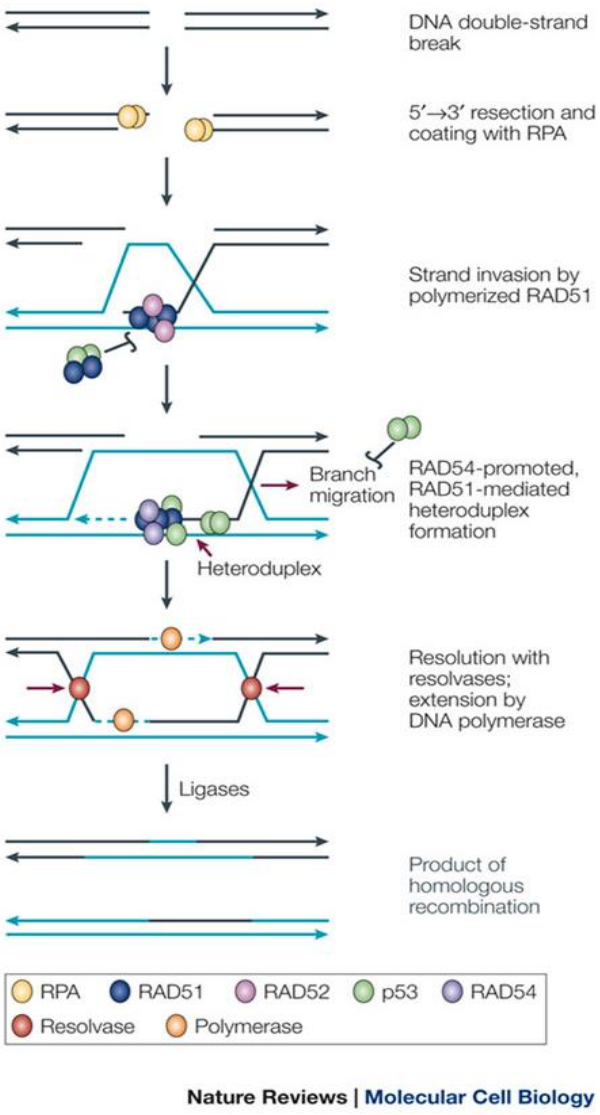


Figure 6. Steps in homologous recombination (details in the text). Reprinted by permission from Macmillan Publishers Ltd: Nature Reviews Molecular Cell Biology [77], copyright 2005.

1.2.5. Alternative End Joining (Alt-EJ), An Error-prone DNA Double Strand Break Repair Pathway Mechanisms of Alt-EJ

While HR and NHEJ are most characterized and major DSB repair pathways, a residual error-prone repair process was reported in yeast that could ligate DSBs in absence of NHEJ factors Ku and LIG4 but strongly required short homologous sequences at the

junctions [78, 79]. Subsequent studies have confirmed existence of this NHEJ-independent DSB repair pathway in Arabidopsis [80], Caenorhabditis elegans [81], Xenopus [82], rodents[83] and human cells [84, 85]. This pathway has been referred as alternative end joining (Alt-EJ), microhomology mediated end joining (MMEJ), or back-up NHEJ (B-NHEJ) [17, 86, 87]; in our studies we prefer to use the more generic term Alt-EJ. It is mechanistically distinct from single strand annealing (SSA), which like HR requires formation of Rad52 nucleofilaments and invasion of the broken DNA termini at the other DSB end that has extended homologous sequence [88]. The general consensus on Alt-EJ is resection of the 5' termini to generate 3' overhangs which are stabilized through annealing at short homologous sequences (microhomology) of length 5-25 bp, followed by trimming of DNA flaps and finally end joining that relies on BER/SSBR DNA ligases, LIG3 or LIG1. Thus, one of the microhomology regions including the intervening sequences would be eliminated during Alt-EJ, leading to extended sequence loss (Figure 7). Investigations on Alt-EJ to date indicate its wide mechanistic diversity [86]. Various Alt-EJ sub-pathways differ in regard to requirement of microhomology, and its *de novo* synthesis, and template-dependent vs. independent synthesis [89-92].

Based on several studies, it is believed that PARP-1 is the early DSB sensor protein in Alt-EJ that can facilitate tethering of DNA ends as well as recruitment of other factors like XRCC1/LIG3, PNK and MRN at the DSB [17, 89, 93, 94]. The initial resection of 5' termini are carried out by MRN and CtIP, similar to that suggested for HR [95-97], while the DNA flaps after annealing through microhomology sequences could be cleaved by endonuclease activity of CtIP or FEN1 [17, 93, 96]. Gap-filling at the breaks is carried out either by BER/SSBR DNA polymerase, Polβ or Polλ [96], or by Polθ through a mechanistically distinct sub-pathway called synthesis-dependent MMEJ (SD-MMEJ) that could cause insertion of nucleotides at the joint site [90-92]. While requirement of XRCC1 for Alt-EJ has been debated [98, 99], the DSB ligation has been indisputably reported to be carried out by LIG1/LIG3 [99-101]. Although, several factors of Alt-EJ has been

reported so far, the precise mechanism(s) of this error-prone repair and the regulating parameters could vary depending upon the genomic landscape and available protein repertoire, which are still poorly understood. PARP1 competes with Ku at the damaged DNA ends, which could possibly regulate the choice between NHEJ and Alt-EJ [102]. Alt-EJ could also act as the last resort due to failure of NHEJ at certain fraction of complex DSBs due to improper end processing or presence of longer ssDNA overhangs which have weaker affinity for Ku compared to duplex DNA ends [14].

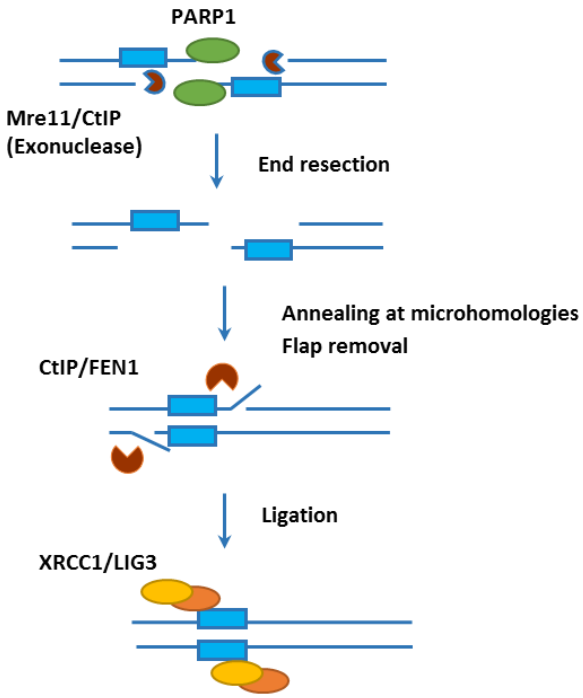


Figure 7. Steps in alternative end joining (details in the text). Modified from [86].

Several studies have implicated Alt-EJ to be a significant contributor towards chromosomal translocations and aberrations resulting in multiple types of leukemia and lymphoma [103-106]. Severe combined immunodeficiency syndrome (SCID), a condition where the immune system is severely affected, is often associated with deficiency in NHEJ factors along with the recombinase RAG [107, 108]. Alt-EJ has been reported to be responsible for frequent tumors in SCID mice with translocations involving IgG locus. Alt-

EJ has also been implicated in oncogenic complex translocations associated with chronic myeloid leukemia and acute myeloid leukemia [109, 110]. Moreover, Alt-EJ has been reported to be the major DSB repair pathway in bladder cancer cells and BRCA1 deficient breast and ovarian cancer cells, conferring high level of genomic instability [111-113].

1.6. SIGNIFICANCE OF THE STUDY

IR-induces complex DNA damage which are difficult to repair and leads to cell death mostly through apoptosis or mitotic catastrophe [114]. However, a certain fraction of cells could evade death, resulting in resistance as observed in cancer cells, enhancing tumor progression. Several mechanisms of cancer resistance have been proposed, one of the most important one being aberrant DSB repair to prevent cell death at the cost of enhancing genomic instability [3, 115]. HR and NHEJ are the major DSB repair pathways in mammalian cells which have been extensively characterized in past several decades, however cancer treatment through therapeutic targeting of DNA repair processes has still remained a major challenge. Although error-prone Alt-EJ was identified as a backup pathway with only minor contribution to DSB repair, only recently its implication in cancer cells has been appreciated which warrants in-depth investigation. Alt-EJ's promiscuous mechanisms for DSB repair makes it an advantageous route for survival of cancer cells. Several DSB repair assays has been developed till date to understand relative contribution of each repair process and factors involved, however, they cannot recapitulate repair of IR-induced DSB lesions with complex termini. Moreover, repair of non-DSB lesions at the clustered damage sites could generate additional DSBs, which has received little attention. The significance of our studies lies in the attempt to assess the contribution of error-prone repair processes for repair of IR-induced DSBs in human cells, which when targeted could enhance their radiosensitivity of surviving cells. We also developed DSB repair assays based on recircularization of linearized plasmid that mimics IR-induced DSB, which could

be utilized to monitor DSB repair mechanisms stimulated in irradiated human cells and understand contribution of various DSB repair factors by their individual depletion/inhibition with siRNA/ small molecule inhibitor. Supported by preliminary observations that early BER protein interact with NHEJ factors, we proposed that there could be cross-talk between the two discreet pathway to ensure genomic stability through hierarchical repair at the clustered DNA damage, which was also investigated in this study. Thus, this study delineates hitherto unknown mechanisms of repair at IR-induced damages in human cells, which could be utilized for developing therapeutic targets for effective sensitization of cancer cells.

1.7. INNOVATION

The innovation of this project lies in the unique hypothesis, the approach and development of a novel DSB repair assay that recapitulates repair of dirty DSBs. In this study we focused on the role of repair complexes that could be induced by dynamic cross-talk between proteins of distinct pathways due to generation of clustered DNA damage in the genome comprising both DSB and non-DSB lesions. This could reveal rather less appreciated contribution of BER/SSBR factors in DSB repair through error-prone repair process(es) of Alt-EJ. The *in cell* repair assay based on linearized plasmid substrate that mimics IR-induced DSB provides a unique proof-of-principle approach to study how dirty termini could impact DSB repair pathway choice. This unique strategy can be further modified to generate plasmid substrates mimicking a variety of clustered damage which would lead to deeper insight on mechanisms to repair such damages *in vivo*. Moreover, our experimental approach provides the opportunity to isolate distinct repair complexes from the human cells and recapitulate DSB repair *in vitro* for detailed biochemical characterization of the repair mechanisms. Furthermore, the hierarchy in repair via NHEJ and BER at clustered genome damage sites is a novel question that is investigated in this

study, as BER at closely spaced non-DSB lesions could generate secondary strand breaks leading to additional loss of sequence. In this context, role of a non-canonical repair protein SAF-A, that belongs to heterogenous nuclear ribonucleoprotein (hnRNP) has been elucidated, which reveals a novel evidences for the role of RNA-binding proteins in DNA repair. Thus, this dissertation research not only aimed to answer several gaps in the current knowledge in repair of IR-induced DNA damage but also to formulate new questions whose answers in the near future could provide in-depth insight into radio-resistance of cancer cells.

1.8. GOALS AND WORKING HYPOTHESIS

There were two major goals of this dissertation project, the first being characterization of the relative contribution of Alt-EJ vs. NHEJ at IR-induced DSBs, and the second was to understand how NHEJ and BER cross-talk at the clustered genome damage induced in the chromatin by IR. Based on previous studies, our preliminary data and expertise of the lab in BER/SSBR, I developed two working hypotheses to pursue these research goals. The first hypothesis was **Alt-EJ has a significant contribution towards repair of IR-induced DSBs due to formation of XRCC1-mediated repair complexes**. To test this hypothesis, I analyzed repair of a novel DSB substrate with blocked termini both *in cell* and *in vitro* with XRCC1-immunocomplex and characterized distinct post-translational modification of XRCC1 that could regulate essential protein-protein interactions required for formation of Alt-EJ proficient repair complexes (Chapter III and IV). My second hypothesis was that **hierarchical coordination at clustered DNA damage is regulated through phosphorylation of SAF-A that acts as a molecular switch between NHEJ and BER**. Preliminary *in vitro* data from our lab indicated that NHEJ affects BER through inhibition of DNA glycosylases such as NEIL1 by Ku. SAF-A, an RNA metabolism protein was found to be a common denominator in both the

pathways; it is phosphorylated by DNA-PK in the NHEJ complex, and it also interacts with NEIL1 to stimulate BER and relieve Ku's inhibition only when non-phosphorylated. In the present study, I analyzed how phosphorylation of SAF-A influences NEIL1 recruitment at the chromatin in irradiated cells to prevent generation of additional DSBs, thus, conferring radio-resistance (Chapter V).

CHAPTER II: METHODS AND REAGENTS

This chapter provides all the buffers, reagents and scientific protocol which has been employed to carry out all the experiments for this dissertation.

2.1. BUFFERS

1. Whole Cell Lysis Buffer:	1X Tris-buffer saline (TBS) solution (50 mM Tris-HCl pH 7.5, 150 mM NaCl), 1% Triton X and one tablet of Pierce™ protease inhibitor cocktail (Thermo Scientific) per 10 ml.
2. Cytoplamic Extraction Buffer:	10 mM Tris-HCl pH 7.9, 0.34 M Sucrose, 3 mM $CaCl_2$, 2mM $MgCl_2$, 0.1 mM EDTA, 1 mM DTT, 0.1% Nonidet P-40, and one tablet Pierce™ protease inhibitor cocktail per 10 ml.
3. Nuclear Extraction Buffer:	20 mM HEPES (4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid) pH 7.9, 3 mM EDTA, 10% Glycerol, 150 mM potassium acetate, 1.5 mM $MgCl_2$, 1.5 mM DTT, 0.5% Nonidet P-40, and one tablet Pierce™ protease inhibitor cocktail per 10 ml.
4. Chromatin Extraction Buffer:	150 mM HEPES pH 7.9, 1.5 mM $MgCl_2$, 10% Glycerol, 150 mM potassium acetate, and one tablet Pierce™ protease inhibitor cocktail per 10 ml.
5. FLAG Co-IP Wash Buffer:	1X TBS, 0.5% Triton X
6. 10X Annealing Buffer:	100 mM Tris-HCl, pH 7.5–8.0, 500 mM NaCl, 10 mM EDTA
7. 10X Plasmid Recircularization Assay Buffer	20mM $MgCl_2$, 600mM NaCl, 500mM HEPES, 20mM DTT, 10 mM ATP, 10mM dNTP, 500 µg/ml BSA
7. 10X CK2 Kinase Buffer:	250 mM MOPS, pH 7.5, 1.5 M NaCl, 50 mM $MgCl_2$, 50 mM $MnCl_2$, 2.5 mM DTT

Table 2: List of Buffers used.

All enzymatic reactions including restriction digestion, polymerase chain reactions and proximity ligation assay were carried out in their respective buffers which were supplied with the enzymes.

2.2. ANTIBODIES

2.2.1. PRIMARY ANTIBODIES:

Mouse monoclonal ANTI-FLAG® M2-Peroxidase (HRP) antibody (A8592, Sigma), mouse monoclonal ANTI-FLAG® M2 antibody (F1804, Sigma), rabbit polyclonal anti-DYKDDDDK tag antibody (#2368, Cell Signaling Technology), mouse monoclonal anti-6X His tag® antibody (ab18184, Abcam), mouse monoclonal anti-XRCC1 antibody (#MS-434-P0, Thermo Scientific), rabbit polyclonal anti-phospho-XRCC1 (S518/T519/T523) antibody (A300-059A, Bethyl Laboratories, Inc.), rabbit polyclonal anti-PARP-1 Antibody (H-300) (sc-25780, Santa Cruz Biotechnology), mouse monoclonal anti-PADPR antibody (ab14459, Abcam), mouse monoclonal anti-DNA Ligase 3 antibody (custom made), rabbit polyclonal anti-Mre11 antibody (#4895, Cell Signaling Technology), rabbit polyclonal anti-CtIP antibody (ab70163, abcam), mouse monoclonal anti-Nbs1 antibody (GTX70224, Genetex), rabbit monoclonal anti-phospho-Histone H2A.X (Ser139) (20E3) (#9718, Cell Signaling Technology), mouse monoclonal anti-phospho-Histone H2A.X (Ser139) antibody (#05-636, EMD Millipore), mouse monoclonal anti-Ku70 + Ku80 antibody (ab80828, Abcam), mouse monoclonal anti-hnRNP-U/SAF-A antibody (ab10297, Abcam), rabbit polyclonal anti-phospho S59 hnRNP-U/SAF-A (custom generated, gifted by Susan Lees-Miller lab [116]), rabbit polyclonal anti-NEIL1 antibody (custom made), rabbit polyclonal anti-H3 antibody (#2650, Cell Signaling Technology), mouse monoclonal anti-β-Actin (A5316, Sigma).

2.2.2. SECONDARY ANTIBODIES:

Amersham ECL rabbit IgG, HRP-linked whole antibody (NA934, GE Healthcare Life Sciences), Amersham ECL mouse IgG, HRP-linked whole antibody (NA931, GE Healthcare Life Sciences), anti-Mouse IgG (H+L) secondary antibody, Texas Red-X conjugate (#T-862, Thermo Scientific), anti-Rabbit IgG (H+L) secondary antibody, Alexa Fluor® 488 conjugate (#A-11008, Thermo Scientific).

2.3. siRNAs

siRNAs for XRCC1, CtIP, Nbs1 and DNA ligase 3 as found in the literature were synthesized from Sigma. XRCC1 3'UTR siRNA was custom-ordered from Dharmacon, GE LifeSciences. SAF-A/hnRNP-U 3'UTR siRNA was a custom siGENOME smartpool synthesis from Dharamcon, GE LifeSciences.

CtIP siRNA 1, [117]	
sense strand sequence:	GCUAAAACAGGAACGAAUC
antisense strand sequence:	GAUUCGUUCCUGUUUUAGC
CtIP siRNA 2, [117]	
sense strand sequence:	UCCACAACAUAAUCCUAAU
antisense strand sequence:	AUUAGGAUUAUGUUGUGGA
DNA Ligase3 siRNA, [118]	
sense strand sequence:	CCACAAAAAAAAUCGAGGAtt
antisense strand sequence:	UCCUCGAUUUUUUUUGUGGtg
Nbs1 siRNA, [119]	
sense strand sequence:	CCAACUAAAUUGCCAAGUAUU
antisense strand sequence:	AAUACUUGGCAAUUUAGUUGG
XRCC1 siRNA, [120]	
sense strand sequence:	GGAAGAUAUAGACAUUGAG[dT][dT]

antisense strand sequence:	CUCAAUGUCUAUAUCUUCC[dT][dT].
XRCC1 3'UTR siRNA, custom designed	
sense strand sequence	ACACACACACGAUGCAUUUUU
antisense strand sequence	AAAAAUGCAUCGUGUGUGUGU

Table 3: List of siRNAs used.

2.4. SMALL MOLECULE INHIBITORS

DNA-PK inhibitor, NU7741 (Tocris Biosciences, Bristol, UK), CK2 inhibitor, CX-4945 (Abcam, US), Mre11 exonuclease inhibitor, Mirin (Sigma-Aldrich, US), Mre11 endonuclease inhibitor, PFM03 (John Tainer Lab, MD Anderson Cancer Center, Houston, TX), PARP1 inhibitor, Rucaparib (Bhuvanesh Dave Lab, Houston Methodist Research Institute, Houston, TX).

2.5. PLASMIDS, SUB-CLONING AND SITE DIRECTED MUTAGENESIS

All oligonucleotide primers for subcloning were purchased from Sigma Aldrich custom DNA synthesis service.

XRCC1^{WT}-6XHis-pCD2E and CK2 non-phosphorylatable mutant XRCC1^{CKM}-6XHis-pCD2E were gifts from Keith Caldecott Lab at University of Sussex, UK.

XRCC1 cDNA sequence was PCR amplified from XRCC1-pCDNA4 using Platinum® Pfx DNA Polymerase (ThermoFisher Scientific) and subcloned in p3X-FLAG-CMV14 at XbaI and ClaI restriction endonuclease sites. p3X-FLAG-CMV14 was a gift from Kenichi Fujise Lab at UTMB, Galveston, TX.

Forward primer: 5'-CCCATCGATATGCCGGAGATCCGCCTCCG-3'

Reverse primer: 5'-CCGTCTAGAGGCTTGCGGCACCACCCCATA-3'

PCR amplification cycle: 4 min 95ºC, followed by 32 cycles of 30 sec at 95ºC, 45 sec at 55ºC and 10 min 65ºC, followed by final extension for 10 min at 68ºC, and cooling at 10ºC. Both the vector and the amplified XRCC1 cDNA sequence were digested with XbaI and ClaI, followed by overnight ligation with T4 DNA ligase (NEB). The ligation mix was used for transformation in *E coli* MAX Efficiency® DH5α™ competent cells (ThermoFisher Scientific) and colonies were checked by sequencing analysis.

Similarly, XRCC1^{CKM} was subcloned similarly from XRCC1^{CKM}-6XHis-pCD2E in p3X-FLAG-CMV14 vector using same set of primers and identical PCR amplification protocol.

XRCC1-S371A and XRCC1-S371D mutants were generated through site directed mutagenesis (SDM) of XRCC1-WT-p3XFLAG-CMV14 using QuikChange II Site-Directed Mutagenesis Kit (Agilent) according to manufacturer's protocol. The oligonucleotides for SDM were designed based on XRCC1 cDNA sequence (NCBI Reference Sequence: NM_006297.2); AGC to GCC for S371A and AGC to GAC for S371D mutation.

F-S371A: 5'-CACCCCCAAGTACGCCCAGGTCCTAGGCCT-3'
R-S371A: 5'-AGGCCTAGGACCTGGGCGTACTTGGGGGTG-3'

F-S371D: 5'-CACCCCCAAGTACGACCAGGTCCTAGGCCT-3'
R-S371D: 5'-AGGCCTAGGACCTGGTCGTACTTGGGGGTG-3'

pEGFPN1 was used as a backbone to generate linearized plasmid substrate, pNS for *in cell* and *in vitro* repair assays (discussed in Chapter III).

2.6. Cell Culture

U2OS cells, A549, and HEK293 cells were grown in Dulbecco's High Glucose Modified Eagles Medium (DMEM) with 4 mM L-Glutamine, without Sodium Pyruvate

(Hyclone, GE Healthcare Life Sciences), supplemented with 10% fetal bovine serum (Hyclone, GE Healthcare Life Sciences) and 1X Penicillin-Streptomycin Solution (Corning cellgro®) at 37ºC in presence of 5% CO_2 in a CO_2 incubator. Cells were washed with Dulbecco's phosphate buffered saline (DPBS, Hyclone, GE Healthcare Life Sciences). Cells were trypsinized using Trypsin-EDTA solution (Sigma-Aldrich). Stable NEIL1-HEK293 cells and stable XRCC1-HEK293 cells were grown in DMEM selection media containing zeocin (Invitrogen) and G418 sulfate solution (Corning cellgro®), respectively.

2.7. IRRADIATION WITH X-RAYS

The cells were irradiated with X-rays in the equipment RS2000 (Rad Source Technologies, Inc., Suwanee, GA). The source of X-rays is an X-ray tube filament that emits 4 pi field of photons at 160 kVp [121]. Tissue culture plates or chamber slides containing cells were placed in the 3rd shelf of the RAD+ chamber within circle 4 where the dose rate is 2.0 Gy/min and the uniformity of does across the field in horizontal plane is >95%. The time of exposure was calculated accordingly for total dose, for example, for 5 Gy dose, time of exposure was 2 mins 30 sec. After irradiation the plates or slides were immediately kept back in the CO_2 incubator or treated according to the experiments.

2.8. RECOMBINANT PROTEINS

Recombinant XRCC1, DNA Ligase 3α and XRCC1/DNA ligase 3α complex were purified by Pavana Dixit at Mitra Lab from cell pellets obtained from Miaw-Sheue Tsai's lab, Lawrence Berkeley National Laboratory, Berkeley, CA.

2.9. TOTAL PROTEIN EXTRACTION FROM CANCER CELLS

2.9.1. WHOLE CELL LYSIS:

Appropriate cells transiently of stably expressing FLAG-tagged protein were washed with Dulbecco's phosphate-buffered saline (DPBS) and harvested with a cell scraper. The cells were pelleted at 800 rpm, 4ºC for 5 min and lysed with whole cell lysis buffer (500 µl per 10 cm plate) and centrifuged at 14000 rpm, 4ºC for 15 min. The supernatant is whole cell lysate.

2.9.1. NUCLEI EXTRACTION:

Extraction of the nuclear fraction was achieved by fusing the following protocol. The cell pellets were resuspended in cytoplasmic extraction buffer (500 µl per 10 cm plate) and mixed by pipetting 10-15 times. The suspension was briefly vortexed and centrifuged at 3500 g. The nuclear pellet was collected and resuspended in whole cell lysis buffer (300 µl per 10 cm plate) centrifuged at 14000 rpm, 4ºC for 15 min. The supernatant is nuclear fraction.

2.9.2. CHROMATIN EXTRACTION:

Extraction of chromatin fraction was achieved by using the following protocol. The cell pellets were resuspended in cytoplasmic extraction buffer (500 µl per 10 cm plate), followed by mixing by pipetting 10-15 times, briefly vortexed and centrifuged at 3500 g. The nuclear pellet was collected and resuspended in nuclear extraction buffer (150 µl per 10cm plate), followed by pipetting 20-50 times, vortexed for 15 mins and centrifuged at 14000 rpm, 4ºC for 15 min. The chromatin pellet was finally resuspended in chromatin extraction buffer (100 µl per 10 cm plate) and pipetted ~80 times. The mix was incubated

with 0.15 U/µl Benzonase (Invitogen) at 37º for 30 min and then centrifuged at 14000 rpm, 4ºC for 15 min. The supernatant is chromatin extract.

2.10. SIZE EXCLUSION CHROMATOGARPHY

U2OS cells were grown in twenty 15 cm plates. 10 plates were irradiated with 5Gy X-ray and harvested along with the untreated cells. Total nuclear extract was prepared as discussed earlier and dialyzed at 4 ºC in a buffer containing 50 mM Tris-HCl pH 7.5, 150 mM NaCl and 10% glycerol. The dialyzed nuclear extract was passed through 0.2 micron filter and 2 ml (2 mg) was loaded in HiPrep 16/60 Sephacryl S-300 HR column through ÄKTA pure chromatography system (GE Healthcare Life Sciences). The fractions were collected in a fraction collector filled with ice and stored at -20 ºC for western blot analysis.

2.11. CO-IMMUNOPRECIPITATION (CO-IP)

Protein concentration of whole cell lysates from appropriate cells were measured through Bio-rad protein assay. ANTI-FLAG® M2 affinity gel beads (Sigma-Aldrich) were washed with cold 1X TBS buffer and mixed with whole cell lysate (10 µl per 1 mg of total protein). The volume was adjusted with 1X TBS to keep the final concentration of Triton-X 0.5%. The beads were incubated 3 hrs at cold room to carry out the co-IP. The beads were washed with FLAG co-IP wash buffer 3 times for 5 min each. The beads were eluted with 2X LDS dye and heated for 1 min at 95ºC.

2.12. PROTEIN TRANSFER AND WESTERN BLOTTING

Protein or co-IP samples were loaded along with Precision Plus Protein™ Dual Color Standards (Bio-rad) in NuPAGE Novex 4-12% Bis-Tris Protein Gels

(Invitrogen) or Criterion™ XT Bis-Tris gels (Bio-rad), and gel electrophoresis were carried out in 1X NuPAGE running buffer (Invitrogen) or MOPS-XT buffer (Bio-rad), respectively. Protein transfer from SDS-PAGE gels to nitrocellulose membrane (Invitrogen) were carried out in 1X NuPAGE transfer buffer (Invitrogen) or 1X Tris-Glycine transfer buffer (Invitrogen) respectively. After transfer, nitrocellulose membrane were appropriately cut according to protein molecular weight size and blocked with 5% skimmed milk (Fisher Scientific) solution in 1% Tris-Buffered Saline and Tween 20 (TBST) buffer (Invitrogen). This was followed by blotting with appropriate primary and secondary antibodies. Washing was done with 1% TBST.

2.13. IMMUNOFLUORESCENCE

U2OS/A549 cells were grown in 8 chamber slides and after appropriate treatment, they were fixed with 4% paraformaldehyde for 15 min, followed by permeabilization with 0.5% Triton-X solution in DPBS for 15 min. Blocking was performed with 3% BSA solution in DPBS with 0.2% Triton-X for 1 hour. The samples were incubated overnight with appropriate primary antibodies diluted in DPBS with 0.2% Triton-X. Thereafter the cells were washed thrice with same buffer and incubated with Texas-red or Alexa-fluor 488 conjugated secondary antibody for 1 hr. The cells were again washed thrice with DPBS with 0.2% Triton-X and now the chambers are opened and the slide is air dried. The slides were mounted with mounting media with DAPI (Duolink) and coverslip. The samples were observed under 60X oil-immersion lens in Nikon upright bright-field/fluorescent microscope and images were captured from five random fields for each sample. The images were merged and analyzed with ImageJ software.

2.14. PROXIMITY LIGATION ASSAY

Proximity ligation assay (PLA) is an immunochemistry based technique for detecting and quantitating *in situ* protein-protein interaction or co-localization (Figure 8). For each assay respective primary antibodies were used raised in different species. PLA was performed by using the Duolink kit (Olink Bioscience) following the manufacturer's protocol for buffers and reagents provided in the kit. U2OS/HEK293 cells grown in 8 chamber slides with appropriate treatment were fixed with 4% paraformaldehyde and permeabilized with 0.5% Triton-X solution in DPBS. The cells were blocked with blocking solution (Duolink) for 30 mins at 37ºC in a CO_2 incubator. Appropriate two primary antibodies for each experiment, were diluted in Antibody Diluent Solution (Duolink) and added to the wells and incubated overnight at 4ºC. Next day, the wells were washed 2 times with PLA Wash Buffer A (Duolink), 5 min each. The samples were incubated with Duolink plus and minus probes diluted appropriately in Antibody Diluent Solution and incubated for 1 hr at 37ºC in a CO_2 incubator. The samples were again washed with Wash Buffer A twice and Ligation was carried out by incubating the samples with 0.3 µl Ligase per well diluted in 30 µl 1X Ligation Stock (Duolink) for 30 mins at 37ºC in a CO_2 incubator. The samples were again washed and Amplification was carried out by addition 0.2 µl Polymerase diluted in 30 µl 1X Amplification Stock (Duolink) and incubating 2 hrs at 37ºC in a CO_2 incubator. Amplification step was carried in dark and the slides are protected from light thereafter. Finally the slides were washed with Wash Buffer B (Duolink) twice for 10 min and once with 0.01X Wash Buffer B for 1 min. The slides were air dried and mounted with mounting media with DAPI (Duolink) and coverslip. The slides were observed under 60X oil-immersion lens in Nikon upright bright-field/fluorescent microscope and images were captured from five random fields for each sample. The images were merged and analyzed with ImageJ software.

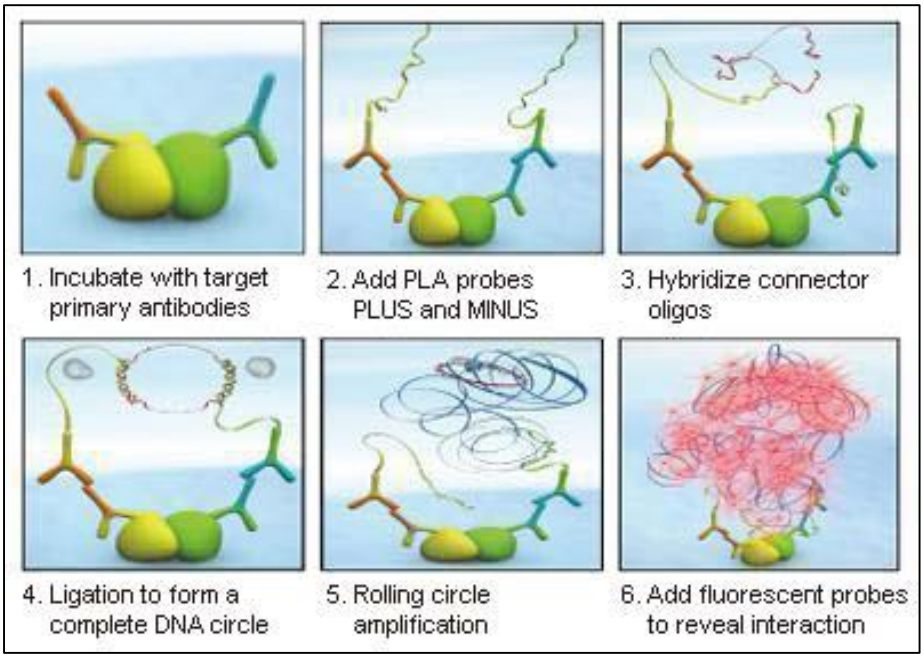


Figure 8. Cartoon representation of steps in proximity ligation assay, Duolink (O-link Biosciences). Fixed and permeabilized cells on glass slide are incubated with two antibodies against proteins of interest, raised is different species (rabbit and mice) (1). Next, the cells are incubated with Duolink PLA probe plus and minus which are anti-mice and anti-rabbit secondary antibodies which are conjugated with a pair of complementary oligonucleotides (2) that can anneal when in close proximity to form an open circular DNA (3). After thorough washing to remove nonspecifically bound probes, Duolink Ligase mix is added to ligate the duplex open circular DNA (4), followed by addition of Duolink Polymerase mix to amplify the DNA by rolling circle mechanism (5). The Polymerase mix contains fluorescent oligonucleotide probes which hybridize with the amplified contameric DNA generating fluorescent foci that can be observed microscopically (6).

2.15. *IN CELL* PLASMID RECIRCULARIZATION ASSAY

Control or appropriately treated U2OS/A549 cells were transfected with 100 ng repair substrate, pNS using Lipofectamine 2000 and incubated overnight. Next day, the cells were checked for GFP expression and plasmids were isolated using Qiagen plasmid miniprep kit [122]. 5 µl of plasmid extract was transfected in XL10-gold ultracompetent cells using manufacturer protocol and plated in 50 µg/ml Kanamycin containing LB-agar plates. The bacterial plates were sent to GENEWIZ, South Plainfield, NJ for sequencing of randomly chosen 40 colonies using CMV-F primer. The sequences were aligned using

'Multalin interface page - Inra'. Relative percentage of repaired joints by Alt-EJ or NHEJ was plotted and statistical analysis was performed by two-tailed Fisher's extact t test (Graphpad).

2.16. *IN VITRO* PLASMID RECIRCULARIZATION ASSAY

Exponentially growing U2OS cells transiently expressing XRCC1-FLAG were treated with inhibitors/ siRNAs as indicated and irradiated with a dose of 3 Gy X-rays. After 1 hour, the cells were harvested along with untreated cells for nuclear extraction. XRCC1-FLAG co-IP was performed by incubating 1.5 mg of nuclear extract with FLAG-M2 agarose beads for 2 h at 4°C. The beads were directly incubated 30 mins with 5ng pNS in a reaction buffer containing 2mM $MgCl_2$, 60mM NaCl, 50mM HEPES, 2mM DTT, 1 mM ATP, 1mM dNTP and 50 μg/ml BSA with mild shaking. This was followed by addition of 14 ng XRCC1-DNA ligase 3α recombinant protein complex in the reaction mix for incubation overnight at 16°C. The beads were spun down and 5 μl of the reaction mix was used to transform XL10-gold ultracompetent cells and sequence analysis of colonies were performed as discussed earlier. Number of colonies obtained from each experiment was plotted and statistical analysis was performed by two-tailed Fisher's extact t test (Graphpad). The beads were eluted with 4X LDS loading buffer and analyzed by western blotting.

2.17. γH2AX FOCI FORMATION ASSAY

U2OS cells with appropriate siRNA treatment were replated in 8-chamber slides and incubated overnight. Next day the cells were treated with appropriate inhibitors (NU7441 for 1hr, Rucaparib and CX4945 for 2 hr) prior to X-ray irradiation. After 1 hour incubation the cells were fixed with 4% paraformaldehyde for 15 minutes, followed by

permeabilization with 0.5% Triton-X solution in DPBS for 15 min. Blocking was performed with 3% BSA solution in DPBS for 1 hour. The samples were incubated with anti-phospho-serine H2A.X antibody diluted 1:500 in DPBS with 0.2% Triton-X for 2 hrs at room temperature. After washing three times with DPBS with 0.2% Triton-X, the cells were incubated with Texas-red conjugated secondary antibody (1:500). After the final washes the slides were dried for 5-10 mins in 37°C incubator and mounted with mounting media containing DAPI (Duolink) and coverslips. The samples were observed under 60X oil-immersion lens in Nikon upright bright-field/fluorescent and images were captured from five random fields for each sample. The images were merged and foci were counted through ImageJ software. The number of γH2AX foci per cell were plotted.

2.18. CLONOGENIC ASSAY

U2OS cells were transfected with 100nM control siRNA or XRCC1 siRNA and incubated 72 hrs. The cells were treated with 10 μM NU7441/ DMSO for 1hr. Thereafter the cells exposed to various dose of X-rays (0, 3, 6, 9 Gy). The cells were trypsinized and 300 cells from each sample were plated in quadruplicate in 6 well plates. The NU7441 pre-treated cells were replated in DMEM containing 10μM NU7441. After 10 days the plates were harvested and the colonies were stained with 0.5% crystal violet solution in 50/50 methanol/water for 15 mins. The plates were washed gently in water and air-dried for counting the colonies.

2.19. XRCC1 PHOSPHORYLATION BY CASEIN KINASE 2, *IN VITRO*

Phosphorylation of purified XRCC1 was carried out by incubating 4μg recombinant XRCC1 protein with 200 ng CK2α2, active, GST-tagged, human PRECISIO® Kinase recombinant protein (Sigma-Aldrich), in a kinase buffer containing 25 mM MOPS,

pH 7.5, 150 mM NaCl, 5 mM $MnCl_2$, 5 mM $MgCl_2$, 1 mM ATP, and 0.25 mM DTT, in 25 µl reaction volume at 37 ºC for 90 min. ATP was not added in the control reaction mix. Thereafter the GST tagged CK2α was removed from the reaction mix by incubating the reaction mix with 10 µl EZview™ Red Glutathione Affinity Gel (Sigma-Aldrich) in a total volume of 50 ul for 1 hr at 4 ºC, followed by removal of the CK2α2-GST/Glutathione beads by centrifuging at 13k rpm. XRCC1 phoshphorylation was confirmed by western blotting using phospho-XRCC1 (S518/T519/T523) antibody.

CHAPTER III: NOVEL REPORTER PLASMID RECIRCULARIZATION ASSAYS TO CHARACTERIZE ALTERNATIVE END JOINING VS. NON-HOMOLOGOUS END JOINING, *IN CELL* AND *IN VITRO*

3.1. INTRODUCTION

In mammals, DNA double strand breaks (DSB) are the most lethal damage in the genome which must be repaired in order to maintain genomic stability and prevent the cell death. NHEJ is the predominant repair pathway for DSB repair throughout the cell cycle while HR is carried out in S/G2 phase of the replicating cells [40]. Alt-EJ was initially found to act only as a back-up to NHEJ, while current studies are emphasizing its significant role in DSB repair similar to other pathways [123, 124]. The key components of NHEJ and HR were identified based on genetic and biochemical studies. Pfeiffer and Vielmetter first showed that linearized plasmids could be recircularized *in vitro* with Xenopus egg extracts [125], followed by North et. al. using human cell extracts [126]. Early studies of DSB repair involved *in vitro* end joining of duplex oligonucleotides or restriction enzyme cut plasmid DNA or those treated with radiomimetic drug like bleomycin, with human cell or xenopus egg extract [127, 128]. Methods for detection of the repaired products were southern blotting with radiolabeled oligonucleotide probes, ethidium bromide staining, autoradiography of radiolabeled substrate, or β-glycosidase dependent bacterial mutagenesis assays through transformation of the repaired plasmid in *E. coli*; other methods for detection of DSB repair such as asymmetric field inversion gel electrophoresis (AFIGE) and quantitative PCR assay were developed later [129-131]. Furthermore, DSB substrates with chemically incompatible termini such 5'hydroxyl, 3'phosphate and 3'phosphoglycolate were generated in order to mimic physiologically relevant complex DSBs induced by IR or radiomimetic drugs, which revealed importance of end processing enzymes like PNKP in DSB repair [132, 133]. Although several key

factors of DSB repair pathway were characterized via end joining assays with cell free extracts, it couldn't recapitulate repair of DSB generated in the chromatin. Introduction of DSB in the genome by a rare-cutting meganuclease I-Sce I not only allowed monitoring DSB repair *in cell*, but led to identification of several regulatory factors and comparison between relative contribution between NHEJ and HR at the chromatin [75, 134, 135]. More recently, I-Sce I based repair assays have been developed that could measure the mutagenic NHEJ and microhomology based end-joining processes [136, 137]. Although this tool gave a huge advantage of studying various DSB repair pathways at the chromatin, I-Sce I generated DSB doesn't represent the complexity of IR-induced damage. Due to the technical challenge of generation of blocked DSBs at the chromatin, we designed linearized plasmids with blocked termini whose *in cell* repair could reveal DSB repair pathway choice in irradiated human cells.

3.2. A Novel Linearized Plasmid Substrate With Blocked Termini, Mimicking X-ray-induced DNA Double Strand Break

In order to study how X-ray-induced DSBs are repaired in cancer cells, we designed a linearized plasmid substrate, pNS, that mimics IR-induced complex DSB, and could be transfected in cancer cell lines, and extracted after repair for sequence analysis at the site of repair. pNS has 2 nt long 5' overhangs and 3'phosphate blocked ends, both of which needs processing for plasmid recircularization (Figure 9). Moreover, to characterize NHEJ vs. Alt-EJ modes of repair, we introduced 5 nt long microhomology sequences flanking the DSB, which has been deemed as the optimum length of microhomology required for Alt-EJ in the literature [93, 138]. Alt-EJ would lead to deletion of one of the microhomology arms flanking the DSB (as explained in section 3.2.2. and illustrated in Figure 12C), while NHEJ should repair the DSB with minimal deletion or insertion. Thus,

we could evaluate relative contribution of NHEJ and Alt-EJ to repair X-ray-induced blocked DNA DSBs by our novel *in cell* repair assays.

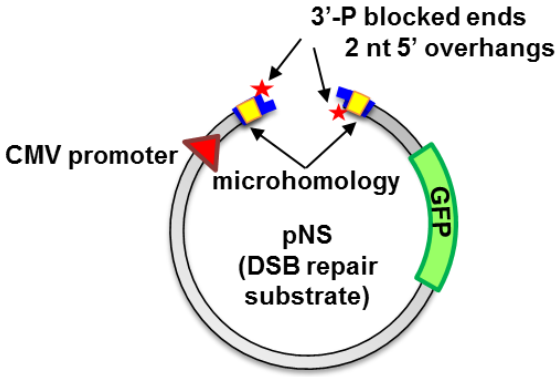


Figure 9. Cartoon of linearized reporter plasmid substrate, pNS, that mimics X-ray induced DSB.

3.2.1. Preparation of the Linearized Plasmid Substrate, pNS With 3' Phosphate Termini

A strand break with 3'-P blocked ends could be generated at a uracil (U) containing duplex oligonucleotide by treating with uracil DNA glycosylase (UDG) and fapyG DNA glycosylase (FpG). UDG excises the U at U:A mismatch to create an AP site followed by strand scission by APlyase activity of FpG that leaves 3'-P and 5'-P termini. In order to generate a DSB containing 3'-P termini via aforesaid technique, we aimed to introduce two bistranded U residues in a 2-nt staggered fashion in pEGFPN1 plasmid backbone. Direct ligation of U containing duplex oligonucleotide to pEGFPN1 backbone gave a very low yield of the final product which would not have been sufficient for performing the repair assays. To increase the yield of plasmid substrates we adopted a methodology where at a time each U containing oligonucleotide at the target site could be introduced with the complementary strand providing template for annealing, followed by nick sealing by T4 DNA ligase. Nicking endonucleases Nt.BbvCI and Nb.BbvCI (NEB) are a pair of

neoisoschizomers that identify the same heptanucleotide sequence, 5'-CCTCAGC-3' but create nick on complementary strands. At first a duplex oligonucleotide (41 nts) containing two CCTCAGC sequence flanking the target site were cloned between EcoR1 and Xho1 within pEGFPN1-MCS. This modified pEGFPN1 vector was named pNTNB (Figure 10A).

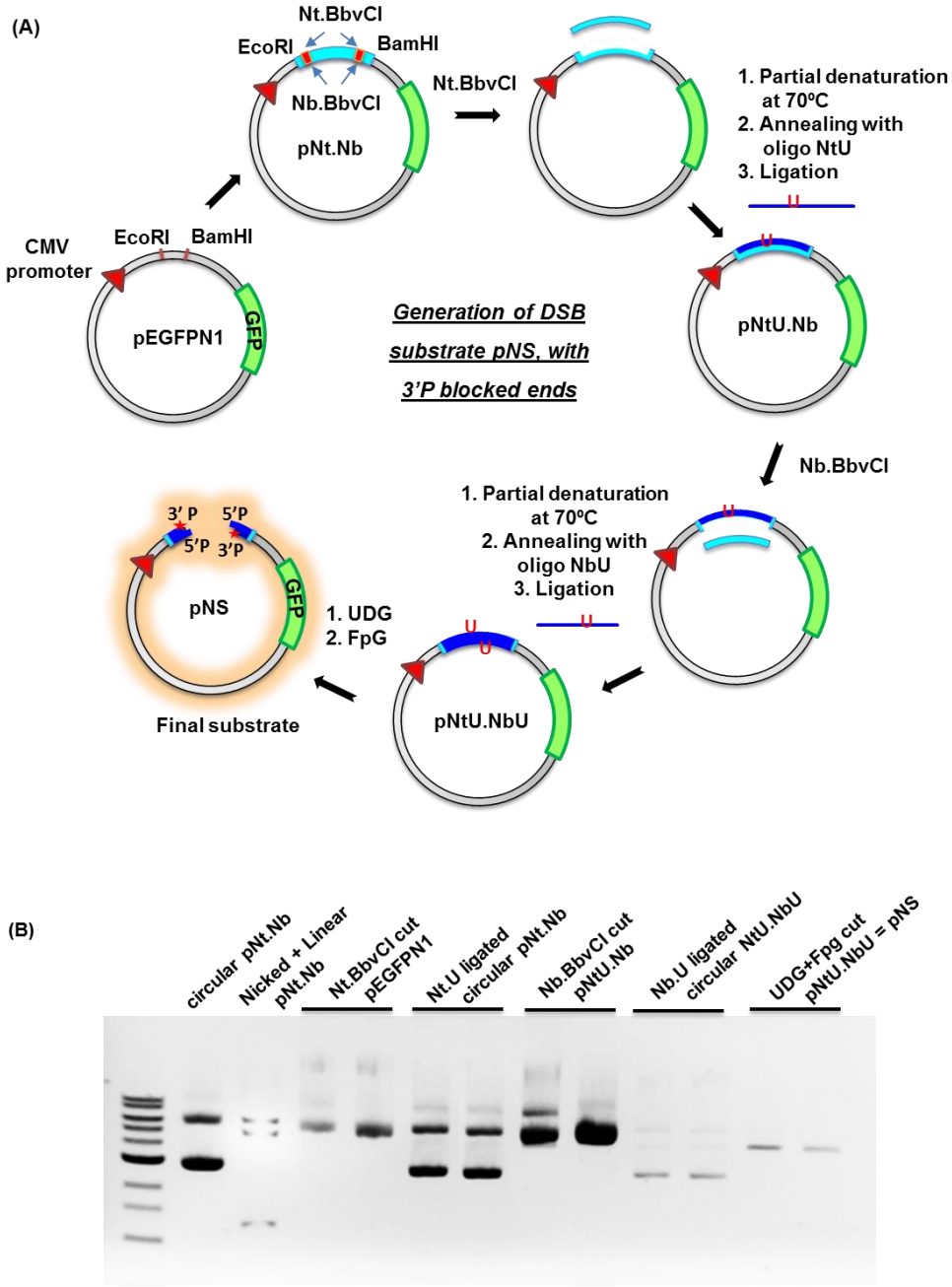


Figure 10. Preparation of plasmid substrate, pNS, (A) Schematic representation of the steps. Duplex oligonucleotide with nicking sites for Nt.BbvCI/Nb.BbvCI were cloned in pEGFPN1 at EcoRI and BamHI. First digestion was carried out with Nt.BbvCI followed by partial denaturation at 70ºC for 10 mins, annealing with the complementary U containing oligonucleotide, NtU and overnight ligation at 16ºC. After purification of the ligated product, second digestion was carried out with Nb.BbvCI, followed by partial denaturation at 70ºC for 10 mins, annealing with the complementary U containing oligonucleotide, NbU and overnight ligation at 16ºC. The ligated product was gel purified and digested with UDG and FpG, followed by purification of the linearized plasmid in 1% agarose gel. (B) Plasmid substrate intermediates and the final product analyzed in 1% agarose gel.

pNTNB was nicked by Nt.BbvCI, followed by removal of the enzyme through column purification with Qiagen PCR purification kit. The nicked strand was then stripped off by partial denaturation at 65°C for 10 minutes followed by annealing with 100 fold molar excess of the first U containing oligonucleotide. The intermediate was ligated overnight at 16°C with T4 DNA ligase (NEB), followed by column purification of the ligated plasmid. This was then nicked with Nb.BbvCI on the opposite strand followed by introduction of the second U containing oligonucleotide similarly. The final ligation product with bistranded U was gel purified and then treated with UDG in 1X UDG buffer followed by addition of FpG and 1X FpG buffer (Figure 10A). The linearized plasmid was gel purified twice to remove contamination of nicked fraction. All intermediate products were checked with 1% agarose gel electrophoresis (Figure 10B).

3.2.2. IN CELL PLASMID RECIRCULRIZATION ASSAY

Exponentially growing cancer cell lines, U2OS and A549 were transfected with pNS. Only recircularized plasmids would lead to GFP expression which could be observed within 6 hrs after transfection (Figure 11). After overnight incubation of the transfected cells, low molecular weight DNA was extracted from these cell and used for transformation of *E.coli,* in which the non-circularized plasmids are degraded by RecBC nuclease [139] and only circular plasmids will be individually scored, based on antibiotic resistance. Thus

each colony would represent a single DSB repair event which are then analyzed by sequencing of the rejoined break site. For each assay we screened at least 40 bacterial colony for sequencing analysis of the repaired plasmid and calculated the relative frequency of Alt-EJ vs. NHEJ. Sequences with very long deletions were ignored as they could be due to extensive non-specific exonuclease activity at the DSB ends.

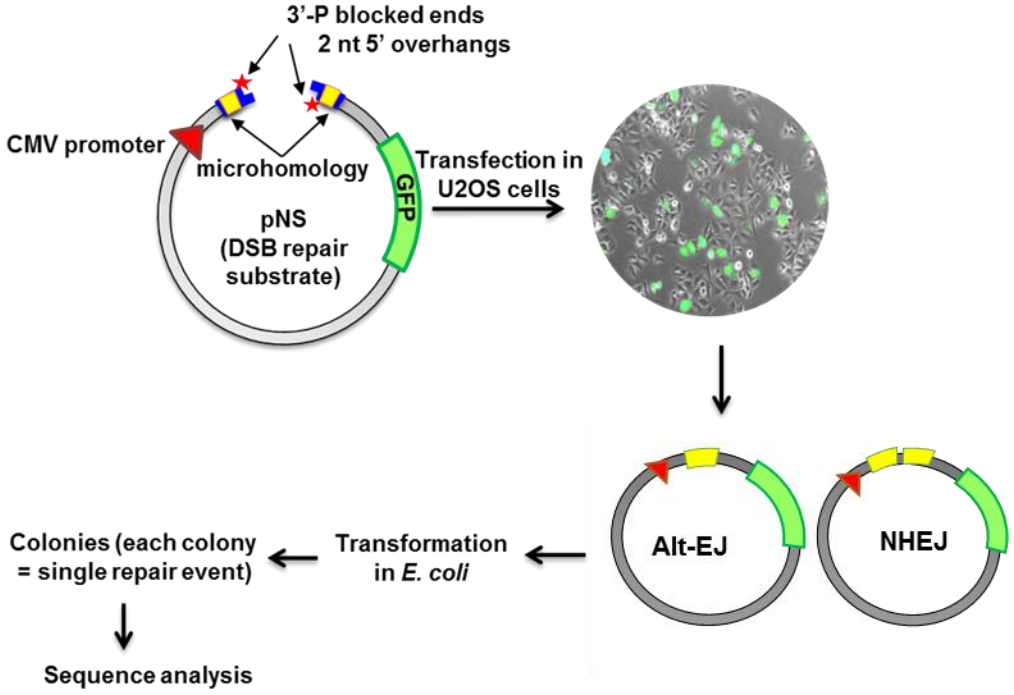
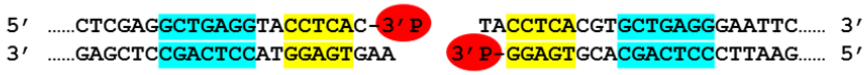


Figure 11. Schematic representation of *in cell* repair assay. pNS was transfected in U2OS or A549 cells using Lipofectamine and incubated overnight. Recircularization of the plasmid could take place through NHEJ or Alt-EJ and would lead to GFP expression. Unlike NHEJ, Alt-EJ will result in loss of one of the microhomology regions. After extraction of the episomal DNA from the cells they were transformed in E coli XL10 gold ultracompetent cells and colonies were screened for sequencing analysis of the repaired plasmid.

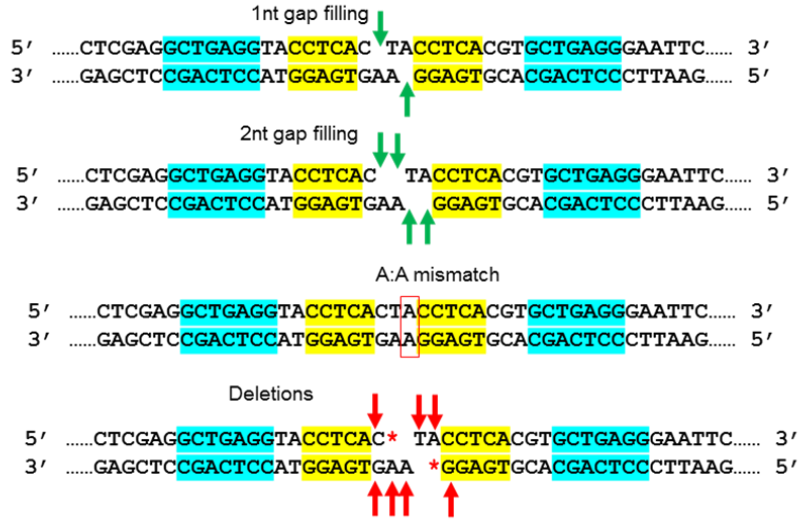
pNS has 2nt overhang with single base complementarity (Figure 12A). We could assume that accurate repair of a dirty DSB by NHEJ would depend upon how the ends are aligned (Figure 12B). DNA-PK recruits human polynucleotide kinase/phosphatase (PNKP) which removes 3'-P to make the ends compatible for nucleotide addition by a polymerase or ligation by a ligase [140, 141]. The DNA-PK heterotrimer complex could

stabilize the broken DNA ends, where the terminal A and T on the opposite strands on either side of the DSB could pair, followed by gap filling and ligation, resulting in accurate repair (Figure 12B.i). Alternatively, there could be gap filling at the 2 nt overhangs followed by ligation of the blunt ends, leading to 1 nt insertion (Figure 13B.ii). Another possibility is base pairing of the second terminal A on the 3' strand with the terminal T on 5' strand leading to a A:A mispair which when repaired would lead to loss of 1 nt (Figure 12B.iii). Sequence analysis of the repaired plasmids transfected in A549 cells showed that majority of repair events were NHEJ as described and some with 2-3 nucletiodes deletions (Figure 12B.iv). However, ~10% of repaired plasmid had extended loss of sequences between the microhomology regions which represent Alt-EJ (Figure 12B). This is in agreement with previous reports that NHEJ is the predominant repair pathway even in replicating cells [142]. In this case, Alt-EJ could occur utilizing either the pair of proximal microhomology sequences (CCTCA) resulting in shorter deletion, that can be termed proximal Alt-EJ, or utilizing the distal microhomology sequences (CTGAGG) causing a larger deletion and called distal Alt-EJ; the latter was found to be rarer than the former.

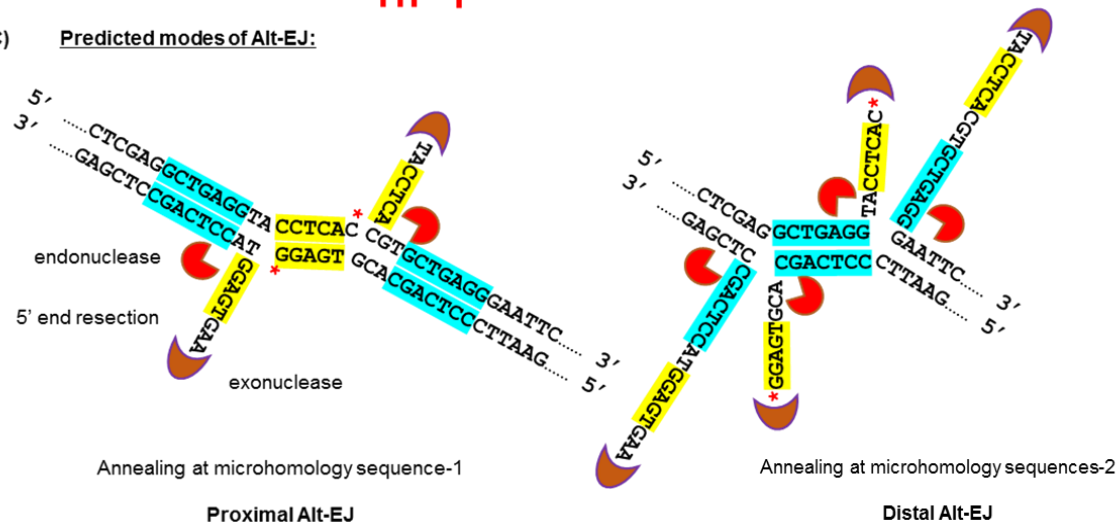
(A) **Sequence of pNS at double strand break:**



(B) **Predicted modes of NHEJ:**



(C) **Predicted modes of Alt-EJ:**



(D) **Post repair sequences:**

NHEJ	CTCGAGGCTGAGGTACCTCACTTTACCTCACGTGCTGAGGGAATTC	1 nt insertion
	CTCGAGGCTGAGGTACCTCACTTACCTCACGTGCTGAGGGAATTC	Error-free
	CTCGAGGCTGAGGTACCTCACT-ACCTCACGTGCTGAGGGAATTC	1 nt deletion
	CTCGAGGCTGAGGTACCTCACTT--CTCACGTGCTGAGGGAATTC	2 nt deletion
	CTCGAGGCTGAGGTACCTCAC----CTCACGTGCTGAGGGAATTC	3 nt deletion
	CTCGAGGCTGAGGTACCTC-----CCTCACGTGCTGAGGGAATTC	4 nt deletion
Alt-EJ		
Proximal:	CTCGAGGCTGAGGT---------ACCTCACGTGCTGAGGGAATTC	Smaller deletion
Distal:	CTCGAG-------------------------GCTGAGGGAATTC	Larger deletion

Figure 12. Modes of repair of pNS through NHEJ and Alt-EJ. (A) Sequence of pNS containing the DSB, 5-nt sequences highlighted yellow is microhomology sequence-1, 7-nt nicking endonuclease sequence (see Figure S1) highlighted in cyan is microhomology sequence-2, 3'-dirty termini are highlighted in red circles, (B) Possible modes of NHEJ, with 1 or 2 nt gap-filling, post-DSB end-joining A:A mismatch repair or deletion of terminal bases by exonuclease to generate flush ends, (C) Possible modes of Alt-EJ, the 3' overhangs could be annealed through microhomology sequence-1 or 2 with exo-/endonucleolytic processing of the intervening sequences, (D) Sequences at repaired joints of recircularized pNS after *in cell* repair.

3.3. RESULTS

3.3.1. ALT-EJ OF COMPLEX DNA DOUBLE STRAND BREAKS IS ENHANCED WHEN END CLEANING IS INHIBITED

Because pNS has 3'-P termini which requires end processing by PNKP prior to DSB repair, we tested how end joining is affected in cells with inhibition of end processing by depleting PNKP. The relative frequency of repaired joints mediated by Alt-EJ vs. NHEJ obtained after *in cell* repair of pNS in A549 cells and those with stable shRNA mediated depletion of PNKP was plotted. Alt-EJ was relatively greater by ~3 folds, while NHEJ was reduced in PNKP depleted cells compared to that in control A549 cells, which was highly statistically significant (P=0.002) (Figure 13A). Relative enhancement of Alt-EJ could also be reduced by ectopic shRNA resistant PNKP(WT) but not PNKP(phosphatase-kinase mutant) in shPNKP-A549 cells (Figure 13B). This suggests PNKP depletion affects NHEJ resulting in relative enhancement of microhomology dependent repair through Alt-EJ. These results are consistent with previous studies showing that PNKP interacts with the NHEJ complex mediated by XRCC4, and is required for efficient NHEJ [140, 143]. Although *in vitro* studies have implicated role of PNKP in a PARP1-dependent mode of Alt-EJ [144], it's *in vivo* occurence is not yet characterized. Moreover, this PARP1-dependent DSBR does not suggest requirement of microhomology, and hence should not result in a loss of DNA sequences [89, 144]. In our studies we have primarily focused on the deletion-prone microhomology dependent mode of Alt-EJ or MMEJ.

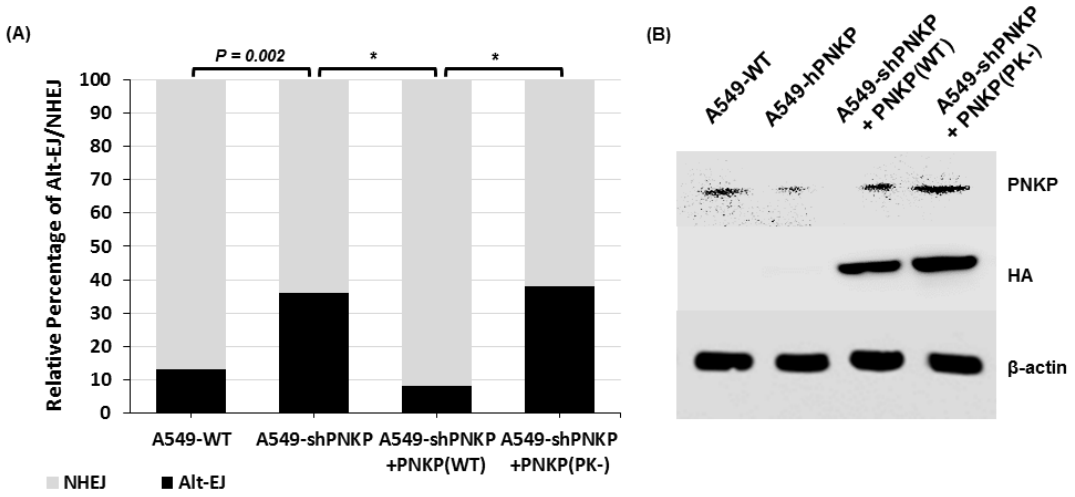


Figure 13. Requirement of PNKP during repair of 3'P containing DSB. (A) *In cell* repair of pNS in A549-WT, stable shRNA-mediated PNKP depleted cells and expressing shRNA resistant PNKP(WT) or PNKP(phosphatase-kinase mutant). Relative percentage of Alt-EJ and NHEJ repaired joints has been plotted (* P<0.0001). (B) Western blot analysis of expression of endogenous PNKP and transiently expressing HA-tagged PNKP(WT) or PNKP(phosphatase-kinase mutant) in A549-WT and A549-shPNKP cells.

3.3.2. ALT-EJ IS ENHANCED AFTER IRRADIATION OF CANCER CELLS

Next, we asked how Alt-EJ or NHEJ is affected in the cells with X-ray-induced activation of DNA damage response. DSB joining in linearized plasmids were performed in A549 and U2OS cells which were treated with increasing dose of X-rays immediately prior to transfection with pNS and found that the relative percentage of Alt-EJ was enhanced by at least 3 folds after X-ray treatment in both the cell lines with P value of 0.004 or less (Figure 14A, 14B). This increase in Alt-EJ in irradiated cells was enhanced at as low as 0.5 Gy which didn't increase with higher doses, indicating DNA damage response elicited by low dose of X-rays is enough to activate Alt-EJ in human cells. Our results support a previous report that MMEJ is induced in yeast and mammalian cells after irradiation, however they didn't characterize the underlying molecular mechanism of this observation [138].

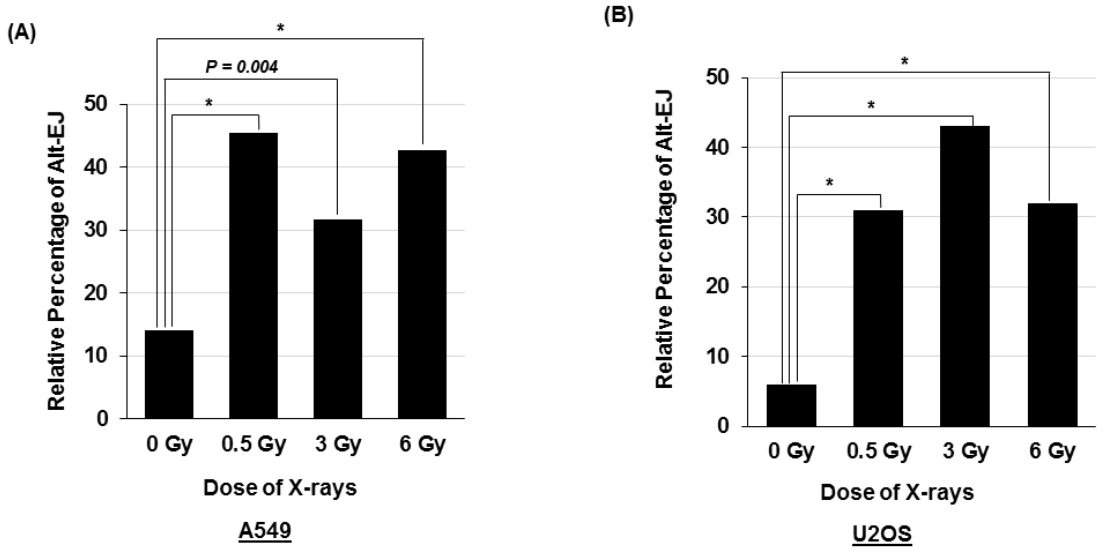


Figure 14. Alt-EJ of pNS enhances after irradiation. Relative percentage of Alt-EJ in control A549 and U2OS cells compared to those treated with increasing dose of X-rays - 0.5, 3, 6 Gy (* P<0.0001).

3.3.3. ENHANCEMENT OF ALT-EJ AFTER IRRADIATION REQUIRES XRCC1, PARP1, MRE11 AND CTIP

Next, we performed *in cell* DSB repair assays in U2OS cells with siRNA mediated depletion or inhibition of specific DNA repair proteins to characterize the factors required for enhancement of Alt-EJ in irradiated cells and found XRCC1, PARP1, CtIP and Mre11 are crucial for Alt-EJ (Figure 15A). Alt-EJ was the major repair process in DNA-PK inhibited cells, confirming that Alt-EJ doesn't require DNA-PK and was rather enhanced as NHEJ was severely affected in DNA-PK treated cells (Figure 15A, last column).

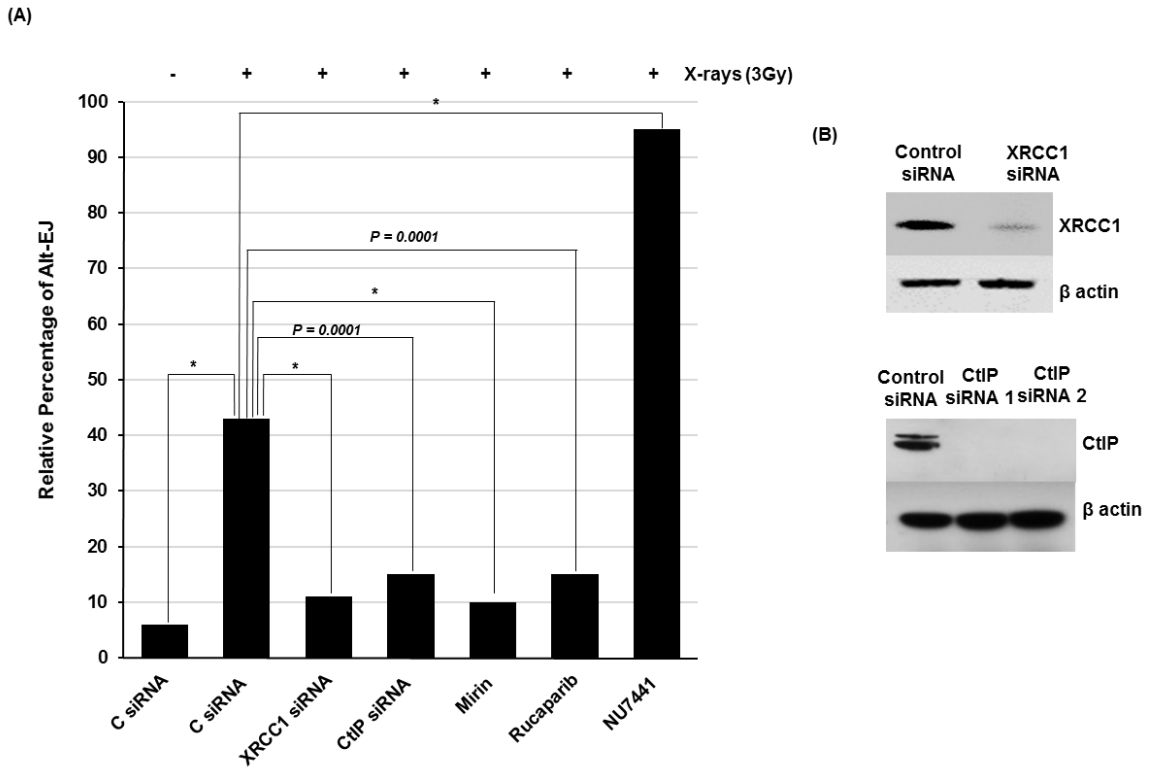


Figure 15. Alt-EJ requires XRCC1, PARP1, Mre11 and CtIP but not DNA-PK. (A) pNS was transfected into control U2OS cells or those treated XRCC1 siRNA (100 nM,72 h), CtIP siRNA (100 nM, 72 h), Mre11 inhibitor mirin (100 μM, 1h), PARP inhibitor (50 μM, 2h) or DNA-PK inhibitor NU7441 (10 μM, 1h) with X-ray treatment as indicated. Relative percentage of Alt-EJ repaired joints obtained from sequence analysis are plotted (*P<0.0001). (B) Western blot analysis for siRNA mediated depletion of XRCC1 and CtIP.

3.3. DISCUSSION

In our study we have established a novel tool to recapitulate *in cell* repair of DSBs with non-ligatable termini and assessed relative contribution of NHEJ vs. Alt-EJ, based on requirement of microhomology sequences at the broken ends. Although plasmid recircularization based assays do not completely mimic DSB repair at the chromatin, it is useful to delineate the relative contribution of distinct repair complexes activated during the X-ray-induced DNA damage response. This assay cannot detect HR, due to lack of an intact DNA sequence homologous to the region spanning the break site that is normally

provided by the sister chromatid during S/G2 phase of cell cycle. However, HR is a slow process compared to both NHEJ and Alt-EJ, and our focus was to compare the immediate repair choices at DSBs with dirty termini when microhomology sequences are available. It was found that in spite of presence of microhomology sequences close to the DSB, NHEJ was robust and major process to repair DSBs in human cells that is error-free or could induced minor deletions/insertions. However, this requires processing of the blocked ends such as 3'P by a specific enzyme, PNKP that could be joined through NHEJ. In absence of proper end processing, NHEJ could fail for a significant fraction of DSBs which are repaired via the back-up pathway of Alt-EJ. Although PNKP interacts with XRCC1 to stimulate BER/SSBR as well as Alt-EJ [39, 144, 145], its depletion didn't affect rather relatively increased Alt-EJ of 3'P containing dirty DSBs, suggesting it could be dispensable for Alt-EJ.

Interestingly, while Alt-EJ mainly served as a backup to NHEJ in normal cells, we found that irradiation of human cells with X-rays enhanced Alt-EJ up to five-fold which was observed in two distinct cancer cell lines, and could be supported by an earlier report [138]. Although our assays provide only a semi-quantitative estimation of Alt-EJ, it is likely that this is due to activation of Alt-EJ pathway itself rather than a relative effect due to inhibition of NHEJ. To get further insights on the molecular mechanism regulating enhancement of Alt-EJ, we studied role of distinct protein-protein interactions and repair complexes that are enhanced after irradiation, as discussed in Chapter IV.

CHAPTER IV: ROLE OF XRCC1 IN ALTERNATIVE END JOINING

4.1. INTRODUCTION

XRCC1 is a scaffold protein that forms multiprotein repair complexes which is critical for base excision repair (BER) and SSB repair (SSBR) [146, 147]. Several groups have identified XRCC1 to be one of the key players in Alt-EJ [93, 148], although it was shown to be dispensable for Alt-EJ mediated class-switch recombination in B lymphocyte [98, 99]. Because we found XRCC1 depletion affected Alt-EJ in human cancer cells, we hypothesized that high level of base lesions and SSBs induced by IR that leads to recruitment of XRCC1/PARP1 complexes at the damaged chromatin [149, 150], could contribute significantly towards Alt-EJ mediated DSB repair through dynamic interaction between SSB and DSB repair proteins, which could be regulated through post-translational modifications. Interaction of XRCC1 with several SSBR proteins like PNKP, Aprataxin and LIG3 are stabilized through phosphorylation of XRCC1 by CK2, which is activated during oxidative stress and is widely overexpressed in tumors [38, 39, 151]. It was tested if CK2 mediated XRCC1 phosphorylation also facilitates XRCC1's interaction with Mre11 and CtIP, the end resection enzymes to promote Alt-EJ.

4.1.1. X-RAY REPAIR CROSS-COMPLEMENTING PROTEIN 1 (XRCC1)

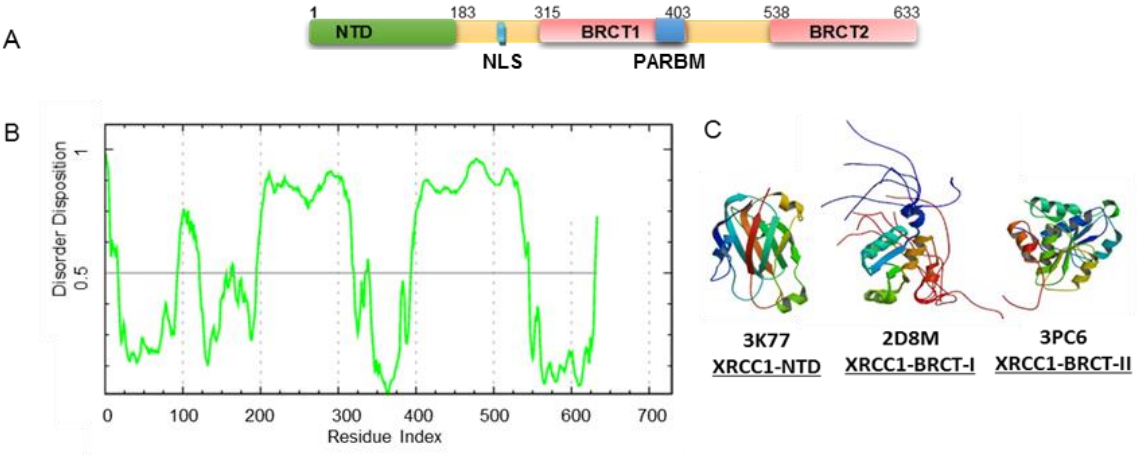
XRCC1 cDNA was discovered by Thompson et. al. based on the observation that deficiency in XRCC1 protein significantly reduced SSBR capacity of the Chinese hamster ovary (CHO) cells and increased sensitivity toward alkylating agents, X-ray and UV irradiation and increased sister chromatid exchange (SCE) [152]. XRCC1 downregulation in human cells, particularly in BRCA2 deficient cell lines enhanced sensitivity towards alkylating agents through generation of single strand breaks [120]. XRCC1 null mouse are embryonic lethal, indicating that it is required for early development, which could be

rescued by XRCC1 transgene complementation [153, 154]. XRCC1 *Nes-cre* conditional knockout in neuronal cells resulted in viable offsprings which lived upto 4 months, however their growth and brain size was seriously affected with loss of cerebellar interneurons and abnormal hippocampal functions [155]. XRCC1 haplo-insufficient mice when exposed to alkylating agents such as azoxymethane induced high liver toxicity and precancerous lesions in the colon compared to control animals [156]. Clinicopathological studies have shown XRCC1 to be predictive biomarker for ovarian cancer with reduced XRCC1 expression associated with sensitivity towards cisplaitin-based drugs [157] and induced synthetic lethality with ATM and DNA-PK inhibitors in BRCA-deficient breast cancer [158].

4.1.1.1. Structure of XRCC1

Human XRCC1 is 633 amino acid (aa) long protein with a molecular weight of 69.51 kDa (UniProtKB - P18887) that is encoded by the *Xrcc1* gene mapped at chromosome 19q13.3–13.3 (Ensembl ID: ENSG00000073050). It has a nuclear localization signal (NLS; 239-266 aa) and three conserved domains, an N-terminal domain (NTD) domain, spanning from 1-188 aa, and two BRCT domains – BRCT1 and BRCT2, spanning from 315-403 aa and 536-633 aa respectively (Figure 16A) [147]. Solution structure of NTD suggests that it has a core of beta-sandwich structure that surrounds the DNA at the lesion and interacts with the palm-thumb domains of Polβ [159]. Moreover, formation of an oxidation dependent transient disulfide bond in NTD has been suggested to provide added stability to XRCC1-Polβ interaction that could be regulated through redox pathways of cellular metabolism [160]. The BRCT domains of XRCC1 provide a hub for interaction with several DNA repair factors. The BRCT1 domain interacts with another key early BER/SSBR factors poly(ADP-ribose) polymerase 1 (PARP1) through its BRCT domain [161, 162] and also has a putative poly(ADP-ribose) binding motif (PARBM, 379-

400 aa) through which it is recruited at the DNA damage site [33, 149]. BRCT2 domain of XRCC1 binds to DNA ligase 3 (LIG3) through its BRCT domain, and stabilizes the latter's intracellular level [163, 164]. XRCC1 can form homodimer through BRCT1 domains [165], and multiple interactions with other proteins like LIG3 and PARP1 could lead to formation of multimeric protein structures [147, 162]. Predictor of Natural Disordered Regions (PONDR) [166] shows that the conserved domains of XRCC1 are linked by two intrinsically disordered linker regions (183-315 aa and 403-538 aa), which couldn't be crystalized or detected through solution structure studies (Figure 16B) [21]. These linker region which could get phosphorylated by protein kinases kinases CHK2 and CK2 provide added flexibility to XRCC1 and interaction regions for multiple repair proteins (Figure 17). The first linker region of XRCC1 is required for its interaction with PCNA, and the DNA glycosylases, endonuclease VIII-like 2 (NIEL2), nth endonuclease III-like 1 (NTH1), 8-oxoguanine DNA glycosylase (OGG1), and Uracil-DNA glycosylase 2 (UNG2) [167-170]. The second linker region between BRCT1 and BRCT2 interacts with aprataxin (APTX) through its forkhead-associated (FHA) domain, polynucleotide kinase/phosphatase (PNKP), and aprataxin and PNKP like factor (APLF) [39, 171, 172]. XRCC1 also stimulates PNKP's phosphatase and kinase activities which are required during SSBR [173]. Based on several studies, Hanssen-Bauer et. al. has reviewed that XRCC1 could facilitate formation of distinct multi-protein complexes associated with short patch BER/SSBR, repair of complex damage, or replication associated BER [147].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XRCC1-BRCT-I
3PC6
XRCC1-BRCT-II

Figure 16. Structure of XRCC1. (A) Schematic representation of conserved domains of XRCC1: N terminal domain (NTD), a nuclear localization signal (NLS), two conserved BRCA1 C Terminus (BRCT) domains – BRCT1 and BRCT2, BRCT1 has a putative polyADP ribose chain binding motif (PAR-BM); conserved phosphorylation sites T284, S371, S518, S519, T523 are indicated with red arrows, (B) PONDR (Predictor of Natural Disordered Regions) plot for XRCC1 [21], (C) PDB structures of conserved domains – NTD (3K77), BRCT1 (2D8M) and BRCT2 (3PC6) (D) Multiple sequence alignment of XRCC1 protein sequences of Homo sapiens (Q00839.6), Pan troglodytes (NP_001267207), Pongo abelii (XP_002829368.2), Macaca mulatta (AFJ71049.1), Mus musculus (Q8VEK3), Rattus norvegicus (NP_476480.2), Xenopus laevis (AAH84742.1), and Danio rerio (NP_001003988.2) (Multalin interface page – Inra).

4.1.1.2. Post-translation modifications of XRCC1

Post-translational modifications such as phosphorylation, acetylation, mono-ubiquitination and SUMOylation could regulate functions of a protein by either affecting its conformation and hence interaction with partner proteins or providing stability against proteasomal degradation [174]. XRCC1 could be phosphorylated at as many as 30 serine/ threonine residues [147]. CHK2 has been reported to promote BER by phosphorylating XRCC1 at Thr284, which is conserved only in higher mammals (Figure 17) [175]. DNA-PK phosphorylates XRCC1 at Ser371 which is located in the BRCT1 domain and is conserved among vertebrates (Figure 17). Phosphorylation of Ser371 has been shown to disrupt XRCC1 homo-dimerization [165]. This suggests a cross-talk between NHEJ and BER and that the former could affect the latter at clustered damages in the genome induced by IR, which demands further investigation. Casein kinase 2 (CK2), which is a ubiquitous and constitutively active kinase phosphorylates XRCC1 at nine residues at the second linker region between BRCT1 and BRCT2, out of which Ser518, Thr519, and Thr523 are most studied and are conserved among vertebrates (Figure 17). CK2 mediated phosphorylation was shown to be required for XRCC1's interaction with PNKP, APTX and APLF [39, 171, 172] and regulated it dissociation from the DNA after repair through its poly-ubiquitylation [33]. PARP1 mediated PARylation on the other hand prevents its poly-ubiquitylation and enhances its retention until the breaks are repaired [33]. XRCC1 has also been reported to

be a substrate for SUMO modification, however the neither the residues nor its physiological consequences have been characterized yet [176, 177].

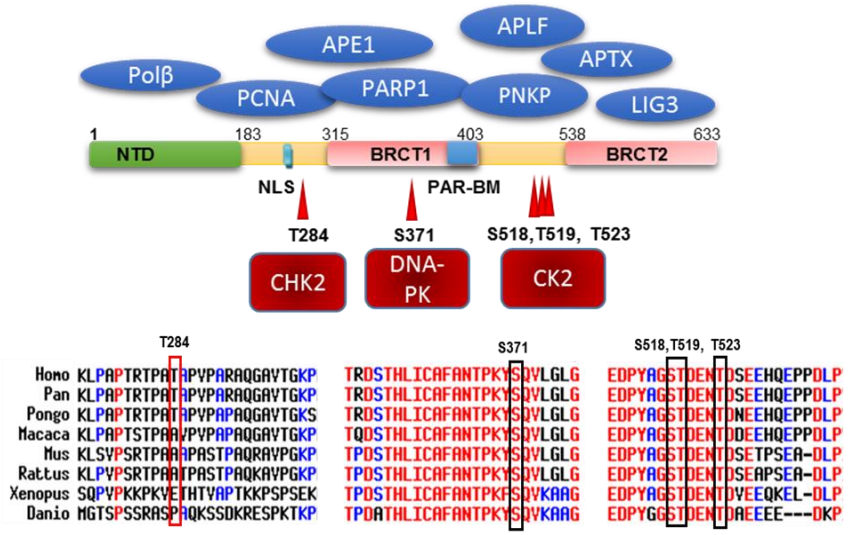


Figure 17. XRCC1 interaction map with different SSBR proteins. XRCC1 interacts with Polβ, PCNA, APE1, PARP1, PNKP, APLF, APTX and LIG3 through direct protein-protein interactions through distinct domains (explained in text). XRCC1 is phosphorylated by CHK2, DNA-PK and CK2 at conserved serine/threonine residues.

4.1.1.3. Polymorphisms in XRCC1 and links to pathogenesis

Three most studied single nucleotide polymorphisms in XRCC1 which have been linked to pathological consequences are Arg194Trp (rs1799782), Arg280His (rs25489), and Arg399Gln (rs25487), albeit with high variability among different ethnic groups [147]. Analysis of sister chromatid exchange and micronuclei formation as marker of genomic instability showed that Arg194Trp could be associated with enhanced genomic instability which could be due to reduced recruitment at the strand breaks or altered protein-protein interactions; Arg280His and Arg399Gln on the other hand variants showed reduced genomic instability [178]. A recent meta-analysis of Arg399 variant has been linked to increased risk of developing breast cancer[179], however it was not found to cause any major structural alteration in the BRCT1 domain [165]. Epidemiological studies have also

linked Arg399Gln to Parkison's disease, sporadic amyoptropic lateral sclerosis, and increased risk of glioma, melanoma and squamous cell carcinoma, while Arg280 His and Arg194Trp variants could be linked to increases risk of melanoma and squamous cell carcinoma respectively [147, 180-184].

4.2. RESULTS

4.2.1. ROLE OF XRCC1 IN DNA DOUBLE STRAND BREAK REPAIR

4.2.1.1. XRCC1 is recruited at X-ray induced DNA double strand breaks

Based on our observation that *in cell* Alt-EJ is compromised when XRCC1 is depleted and also earlier reports showing involvement of XRCC1 in DSBR via Alt-EJ, we tested if XRCC1 is recruited at X-ray-induced DSBs. Formation of DSBs in the genome induce a cascade of signaling events known as DNA damage response that includes phosphorylation and recruitment of histone variant H2AX at the DSBs, producing γH2AX that serve as marker for DSBs [185]. To check if XRCC1 is recruited at the DSBs we performed proximity ligation assay (PLA) using anti-XRCC1 and anti-γH2AX primary antibodies raised in different species. XRCC1-γH2AX PLA foci in irradiated U2OS cells indicates that XRCC1 is recruited at the DSBs, which was significantly enhanced when NHEJ was inhibited with the DNA-PK inhibitor NU7441 (Figure 18). XRCC1 has been earlier shown to localize at IR/ROS-induced SSB sites [39]. However, enhancement of XRCC1-γH2AX interaction in NHEJ compromised cells suggests that this does not reflect XRCC1 recruitment at closely spaced SSBs within X-ray induced damage clusters, rather, its involvement in DSB repair.

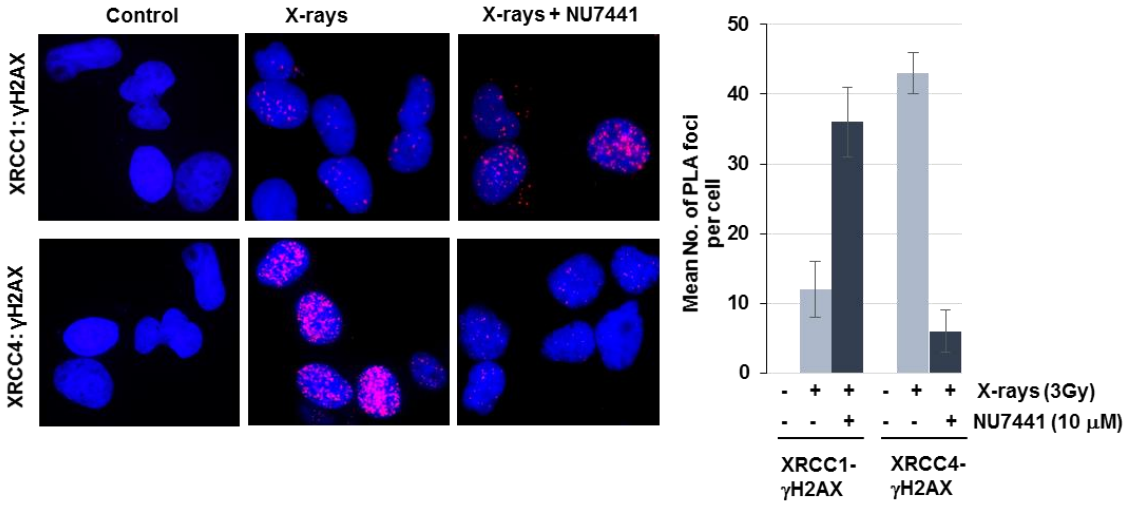


Figure 18. XRCC1 is recruited at X-ray induced DNA double strand breaks. Left panel shows PLA for XRCC1-γH2AX and XRCC4-γH2AX interaction in control U2OS cells, those treated with X-rays only or with 10 μM NU7441 and X-rays. Right panel shows the quantification of mean number of PLA foci per cell.

4.2.1.2. XRCC1 recruitment at DSBs is not dependent upon PARP1

Next, we checked if recruitment of XRCC1 at DSBs is influenced by PARP1-mediated PARylation. PARP1 is an SSB sensor protein which auto-PARylates itself and several DNA repair factors including XRCC1 and facilitates recruitment of XRCC1 at SSBs by both BRCT domain mediated interaction and PAR chains [33]. PARP1 has also been reported to influence DSBR by competing with Ku for DSBs and by PARylating several NHEJ factors including DNA-PK [102, 186]. However, pre-treatment with PARP inhibitor 10 μM rucaparib didn't significantly affect XRCC1-γH2AX PLA foci in X-ray treated U2OS cells, indicating that XRCC1's recruitment at DSBs is independent of PARylation activity of PARP1 (Figure 19A). PARP inhibition in rucaparib treated cells was confirmed by western blot analysis with anti-PAR antibody (Figure 19B). This conclusion is supported by other reports suggesting XRCC1's recruitment at DSBs doesn't require PAR chains or PARP activity [146, 147]. However, with this observation we cannot exclude PARP1's role in Alt-EJ as reported previously [89, 187]; there could be a distinct

sub-pathway, because we also found that PARP inhibition affects Alt-EJ enhancement in irradiated U2OS cells (Figure 15).

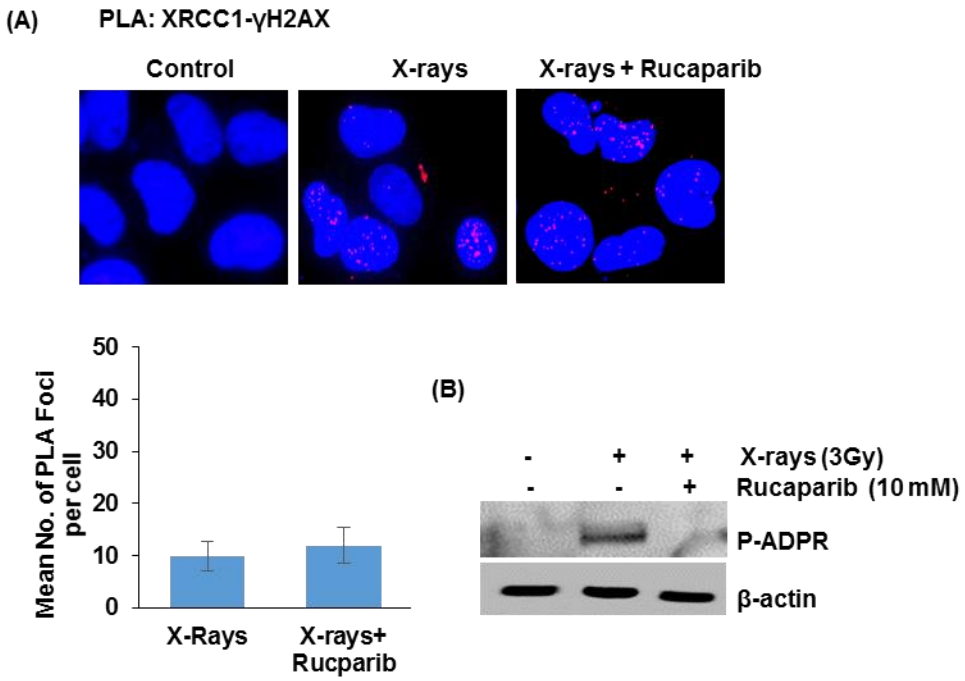


Figure 19. XRCC1 recruitment at DSBs is not dependent upon PARP1. (A) PLA for XRCC1-γH2AX interaction in control U2OS cells, or those treated with X-rays and 10 μM rucaparib and X-rays; quantification of mean number PLA foci per cell is given below. (B) Western blot analysis of the total cell extract from control U2OS cells, or those treated with X-rays and 10 μM rucaparib and X-rays.

4.2.1.3. XRCC1 is required for DSB repair.

DSB marker γH2AX forms distinct foci that can be microscopically visualized through immunocytochemistry to quantitate DSB generated following irradiation or other genotoxic stress [185]. To investigate contribution of XRCC1 to DNA double strand break repair we analyzed γH2AX foci in control or XRCC1 depleted U2OS cells with or without DNA-PK inhibition by NU7441 treatment, after X-rays treatment. We expected to see more γH2AX foci accumulation in NU7441 treated cells due to inhibition of NHEJ [188].

We observed higher number of γH2AX foci in U2OS cells after combined depletion of XRCC1 and DNA-PK inhibition than those after DNA-PK inhibition alone (Figure 20). Previous studies in Arabidopsis showing accumulation of additional DSBs with loss of XRCC1, support our findings in cancer cells [189].

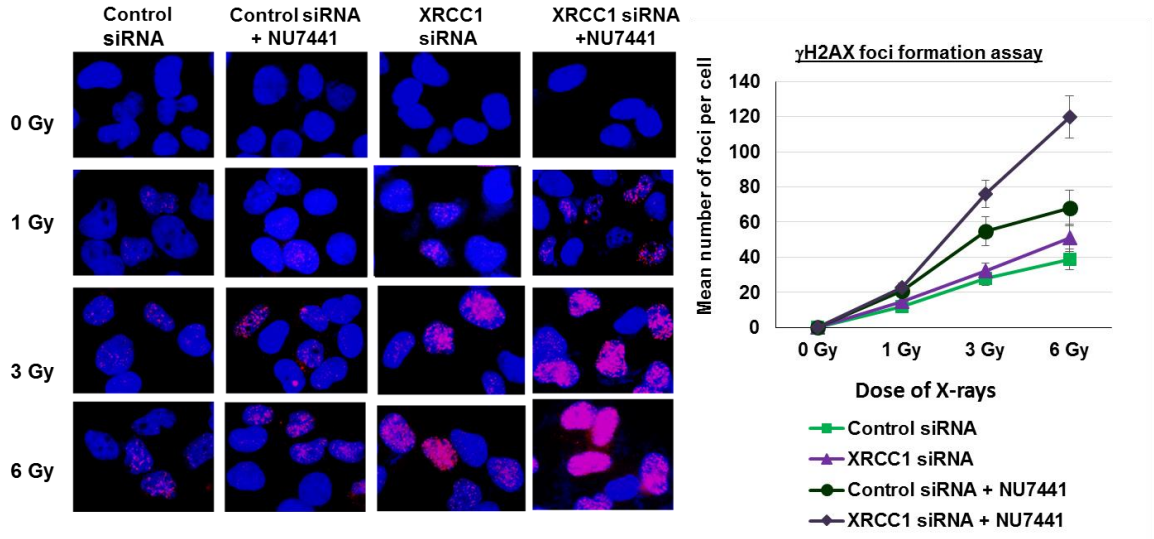


Figure 20. XRCC1 depletion enhances γH2AX foci accumulation in NHEJ inhibited U2OS cells. γH2AX foci formation assay performed in U2OS cells transfected with control siRNA or XRCC1 siRNA with/ without NU7441 treatment, which were exposed to different dose of X-rays (0, 1, 3, 6 Gy).

Next, we analyzed γH2AX foci formation to monitor repair of DSBs as a function of γH2AX foci disappearance. As expected, γH2AX foci reduced to basal level in control siRNA and XRCC1 siRNA treated cells due to presence of active NHEJ. However, we found that residual γH2AX foci accumulated at later time points in U2OS cells which were both XRCC1 depleted and DNA-PK inhibited that that with DNA-PK inhibition alone (Figure 21). The slow repair process in NU7441 treated cells could be via Alt-EJ as has been reported earlier [84, 190]. Further reduction of DSB repair in cells with combined deficiency of XRCC1 and DNA-PK activity, implies XRCC1's role in DSB repair by Alt-EJ.

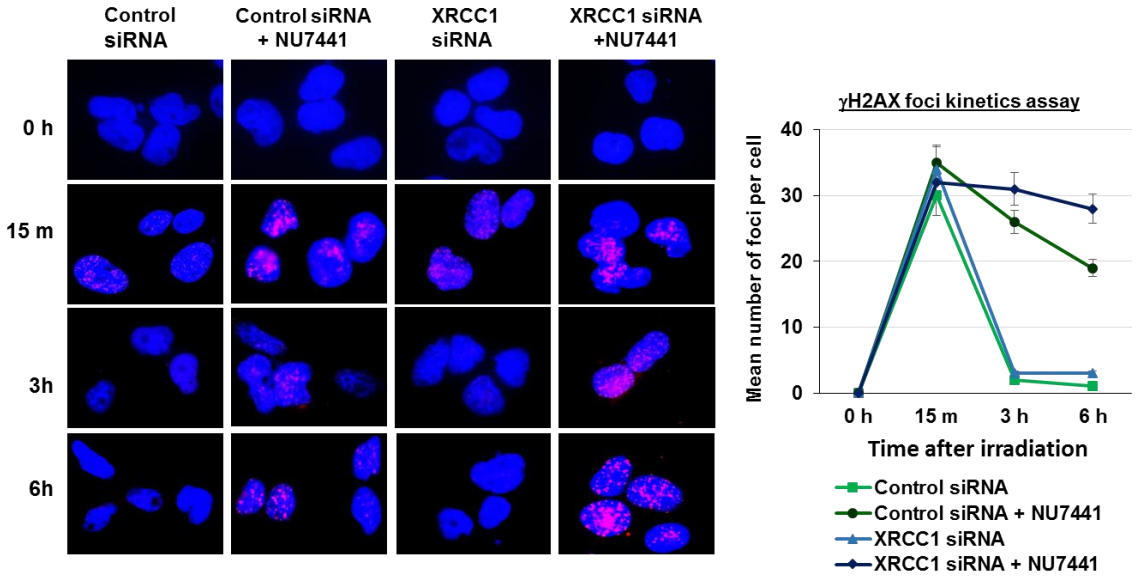


Figure 21. XRCC1 depletion affects γH2AX foci disappearance kinetics. γH2AX foci disappearance kinetics performed in U2OS cells transfected with control siRNA or XRCC1 siRNA with/ without NU7441 treatment, fixed at different time points (15m, 3h, 6h) after treating with 3 Gy X-rays, with an untreated set.

4.2.1.4. XRCC1 confers radio-resistance to U2OS cells

We used clonogenic survival assay to confirm that XRCC1 contributed to radio-resistance of cancer cells. Cancer cells surviving X-ray irradiation would grow to form single colonies that could be visualized by staining the plates with 0.5% crystal violet. U2OS cells treated with scramble or XRCC1 siRNA were treated with DNA-PK inhibitor NU7441 prior to treatment with different dose of X-rays. U2OS cells with XRCC1 depletion and NU7441 treatment showed poor clonogenic survival after irradiation than control cells with NU7441 treatment (Figure 22). This establishes the role of XRCC1 in DSB repair non-epistatic to NHEJ. Previous studies in Arabidopsis showing accumulation of additional DSBs with loss of XRCC1, support our findings in cancer cells [189].

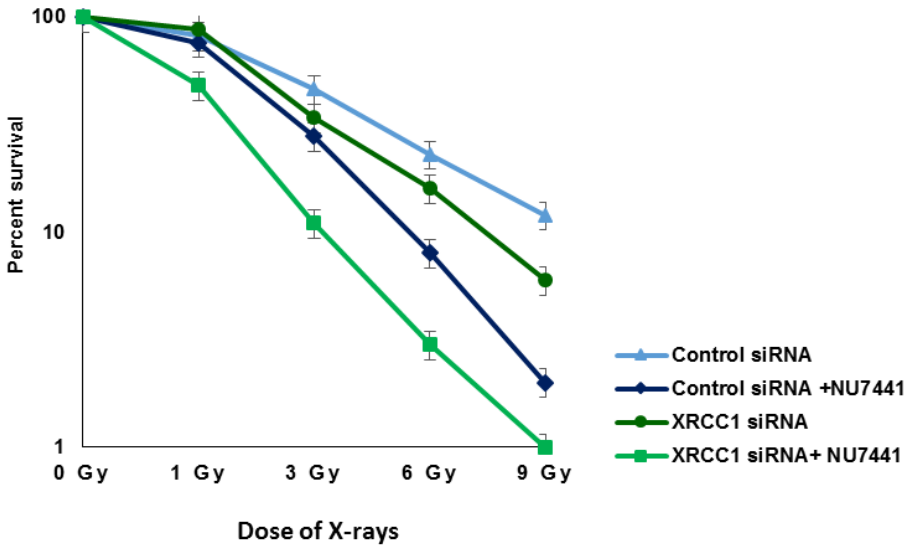


Figure 22. XRCC1 depletion reduces clonogenic survival of U2OS cells. Clonogenic survival assay was performed in U2OS cells transfected with control siRNA or XRCC1 siRNA with/ without NU7441 treatment, which were exposed to increasing dose of X-rays (1, 3, 6 Gy), with untreated control.

4.2.2. Role of CK2-mediated XRCC1 Phosphorylation in Alt-EJ

4.2.2.1. CK2 interacts with and phosphorylates XRCC1 after irradiation

CK2-catalyzed phosphorylation of XRCC1 at S518/T519/T523 residues was found to be critical in promoting SSBR by enhancing its interaction with LIG3 and PNKP [38, 39]. We asked if XRCC1-dependent enhancement of Alt-EJ in irradiated cells is regulated by CK2 mediated phosphorylation. FLAG-coimmunoprecipitation (IP) studies in U2OS cells transiently expressing XRCC1-FLAG showed that CK2 level in XRCC1 IP was significantly enhanced after the cells were irradiated with 3 Gy X-rays (Figure 23A). CK2 is activated by stress signaling [191], and translocates to the nuclei after irradiation [192], where it colocalize with γH2AX [193]. CK2 localizes to U2OS nuclei after irradiation (Figure 23B), and its interaction with XRCC1 in the nucleus increases after X-ray treatment (Figure 23C). Contrary to an earlier suggestion that XRCC1 is phosphorylated by CK2 in

the cytosol before it shuttles to the nucleus [38], we found that XRCC1 level in the nucleus remained unchanged after irradiation (data not shown); thus accumulation of phosphorylated XRCC1 in the nucleus could be attributed to X-ray induced translocation of CK2 in the nucleus. This was further supported by our observation that both exogenously expressed wild type XRCC1^{WT}-FLAG and the phospho-mutant XRCC1^{CKM}-FLAG accumulated in the nucleus (Figure 22D), and previous study showing XRCC1^{CKM} accumulates in the nuclear soluble and chromatin fraction [37].

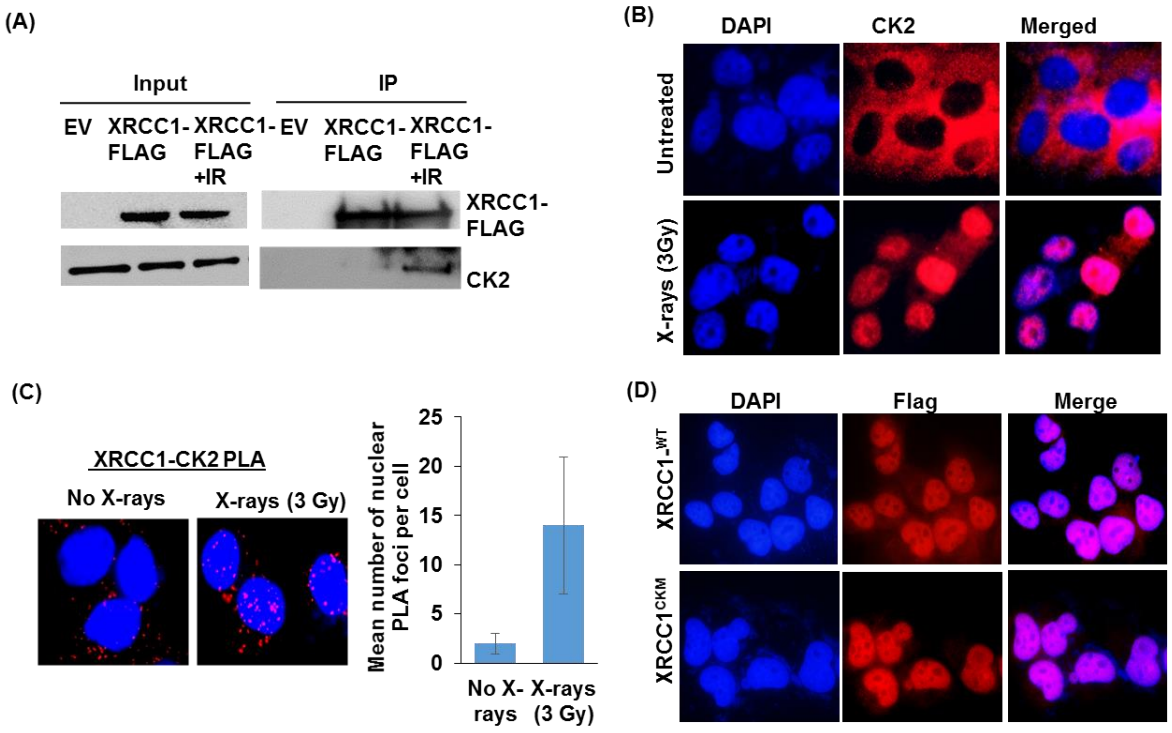


Figure 23. CK2 interacts with XRCC1 after irradiation. (A) XRCC1-FLAG IP from nuclear extract of U2OS cells transiently transfected with XRCC1-FLAG plasmid, with or without X-ray (3 Gy) treatment, (B) PLA for XRCC1-FLAG and CK2 in control and irradiated U2OS cells, (B) CK2 immunostaining in control and X-ray treated U2OS cells, (D) FLAG-immunostaining in U2OS cells transiently expressing XRCC1^{WT}-FLAG and XRCC1^{CKM} -FLAG.

XRCC1 phosphorylation was found to be enhanced after irradiation, which was inhibited in cells treated with CK2 inhibitor, CX-4945 (Figure 24A). Specificity of

phospho-XRCC1 antibody for CK2 mediated phosphorylation was confirmed through *in vitro* phosphorylation of XRCC1 with GST-CK2 recombinant protein (Figure 24B). PLA studies showed that phosphorylated XRCC1 interacts with γH2AX in irradiated U2OS cells, which was affected when cells were pre-treated with 25 μM CX-4945 (Figure 24C). However, both XRCC1^{WT}-FLAG and XRCC1^{CKM}-FLAG could interact with γH2AX with no statistically significant difference, as observed by PLA studies, suggesting that phosphorylation doesn't affect XRCC1's recruitment at or close to DSBs (Figure 24D).

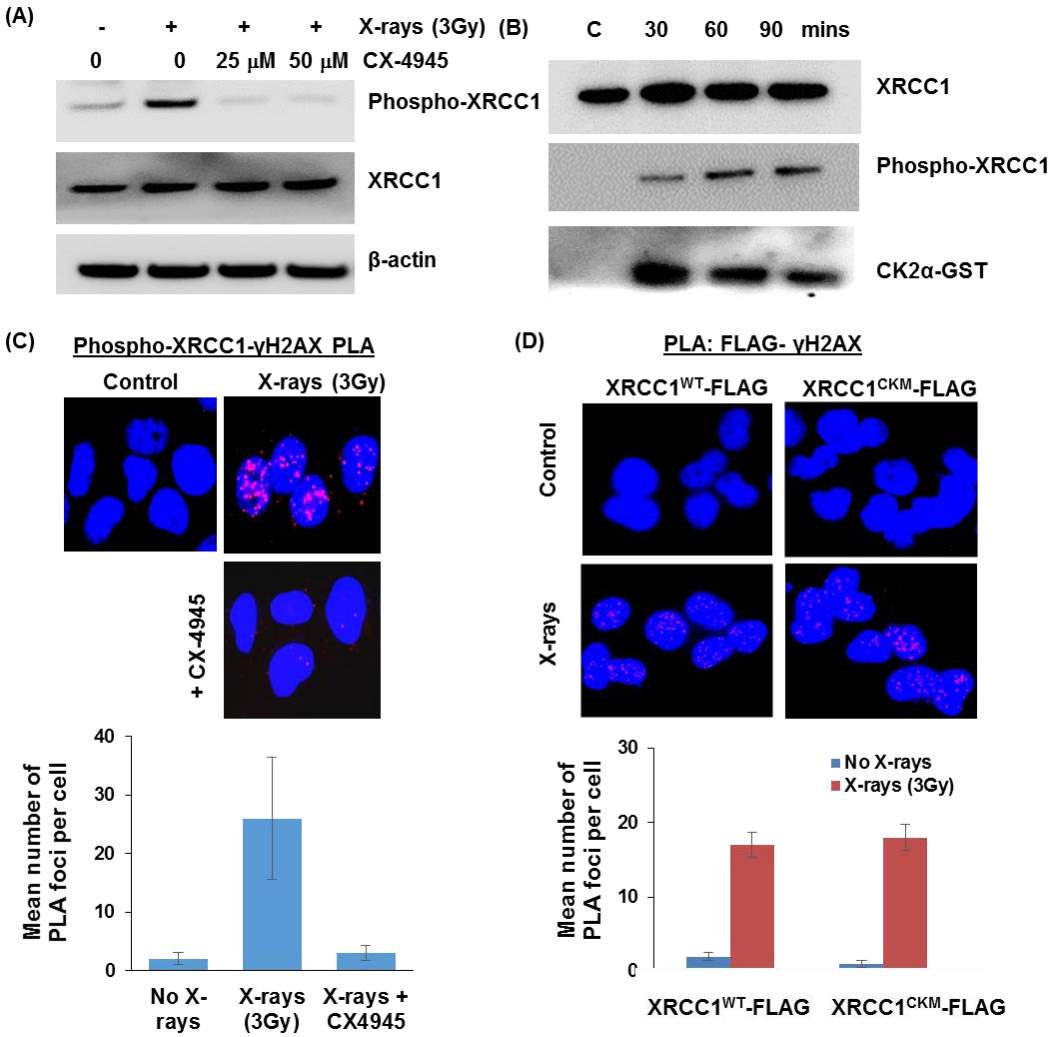


Figure 24. CK2 phosphorylates XRCC1 after irradiation. (A) Western blot analysis of phosphorylated-XRCC1 and total XRCC1 in nuclear extracts from control U2OS cells, and those treated with X-rays and treated with CX-4945 (25 μM, 50 μM) prior to X-ray treatment. (B) Western Blot analysis of the reaction mix after incubating recombinant XRCC1 with purified CK2α-GST protein up to 30, 60, and 90 mins with a control lacking CK2α-GST. (C) PLA for phospho-XRCC1- γH2AX in control U2OS cells and those treated with X-rays and CX-4945 plus X-rays, (D) FLAG-γH2AX PLA in control and X-ray treated U2OS cells transiently expressing FLAG-tagged $XRCC1^{WT}$/$XRCC1^{CKM}$.

4.2.2.2. CK2 promotes Alt-EJ by phosphorylating XRCC1 after irradiation.

In order to test role of CK2 in Alt-EJ, we performed *in cell* repair of pNS in irradiated U2OS cells which were pretreated with CX-4945, along with untreated control. Sequencing analysis of the repaired plasmid clones obtained by transforming the plasmid extract from U2OS cells in *E coli*, showed that enhancement of Alt-EJ was significantly affected in absence of CK2 activity (Figure 25A). This suggests CK2 activity has a crucial role in Alt-EJ which could be through phosphorylation of XRCC1. To confirm that this effect could be due to phosphorylation of XRCC1 by CK2, we performed *in cell* repair assay in U2OS cells with depletion of endogenous XRCC1 by 3'UTR targeting siRNA and transient expression of $XRCC1^{WT}$ or $XRCC1^{CKM}$-FLAG (Figure 25B). Difference between relative fraction of Alt-EJ in XRCC1 depleted cells compared to those expressing $XRCC1^{WT}$ but not $XRCC1^{CKM}$ was statistically significant (P=0.0024 vs. P=0.2278, respectively). Thus, we conclude that $XRCC1^{WT}$ but not $XRCC1^{CKM}$ could restore enhancement of Alt-EJ in XRCC1 depleted cells, confirming that XRCC1 phosphorylation by CK2 is critical for Alt-EJ (Figure 25C).

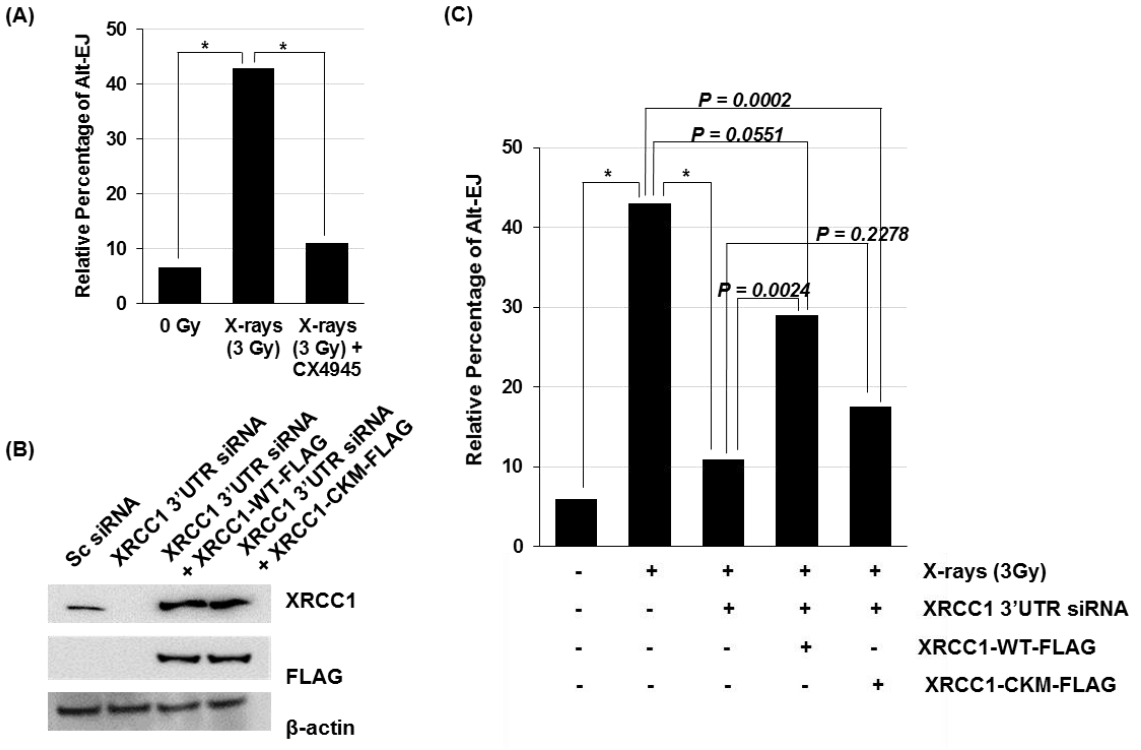


Figure 25. CK2 mediated XRCC1 phosphorylation is required of Alt-EJ. (A) Relative percentage of Alt-EJ in control U2OS cells and those treated with X-rays and CX-4945 plus X-rays. (B) *In cell* DSB repair of pNS was performed in control U2OS cells or those treated with XRCC1-3'UTR siRNA alone or with transient expression of $XRCC1^{WT}$-FLAG or $XRCC1^{CKM}$-FLAG, after X-ray treatment (* P<0.0001). (C) Western blot analysis of endogenous XRCC1 and transiently expressed $XRCC1^{WT}$/$XRCC1^{CKM}$-FLAG.

4.2.3. XRCC1 INTERACTS WITH END RESECTION ENZYMES MRE11 AND CtIP IN IRRADIATED CELLS TO PROMOTE ALT-EJ

4.2.3.1. XRCC1 forms distinct repair complexes with multiple repair proteins

Because distinct repair complexes are formed after X-ray-induced DNA damage, and we and others have found that XRCC1 is required for both SSB and DSB repair, we hypothesized that XRCC1 could form distinct BER/SSBR and Alt-EJ complexes for DSB repair. To test this we performed size fractionation chromatography of nuclear extracts isolated from irradiated U2OS cells to separate distinct protein complexes that could form in response to IR-induced DNA damage. Western analysis of the chromatographic

fractions showed that XRCC1 co-elutes with BER enzyme NEIL1 and DSBR factors Ku80 and Mre11 in distinct fractions that supports its role in multiple DNA repair pathways as a scaffolding protein (Figure 26). This also suggest extensive cross-talk between BER/SSBR and DSBR processes through distinct post-translational modifications, which warrants extensive characterization.

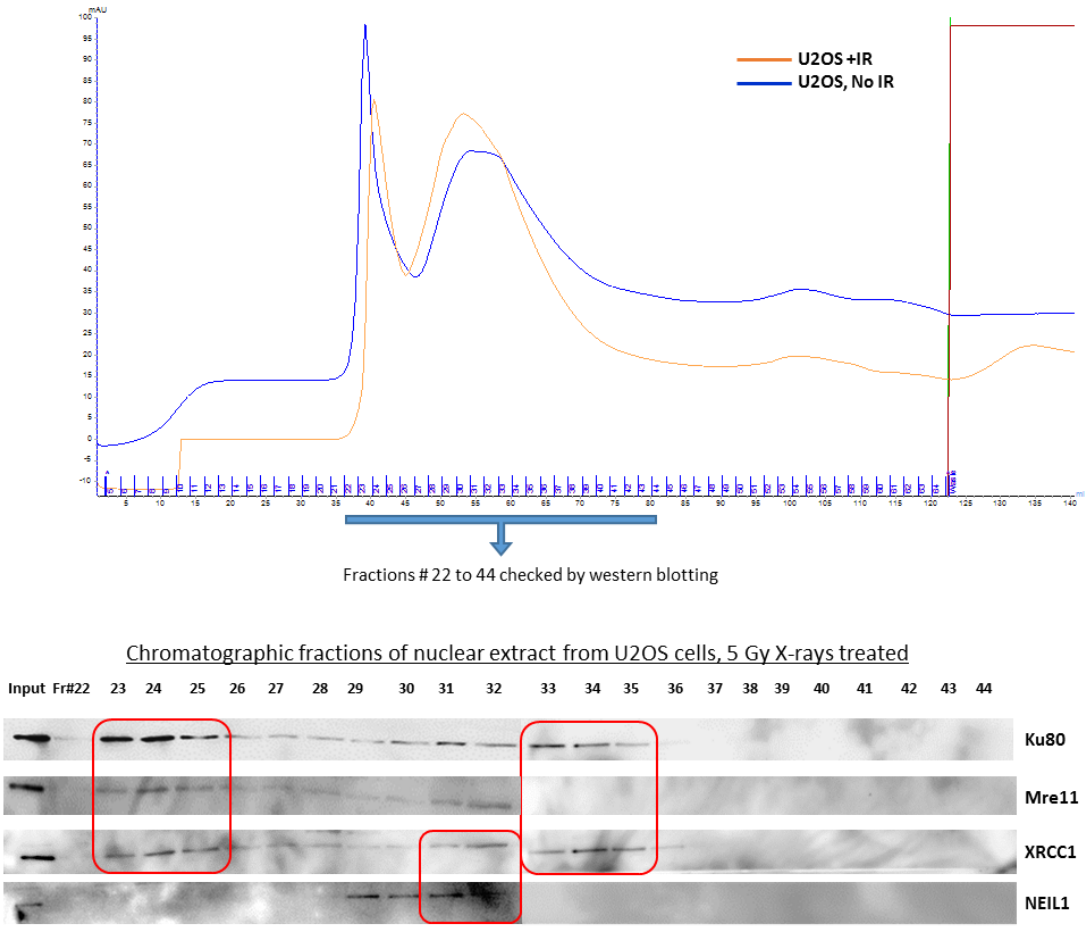


Figure 26. XRCC1 co-elutes with Mre11, Ku80 and NEIL1. (A) Chromatogram for purification of U2OS cell nuclear extract, 5 Gy X-ray treated and without any treatment. 2 mg dialyzed and 0.2 micron filtered nuclear extracts were passed through HiPrep 16/60 Sephacryl S-300 HR with ÄKTA pure chromatography system (GE Healthcare Life Sciences). Fraction number 22 through 44 analyzed through western blotting, (B) Western blot analysis of chromatographic fractions of nuclear extract from U2OS cells, 5 Gy X-rays treated; co-elution of XRCC1 with Mre11, NEIL1 and Ku80 has been highlighted in red circles.

4.2.3.2. Interaction of XRCC1 with Mre11 and CtIP increases after irradiation requiring CK2 mediated phosphorylation of XRCC1

Several studies have implicated Mre11 and CtIP in Alt-EJ where these enzymes generate 3' ssDNA overhangs with their end resection activity [95-97]. The overhang is a prerequisite for stabilizing the DSB termini via microhomology-dependent annealing prior to ligation by XRCC1/LIG3 [100]. However, how the interaction of these end resection enzymes with XRCC1/LIG3 are regulated during Alt-EJ is poorly understood. Since we observed recruitment of XRCC1 at X-ray induced DSBs (Figure 18) and its co-elution with DSBR factors Ku80 and Mre11 (Figure 26), we hypothesized that XRCC1 forms distinct Alt-EJ proficient repair complexes by interaction with other Alt-EJ factors. XRCC1-FLAG co-IP was performed from U2OS cells transiently expressing XRCC1-FLAG, and found that DNA end resection enzymes, Mre11 and CtIP which were earlier implicated in Alt-EJ, increases in XRCC1 co-IP after X-ray treatment. Interestingly Mre11 and CtIP couldn't enhance in XRCC1 co-IP when the U2OS cells were pre-treated with CK2 inhibitor, CX-4945 before X-ray treatment (Figure 27A). This was also verified with corresponding PLA studies (Figure 27B). LIG3 similarly decreased in XRCC1-FLAG co-IP when the cells were treated with CK2 inhibitor, which supports an earlier study [38], however PARP1 level was not affected indicating its interaction with XRCC1 is not regulated through CK2 (Figure 27A).

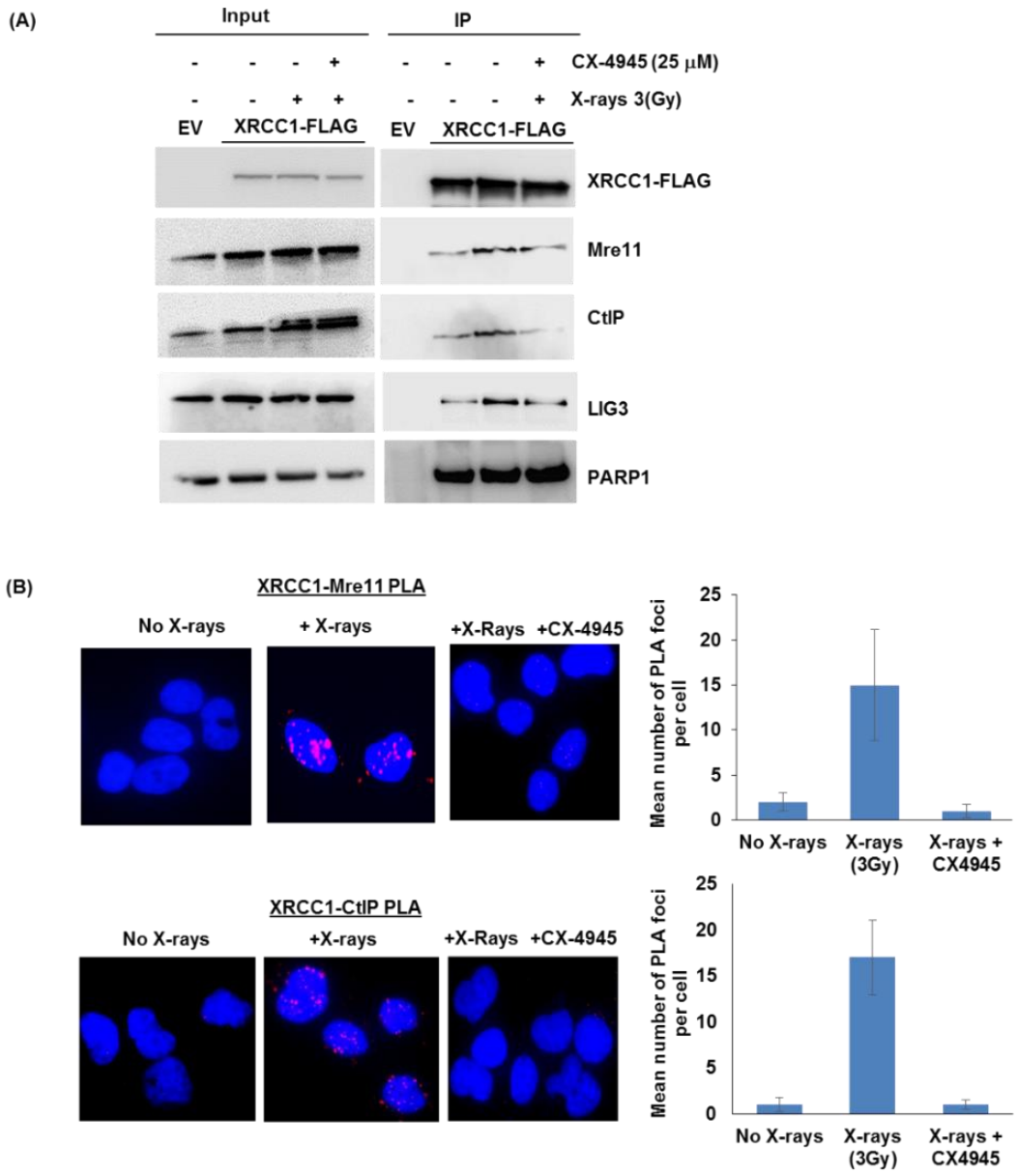


Figure 27. XRCC1 interacts with Mre11 and CtIP after irradiation that requires CK2 activity. (A) XRCC1-FLAG co-IP from control U2OS cells or those treated with 50 μM CX-4945 treatment and X-rays, as indicated, (B) Corresponding PLA studies for XRCC1-Mre11 and XRCC1-CtIP interaction in U2OS cells with similar treatments.

To further confirm that CK2 regulates interaction between XRCC1 and Mre11/CtIP, we performed PLA studies for interaction of XRCC1 WT/phospho-mutant with Mre11 and CtIP in U2OS cells transiently expressing $XRCC1^{WT}$-FLAG/$XRCC1^{CKM}$-FLAG. The results indicate that $XRCC1^{WT}$ but not $XRCC1^{CKM}$ interacts with Mre11 and

CtIP after irradiation (Figure 28). Thus phosphorylation of XRCC1 by CK2 is critical for XRCC1's interaction with end resection proteins Mre11 and CtIP that might have implication in Alt-EJ.

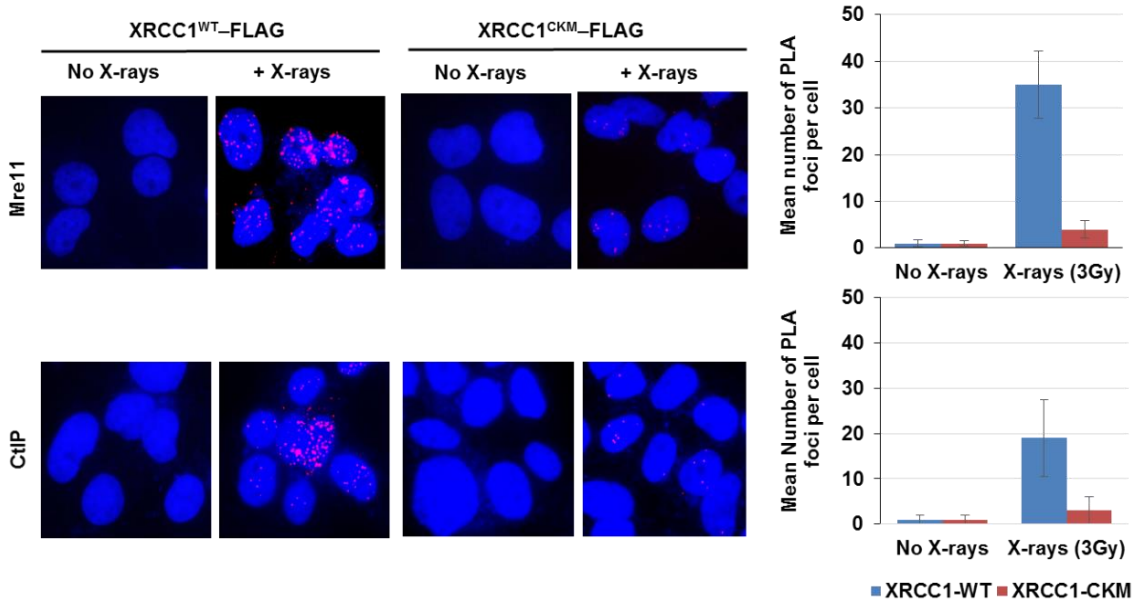


Figure 28. XRCC1^{WT} but not XRCC1^{CKM} interacts with Mre11 and CtIP after irradiation. PLA studies were carried out using mouse anti-FLAG and rabbit anti-Mre11 or anti-CtIP antibodies in U2OS cells transiently expressing XRCC1^{WT}/XRCC1^{CKM}-FLAG, with or without X-ray irradiation (3 Gy).

It was reported earlier that XRCC1/LIG3 interacts with Mre11/Rad50/Nbs1 (MRN) complex via BRCT domain of LIG3 and FHA domain of Nbs1 [100]. We tested if XRCC1-Mre11 interaction is affected when LIG3 and Nbs1 were depleted. FLAG co-IP studies in U2OS cells with siRNA mediated depletion of LIG3 and Nbs1 showed that XRCC1-Mre11 interaction was not hampered in either condition (Figure 29A), indicating that XRCC1 could directly interact with Mre11, which is regulated via phosphorylation of XRCC1 by CK2.

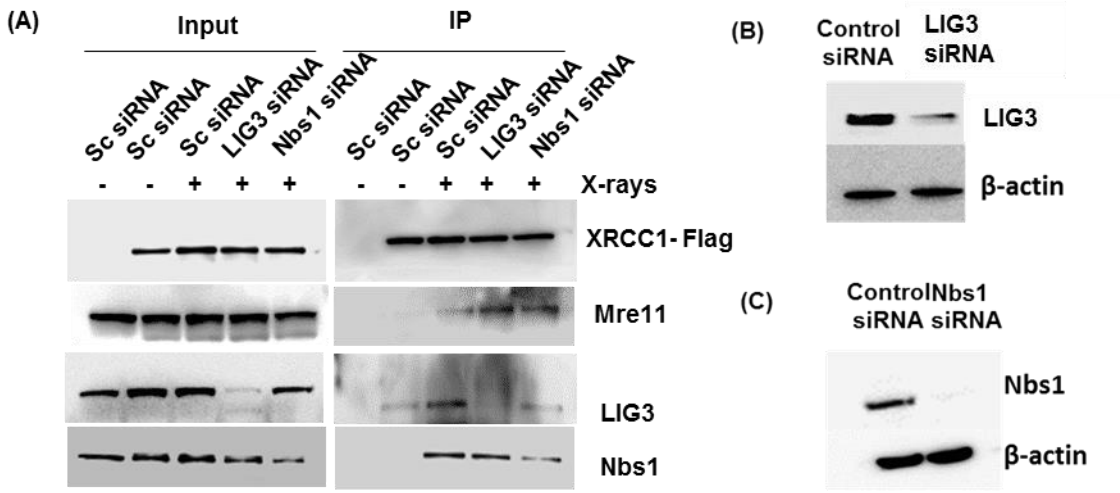


Figure 29. XRCC1^{WT} but not XRCC1^{CKM} interacts with Mre11 and CtIP after irradiation. (A) XRCC1-FLAG co-IP in U2OS cells treated with scramble siRNA (100 nM), LIG3 siRNA (100nM) or Nbs1 siRNA (100 nM) with X-ray treatment as indicated, showing Mre11 increases in XRCC1 IC after irradiation, and was not affected with either LIG3 or Nbs1 depletion. (B) Western blot analysis of total cell extract from U2OS treated 72 hrs with 100 μM control siRNA and DNA Ligase3 siRNA (C) Western blot analysis of total cell extract from U2OS treated 72 h with 100 μM control siRNA and DNA Nbs1 siRNA

4.2.3.2. phospho-XRCC1-FLAG immunocomplex from U2OS cells can carry out Alt-EJ *in vitro*

Several studies have underscored the role of dynamic repair complexes in specific repair processes such as BER, intra-strand cross-link repair, nucleotide excision repair, etc. [194-196]. Formation of such repair complexes via protein-protein interactions that could be regulated through post-translational modifications [174, 197]. XRCC1 is a classic example of a scaffold protein that facilitates formation of multiprotein repair complex for carrying out BER and SSBR [147]. Because we found that XRCC1 interacts with essential Alt-EJ factors Mre11 and CtIP regulated via its phosphorylation by CK2, we asked if XRCC1-FLAG IP could recapitulate Alt-EJ *in vitro*. XRCC1-FLAG co-IP was performed from the nuclear extract of U2OS cells transiently expressing XRCC1-FLAG (Figure 30)

and incubated the IP with the DSB repair substrate, pNS for 30 min at 37 ºC in appropriate buffer and then fortified the repair mix with purified LIG3 and incubated overnight at 16ºC, in order to promote ligation of the repaired joints. Sequence analysis of the plasmids from individual bacterial colonies obtained after transformation of *E coli* XL10 gold ultracompetent cells with the reaction mix showed that XRCC1-FLAG IP was proficient to carry out Alt-EJ *in vitro* (Figure 30, lower panel). However, unlike *in cell* repair of pNS, all *in vitro* repaired plasmids had only one identical joined sequence with loss of one microhomology region, indicating single type of Alt-EJ. This could be due to enhanced pulldown of specific Alt-EJ repair complex(es) or absence of some regulatory factors.

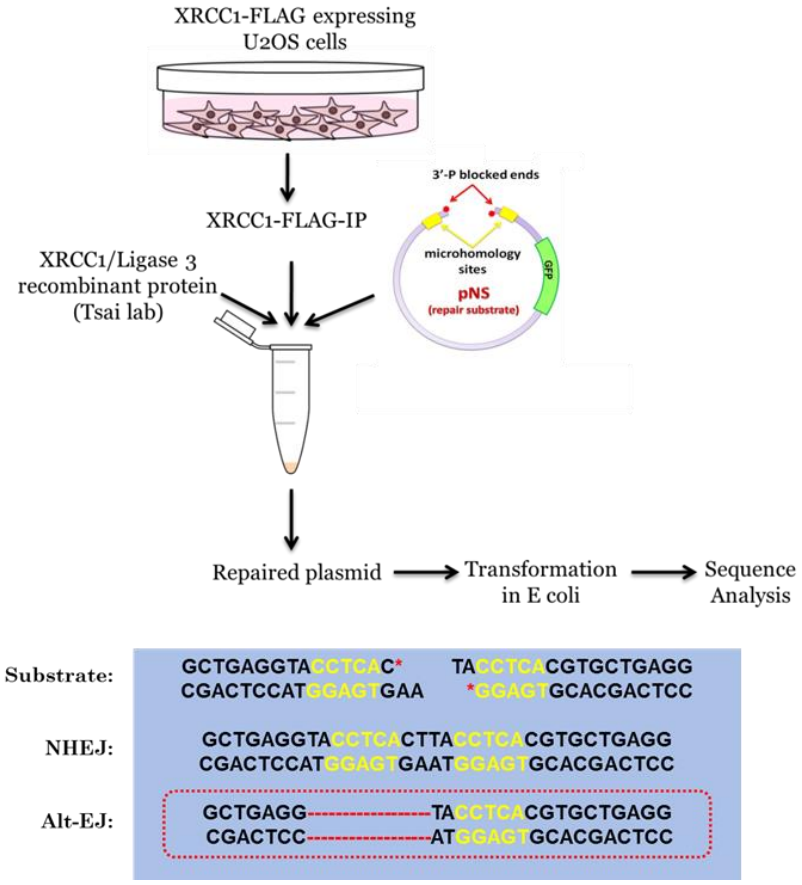


Figure 30. Schematic diagram of *in vitro* repair of DSB repair substrate, pNS with XRCC1-FLAG IP (details in text).

The ability of XRCC1-IP to recircularize the plasmid substrate was characterized as a function of number of resistant colonies obtained after bacterial transformation with the repair mix. Pre-treatment of the U2OS cells with 3 Gy X-rays enhanced repair capacity of XRCC1-FLAG IP, supporting our previous results from *in cell* assays (Figure 31A, 1st and 2nd bar). Pre-treatment of the XRCC1-FLAG IP with Mre11 exonuclease inhibitor, Mirin and Mre11 endonuclease inhibitor PFM03, *in vitro*, drastically affected plasmid recircularization that not only confirms presence of Mre11 in XRCC1 IP and but also requirement of its both endonuclease and 3'→ 5' exonuclease activities in Alt-EJ (Figure 31A, 4th and 5th bar).

Then it was checked if *in vitro* Alt-EJ proficiency XRCC1-FLAG IP is affected due to CK2 inhibition. XRCC1-FLAG IP from CX-4945 treated and irradiated U2OS cells were similarly incubated with pNS, along with control FLAG-IP. Very low level of pNS recircularization by XRCC1-IP from CX-4945 treated cells is consistent with our *in cell* repair data showing XRCC1 phosphorylation by CK2 is essential for Alt-EJ (Figure 31A, 3rd bar). To further confirm that formation of Alt-EJ proficient XRCC1 repair complexes requires CK2 mediated phosphorylation, we performed *in vitro* repair assays with $XRCC1^{WT}$-FLAG and $XRCC1^{CKM}$-FLAG co-IPs extracted from U2OS cells with comparable amount of transient expression of respective proteins. $XRCC1^{WT}$-FLAG IP was able to recircularize pNS via Alt-EJ however there was almost no plasmid repair with $XRCC1^{CKM}$-FLAG IP (Figure 31B, 2nd bar). $XRCC1^{WT}$-FLAG IP from irradiated cells could carry out plasmid recircularization significantly higher than that from control cells as found earlier (P=0.0216, 3rd bar). Although plasmid recircularization with $XRCC1^{CKM}$-FLAG IP from irradiated cells was greater than that from control cells (P=0.0017), the former was significantly lower compared to $XRCC1^{WT}$-FLAG IP from irradiated cells (P=0.001, last bar). This indicates that unlike $XRCC1^{WT}$, $XRCC1^{CKM}$ is inefficient to carry out DSB repair. Thus CK2 mediated phosphorylation of XRCC1 is essential for formation

of Alt-EJ proficient repair complexes through interaction with Mre11, CtIP and LIG3 and plausibly other factors, which are yet to be characterized.

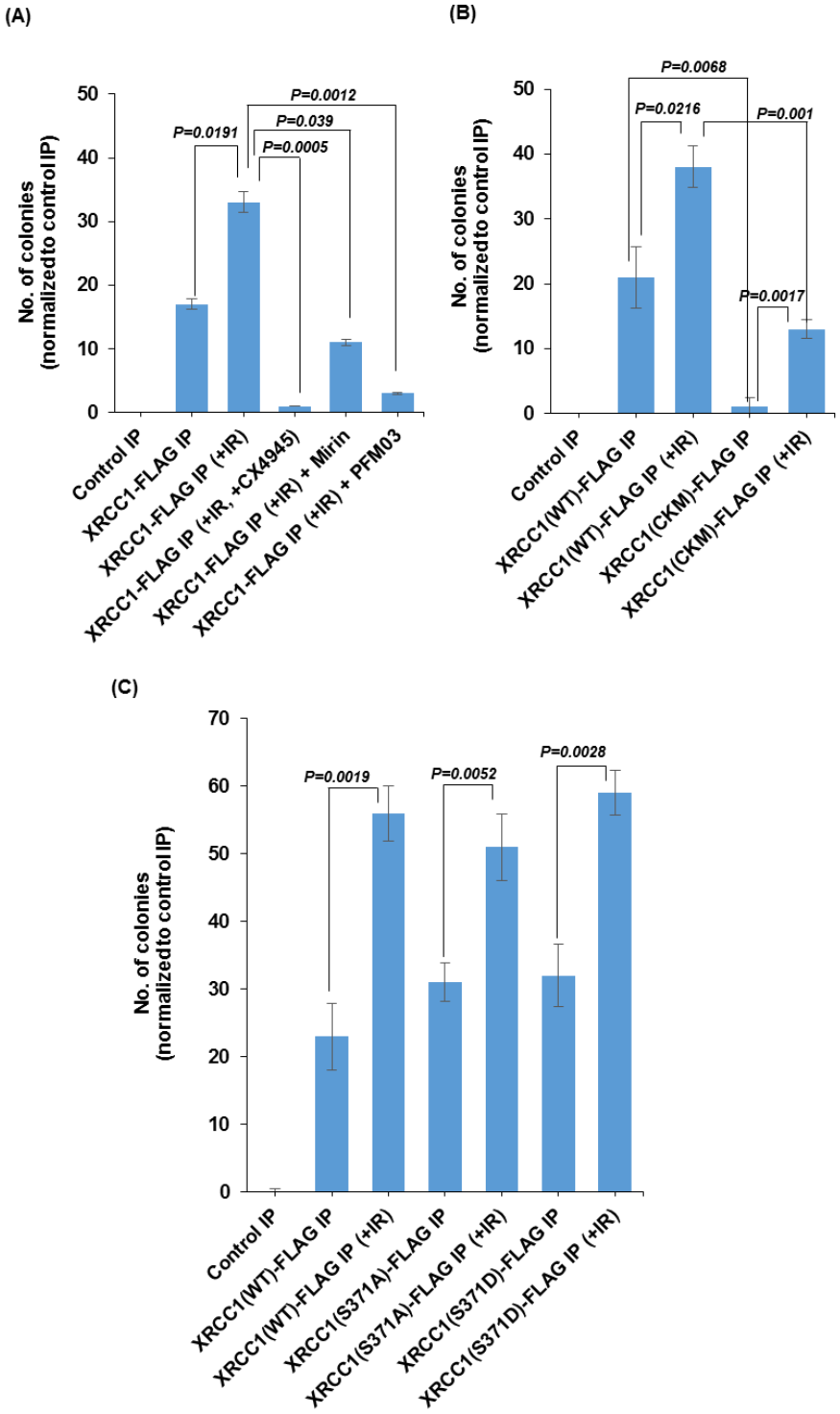


Figure 31. Phospho-XRCC1 immuno-complex can perform Alt-EJ in vitro. (A) Mean number of colonies obtained from the *in vitro* repair assay with XRCC1-FLAG IP from unirradiated or irradiated U2OS cells (1st, 2nd bar), or those treated with 50 mM CX-4945 with irradiation (3rd bar), XRCC1-FLAG IP from irradiated cells incubated with 100 nM Mre11 exonuclease inhibitor, mirin (4th bar) and that incubated with 100 nM Mre11 endonuclease inhibitor, PFM03 (5th bar). (B) Mean number of colonies obtained from the *in vitro* repair assay with $XRCC1^{WT}$/ $XRCC1^{CKM}$-FLAG IP from unirradiated or irradiated U2OS cells. (C) Mean number of colonies obtained from the *in vitro* repair assay with XRCC1-WT/ S371A/S371D-FLAG IP from unirradiated or irradiated U2OS cells.

Furthermore, it was checked if DNA-PK mediated phosphorylation of XRCC1 at Ser371 also affects its Alt-EJ capacity. An earlier report suggested that this phosphorylation results in XRCC1 dimer dissociation [165]. *In vitro* plasmid recircularization assay with XRCC1-WT/S371A/S371D-FLAG IP from transiently expressing U2OS cells showed that both non-phoshpho-mutant XRCC1-S371A and phospho-mimic-mutant XRCC1-S371D IP showed plasmid recirculrization proficiency comparable to XRCC1-WT IP (Figure 31C). This indicates that Ser371 phosphorylation by DNA-PK doesn't affect Alt-EJ capacity of XRCC1 repair complex. This could be also supported by the observation that Mre11 pulldown was almost similar in XRCC1-WT, S371A and S371-D FLAG IPs (data not shown). Thus, CK2 specifically regulates formation of XRCC1-Alt-EJ complexes.

4.3. DISCUSSION

NHEJ has been extensively characterized as the predominant and fast DSB repair pathway in human cells, including replicating cells, that repairs up to 75% of DSBs within 30 mins [16, 40, 88, 198]. However, ionizing radiation, such as X-rays, induce complex genome damage, all of which cannot be processed through NHEJ, and could follow slower repair processes that include HR and the more error-prone Alt-EJ [199]. It is poorly understood how these multiple discrete repair processes interact and which molecular factors make the pathway choice *in vivo*, as this could differ depending upon the

complexity of the damage [200]. Although NHEJ and HR have been considered the major players in DSB repair, Alt-EJ has been recognized recently as another pathway that could contribute to DSB repair more significantly than reported earlier [93, 201, 202]. Microhomology sequence-dependent repair processes of Alt-EJ are more likely to happen in the mammalian genome, which has large repetitive sequences [16, 203, 204], and could be enhanced in cancer cells conferring additional resistance against radiation and chemotherapeutics, as has been frequently reported [205, 206]. Although several factors of Alt-EJ have been identified and many others are yet to be characterized, the prevailing questions of when and how this error-prone pathway kicks into action, were the major goals for investigation in our current study.

We investigated the underlying molecular mechanism behind enhancement of Alt-EJ following irradiation. We and other groups have reported the role of dynamic protein complexes in response to genotoxic stress, for repairing distinct DNA lesions [28, 196, 207]. Formation of such repair complexes are often facilitated through the scaffolding action of certain proteins, and are regulated through post-translational modifications [147, 197]. Since XRCC1 is a widely characterized SSBR scaffold protein that is also required for Alt-EJ in cancer cells, we checked the Alt-EJ proficiency of the XRCC1 complex through *in vitro* assays. It was observed that Alt-EJ-proficient XRCC1 complex(s) are enhanced after irradiation, and that this is regulated by CK2 through phosphorylation at multiple serine and threonine residues located at the disordered region between the two BRCT domains {as reviewed in [21, 147]}. CK2-mediated phosphorylation of XRCC1 not only provides stability against proteasomal degradation [38], but also reduces its affinity for naked DNA [37]. Thus, XRCC1, which is otherwise sequestered at chromatin, could be mobilized for the formation of multi-protein complexes that are required for repair activities. Such multiple interactions are facilitated through the conserved N-terminal and BRCT domains of XRCC1; additionally, the unstructured domain between the two BRCT domains, which harbors CK2 phosphorylation sites, could also attain a dynamic

conformation, thereby aiding unique protein-protein interactions. This concept is supported by several studies underscoring the role of intrinsically disordered regions in facilitating dynamic protein-protein interactions [208], including ours, showing disordered C-terminal domain of NEIL1 is crucial for BERosome complex formation [28]. This demands further structural studies on XRCC1 to characterize its CK2 phosphorylation-dependent interaction with Mre11. Although XRCC1 phosphorylation is significant for the enhancement of Alt-EJ, CK2 could regulate Alt-EJ through multiple substrates, as CK2 inhibition drastically affected Alt-EJ. CK2-mediated MDC1 phosphorylation is required for the recruitment of the MRN complex at DSBs, thus regulating HR [209]; however it would be interesting to characterize its role in Alt-EJ.

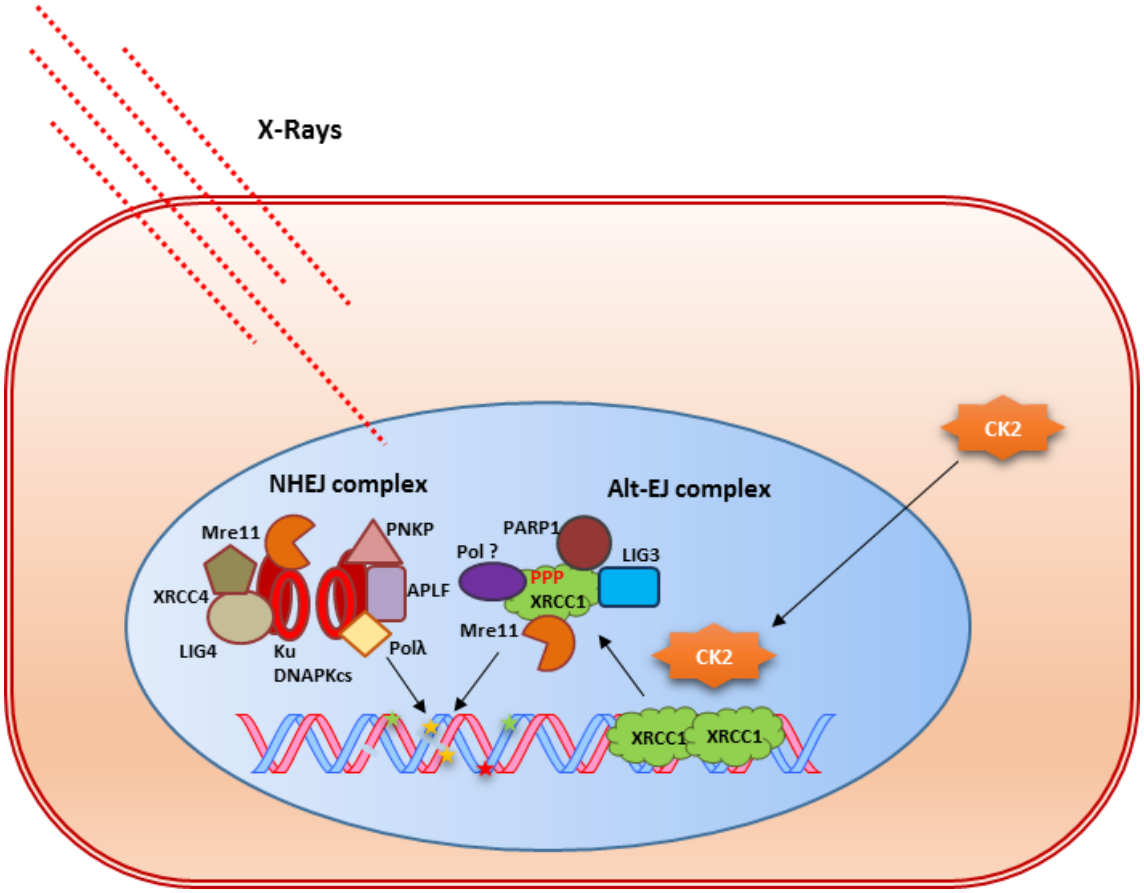


Figure 32. Mechanism for enhancement of Alt-EJ after irradiation. CK2 translocate to nucleus as a stress response when cancer cells are irradiated and along with many substrates phosphorylates XRCC1 bound to the chromatin or in the nuclear matrix. This enhances its interaction with DNA repair proteins including end resection enzymes Mre11 and CtIP, leading to formation of Alt-EJ proficient repair complexes. Complex DSBs which fail to get repaired through NHEJ can undergo extended resection at the ends and repair through Alt-EJ proficient XRCC1 complexes. PARP1 also interferes with Ku at DSBs leading to a competition between Alt-EJ and NHEJ at IR-induced DSBs. Moreover, Alt-J could be preferred at complex DSBs which have random microhomology sequences at the ends.

Some groups have debated the requirement of XRCC1 in Alt-EJ for VDJ recombination and chromosomal translocation [98, 99]; however, Alt-EJ in IR-induced genome damage could utilize a distinct repertoire of proteins. X-rays induce several-fold more number of oxidized bases and SSBs along with DSBs in close proximity in the genome, often referred to as damage clusters [9], thereby leading to the recruitment of both SSBR and DSBR factors at these damage clusters. Moreover, closely spaced SSBs or oxidized bases could lead to generation of secondary DSBs with longer ssDNA overhangs which have poor affinity for Ku [210, 211]. Another possibility is that SSB factors PARP1 and XRCC1 which are recruited as early as 5 mins after damage [33, 150], could prevent access of Ku heterodimer at secondary DSBs [102]. Thus, a significant fraction of complex DSBs and secondary DSBs that fail to undergo NHEJ, could attempt to undergo homology-directed repair that requires generation of 3' overhangs through resection at 5' end [199]. However, extensive end resection (>30 nt) could lead to binding of RPA to the ssDNA, which inhibits Alt-EJ through stabilization of the strands and subsequent replacement of RPA with Rad51 would drive HR [212]. Thus, the pathway choice at the resected ends could be dependent upon the extent of resection, length of the available homology and available repair complexes [213]. HR, which requires extensive end resection initiated by Mre11 and CtIP, followed by Exo1 and BLM1 [68, 214], and the formation of Rad51 dependent pre-synaptic filaments, is a highly coordinated repair process that could take as long as 7 h to complete [16, 74]. The requirement of Alt-EJ for only limited end resection, and DSB rejoining utilizing the limited repertoire of proteins, could be highly advantageous

to cancer cells to evade death by apoptosis induced by DNA damaging agents. It has been suggested that Alt-EJ could be particularly enhanced in specific cancer types due to either defective NHEJ or HR machinery or overexpression of specific Alt-EJ factors, which if targeted could reduce radiation resistance in tumors [112, 113]. However, enhancement of Alt-EJ due to formation of dynamic repair complexes induced by IR-associated DNA damage response is a novel concept. In our study, we provide strong evidence that increase in Alt-EJ after irradiation is a robust event due to CK2 phosphorylation dependent enhancement of XRCC1 repair complexes that could operate parallel to NHEJ or could compete with it (Figure 32). Thus enhanced Alt-EJ can provide additional radiation resistance to cancer cells at the cost of generating further mutations and chromosomal translocation.

CHAPTER V: SCAFFOLD ATTACHMENT FACTOR-A (SAF-A) COORDINATES A TEMPORAL REPAIR AT IONIZING RADIATION INDUCED DAMAGE CLUSTERS IN THE HUMAN GENOME

5.1. INTRODUCTION

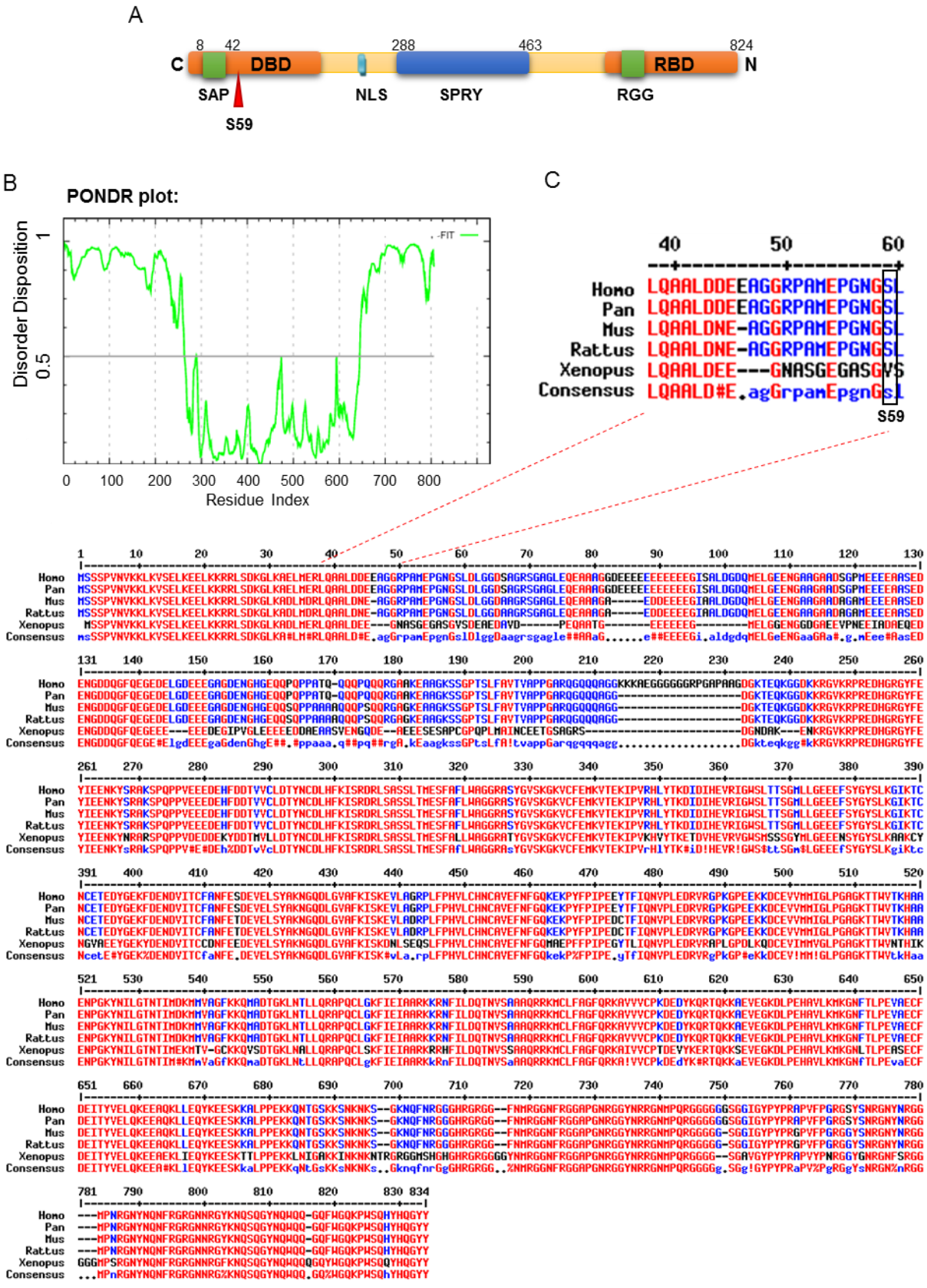
It has been discussed in Chapter I that IR as well as radiomimetic drugs induce clustered genome damage that consists of cytotoxic DSBs with nonligatable termini, and several folds more SSBs, and bi-stranded clusters of AP sites and oxidized bases [215]. Genome damage activates a cascade of signalling that begins with binding of Mre11/Rad50/Nbs1 (MRN) complex to the DSBs followed by activation of protein kinase ATM, which subsequently activates the cell cycle checkpoint and DNA repair pathways [216-218]. DSBs in mammalian cells are preferentially repaired via NHEJ that involves binding of Ku to the DSB site that competes with MRN and limits end resection that is requisite for HR and Alt-EJ. Base lesions and AP sites on the other hand are recognized by specific DNA glycosylases (DG) and APE1 which generate SSB intermediates, resulting in recruitment of PARP-1 and XRCC1/LIG3 that induces formation of BER/SSBR complexes [12, 20, 21]. How multiple functionally distinct repair pathways, particularly NHEJ and BER, are coordinated in repairing IR-induced damage clusters in the mammalian genome have not been investigated. The SSB intermediates generated during repair of oxidized bases and AP sites, if in the proximity of an unrepaired DSB, could cause additional DNA sequence loss [215, 219]. Furthermore, bi-stranded base lesions/AP sites and SSBs could produce secondary DSBs [220-224]. Hence, we hypothesized that NHEJ precedes BER.

Recent studies have documented non-canonical role of heterogeneous nuclear ribonucleoproteins (hnRNPs), a family of RNA/DNA binding protein in IR-induced DNA damage response which couples RNA metabolism pathway to DNA repair [225-227].

Particularly, one of the hnRNP proteins, hnRNP-U also called scaffold attachment factor A (SAF-A), was reported to be phosphorylated by key NHEJ factor DNA-PK and influence DSB repair through association of the NHEJ complex to nuclear matrix attachment DNA sequences at the break site [225, 228]. Our group characterized the presence of SAF-A in the IPs of both the DGs NEIL1 and NEIL2 and its functional implication in stimulating BER [30, 229]. SAF-A's role in repair of both DSB and oxidative base lesions led us to hypothesize if could regulate temporal coordination between the two pathways at IR-induced damage clusters, in order to maintain genomic stability.

5.1.1. SCAFFOLD ATTACHMENT FACTOR-A (SAF-A), A NON-CANONICAL DNA REPAIR PROTEIN

SAF-A, also known as hnRNP-U, belongs to a ubiquitously expressed family of RNA/DNA binding protein, heterogeneous ribonucleoprotein, 50% of which is tightly bound to the nuclear matrix [230]. It has been reported to participate in various facets of RNA metabolism like alternative splicing, mRNA transport and stability [231-233] and regulate gene activation processes [234, 235]. Hypomorphic SAF-A mutant mice are embryonic lethal indicating that it is an essential gene for cell viability [236]. SAF-A preferentially binds to A/T rich double stranded DNA or scaffold attachment regions in the chromatin through its N-terminal DNA binding domain (DBD) that contains SAP motif and to G/U rich heterogenous RNA through its C-terminal RNA binding domain (RBD) with RGG motif (Figure 33A). Both these conserved nucleic acid binding domains are highly disordered (Figure 33B) [21]. SAF-A also has a nuclear localization signal and a SPRY (*SPore lysis A* and *RYanodine receptor*) domain, whose function has not been characterized yet.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Mus LQAALDNE-AGGRPAMEPGNGSL
Rattus LQAALDNE-AGGRPAMEPGNGSL
Xenopus LQAALDEE---GNASGEGASGVS
Consensus LQAALD#E.agGrpamEpgnGsl
S59

Figure 33. Conserved domains and DNA-PK phosphorylation site of SAF-A. (A) Schematic representation of conserved domains of SAF-A: a putative DNA-binding domain (DBD) containing SAP motif and Serine59 residue, phosphorylated by DNA-PK/PLK1, a nuclear localization signal (NLS), a SPRY (*SPore lysis A* and *RYanodine receptor*) domain, and a RNA-binding domain (RBD) containing RGG motif, (B) PONDR (Predictor of Natural Disordered Regions) plot for SAF-A [21], (C) Multiple sequence alignment of SAF-A protein sequences of Homo sapiens (Q00839.6), Pan troglodytes (NP_001267207), Mus musculus (Q8VEK3), Rattus norvegicus (NP_476480.2) and Xenopus laevis (AAH84742.1) *(Multalin interface page – Inra);* zoomed region showing Serine59 residue is conserved among mammals.

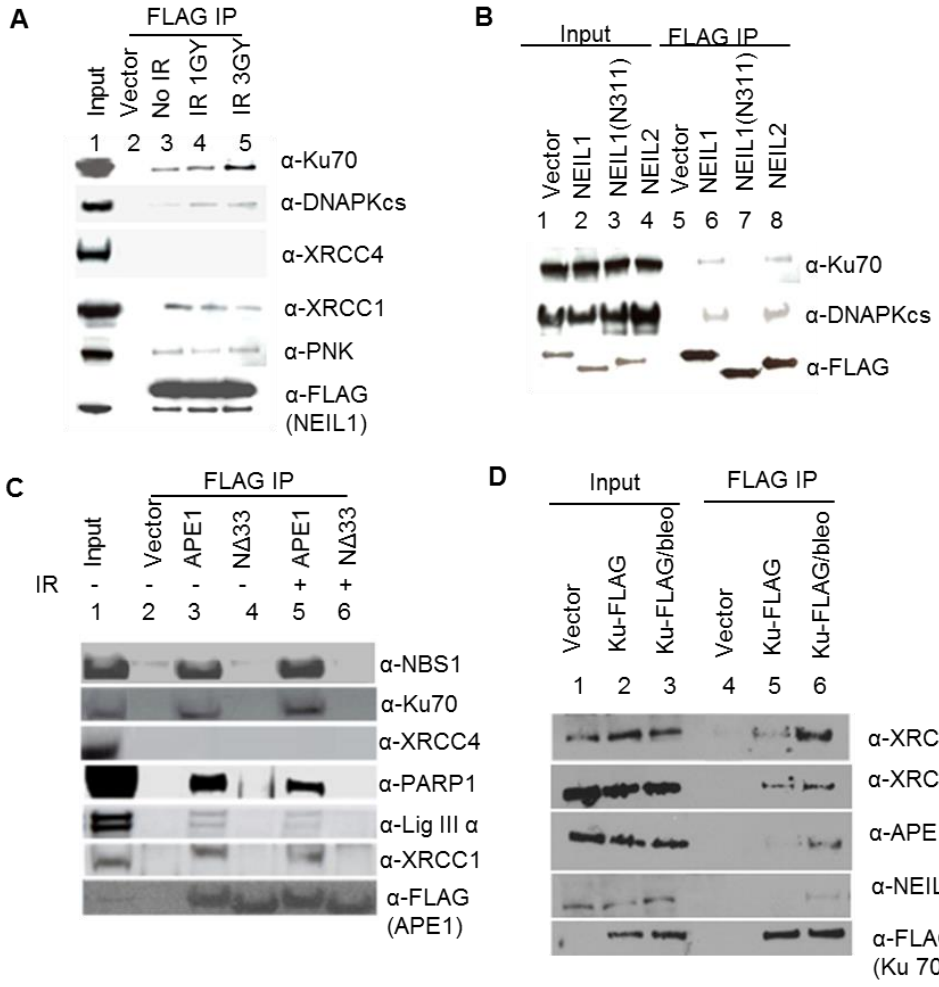
DNA-PK phosphorylates itself and myriad of proteins that not only include NHEJ factors like Artemis and XRCC4, but other DNA damage response and repair proteins like H2A.X, RPA and WRN, RNA metabolism proteins, RNA Pol I, Oct-1, c-fos and c-jun, etc [237]. SAF-A was reported to be one of many substrates of DNA-PK in two independent studies by Berglund et. al. and Britton et. al. [225, 228]. DNA-PK phosphorylates SAF-A in response to DNA damage at serine 59 residue which is conserved in mammals but not in lower vertebrates (Figure 33). However how SAF-A could influence DSB repair is unknown. Recently Douglas et. al. showed that SAF-A is also phosphorylated at serine 59 residue by a mitotic protein kinase, Polo-like kinase 1 (PLK1) and implicated its role in mitosis [116].

5.1.2. KU INHIBITS EARLY BER PROTEINS VIA DIRECT INTERACTION

Previously, we found that NHEJ proteins Ku70 and DNA-PKcs are present in the IP of FLAG-tagged early BER proteins NEIL1 and APE1 from HEK293 cell extracts, which enhanced after irradiation (Figure 34A). Pre-treatment of the cell extract with ethidium bromide ensured these interactions are not through nucleic acids. Moreover, C-terminal domain truncated mutant of NEIL1, NEIL1 (1-311) and N-terminal deletion mutant of APE1, APE1-NΔ33 couldn't pulldown Ku, confirming both NEIL1 and APE1 could directly interact with Ku. Similarly, co-IP of NEIL2, a related DG that serves as the

primary back up for NEIL1 [27], also contained Ku70 and DNA-PKcs (Figure 34B, 34C). XRCC4, a late NHEJ protein, was not detected in the IP of either NEIL1 or APE1. The FLAG IP from HEK293 cells transiently expressing FLAG-Ku provided confirmatory evidence for Ku's interaction with the BER proteins NEIL1 and APE1. These interactions were enhanced after treatment with radiomimetic bleomycin (Figure 34D). These observations were further confirmed through *in situ* PLA studies and *in vitro* pulldown studies with recombinant Ku, NEIL1 and NEIL1 truncated domains (data not shown).

Next, it was tested if interaction with Ku affected the activities of NEIL1 or APE1 using 5-OHU or THF-containing duplex oligonucleotide substrates, respectively, as previously described [238]. The Ku70/80 heterodimer inhibited base excision/strand cleavage activity of full-length NEIL1 in a dose-dependent manner but not the N311 mutant (Figure 34E). This result indicates that inhibition of NEIL1 by Ku requires its physical interaction with NEIL1. Similarly, Ku was also found to inhibit other oxidized base-specific DGs NEIL2 and OGG1 (data not shown). Furthermore, Ku inhibited APE1's AP endonuclease activity with a THF-containing substrate (Figure 34F), and 3'dRP lyase activity at an SSB site in a duplex oligonucleotide substrate (data not shown). These preliminary studies suggested NHEJ should inhibit BER at IR-induced damage clusters.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1 2 3 4 5 6
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α-APE1
α-NEIL1
α-FLAG
(Ku 70)
E
5'-[32P]
(5-OHU)
Ku70/80
NEIL1
NEIL1 (N311)
1 2 3 4 5 6 7 8 9 10
S (51nt) -
P (25nt) -
F
5'-[32P]
(THF)
APE1
Ku70/80
(1-10nM)
1 2 3 4 5 6 7
S (43nt)
P (31nt)

Figure 34. Ku interacts with early BER proteins NEIL1 and APE1 and inhibits their activity. (A) Western blot analysis of Ku70, DNAPKcs, XRCC4, XRCC1 and PNK in FLAG-NEIL1 co-IP from HEK293 cells. (B) FLAG-NEIL1, FLAG-NEIL1(N311) and FLAG-NEIL2 co-IP from HEK293 cells with comparable transient expression to check Ku70 and DNA-PKcs levels. (C) FLAG-APE1 (WT) and FLAG-APE1(NΔ33) co-IP from HEK293 cells with comparable transient expression to check levels of Nbs1, Ku70, XRCC4, PARP1, LIG3, and XRCC1. (D) FLAG-Ku co-IP from control or bleomycin treated HEK293 cells to check levels of XRCC4, XRCC1, APE1, and NEIL1. (E) NEIL1 activity assay to cleave a radiolabelled 51 nt long duplex oligonucleotide containing 5-OHU, with recombinant NEIL1 or NEIL1(N311) and increasing dose of Ku70/Ku80, as indicated. Product and substrates are separated in 10% TBE urea gel and exposed to phosphorimager screen which was analysed by Typhoon FLA7000. (F) APE1 activity assay to cleave a radiolabelled 51 nt long duplex oligonucleotide containing THF, with recombinant APE1 and increasing dose of Ku70/Ku80 as indicated and analysed as mentioned above. *(unpublished data, Hegde M.L.)*.

5.1.3. SAF-A RELIEVES KU INHIBITION OF NEIL1

Since we previously found that SAF-A interact with NEIL1 and stimulates its activity [229], effect of SAF-A phosphorylation (at S59) by DNA-PK on BER was characterized. Fluorescence and affinity co-elution studies indicated that NEIL1 has 10-fold stronger affinity for WT SAF-A than S59D mutant, indicating that phosphorylation negatively impacts NEIL1-SAF-A interaction (Figure 35A, 35B). In contrast, Ku binding to the S59D mutant was higher compared to the WT SAF-A polypeptide. Thereafter, the effect of SAF-A phosphorylation on NEIL1's DG activity was analysed through *in vitro* repair assays. WT SAF-A but not S59D SAF-A stimulated NEIL1 activity, and could override Ku inhibition of NEIL1 (Figure 35C, 35D). Together, these results suggested that phosphorylated SAF-A, is a component of the NHEJ complex that ensures Ku inhibition of NEIL1 and may also contribute to NEIL1's dissociation from chromatin in order to prevent BER. SAF-A, dephosphorylated after completion of NHEJ, stimulates NEIL1 even in the presence of Ku, and acts as a molecular switch for the NHEJ-to-BER transition.

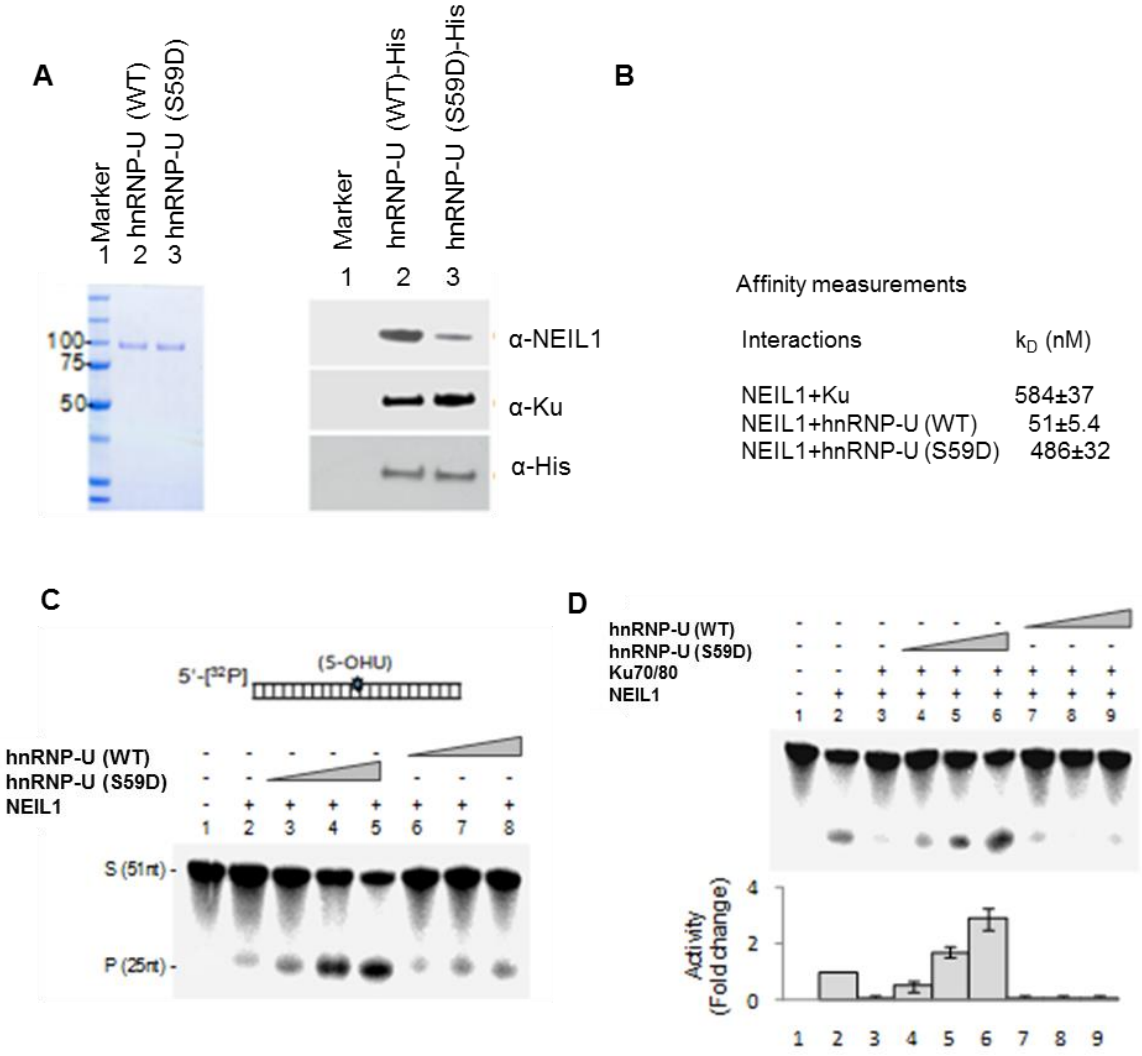

Figure 35. WT SAF-A but not S59D mutant overrides NEIL1 inhibition by Ku. (A) Purification of WT and S59D SAF-A to near homogeneity (left panel after Coomassie staining). *In vitro* His-pull down of untagged NEIL1 or Ku by His-tagged SAF-A (WT vs. S59D) bound to Ni-beads. (B) Affinity measurement of NEIL1 for Ku, WT SAF-A and the S59D mutant by fluorescence analysis. (C) NEIL1 activity assay to cleave a radiolabelled 51 nt long duplex oligonucleotide containing 5-OHU, with recombinant NEIL1 and increasing dose of either hnRNP-U(WT) or hnRNP-U(S59D), as indicated. Product and substrates are separated in 10% TBE urea gel and exposed to phosphorimager screen which was analysed by Typhoon FLA7000. (D) NEIL1 activity assay in presence of Ku70/Ku80 and increasing dose of either hnRNP-U(WT) or hnRNP-U(S59D), as indicated. *(unpublished data, Hegde M.L.).*

5.2. RESULTS

5.2.1. PHOSPHORYLATION OF SAF-A AT SER59 REDUCES NEIL1 RECRUITMENT AT THE CHROMATIN AT EARLY TIME POINTS AFTER X-RAY INDUCED DAMAGE

Since SAF-A is recruited and phosphorylated at the DSBs by NHEJ complex, and phosphorylated SAF-A cannot relieve Ku inhibition of NEIL1, we studied the kinetics of SAF-A phosphorylation and how it affects NEIL1 recruitment at the chromatin after IR-induced damage. Western blot analysis of chromatin extracts from HEK293 cells at different time points after irradiation showed that phosphorylation of SAF-A correlated with reduction of NEIL1 level at early time points of 15-30 mins (Figure 36A). This transient removal of NEIL1 from the chromatin was absent in chromatin extracts from DNA-PK inhibitor treated cells, where SAF-A was not phosphorylated. NHEJ of DSBs directly induced by IR occurs in the same 15-60 min window after irradiation, as indicated by an increase in 53BP1 levels in the chromatin fractions (Figure 36A) which is consistent with previous studies [217]. This suggests NEIL1 is restored to the chromatin at 1 h after irradiation, when DSB repair is mostly completed. To further confirm this observation, we analysed the chromatin extracts from HEK293 cells with 3'UTR-specific siRNA mediated knockdown of endogenous SAF-A and ectopically expressed WT SAF-A or the non-phosphorylable mutant. We observed release of chromatin-bound NEIL1 at early time points only in WT SAF-A expressing cell (Figure 36B). Additionally, PLA analysis showed *in-cell* interaction of NEIL1 with SAF-A in HEK293 cells at 30 min post-irradiation was significantly higher for WT SAF-A or non-phosphorylable S59A mutant compared to the phosphomimetic S59D mutant. This further confirms that NEIL1 dissociates from the chromatin 30 mins after irradiation due to phosphorylation of SAF-A, which was compromised when the cells expressed non-phosphorylable S59A mutant (Figure 36C). This suggests a direct link between SAF-A phosphorylation and NEIL1's dissociation from chromatin to prevent BER initiation until DSB repair by NHEJ is complete. Our results thus provide strong support for temporal regulation of NHEJ and

BER after treatment with IR, coordinated by SAF-A phosphorylation and regulating release of chromatin-bound NEIL1.

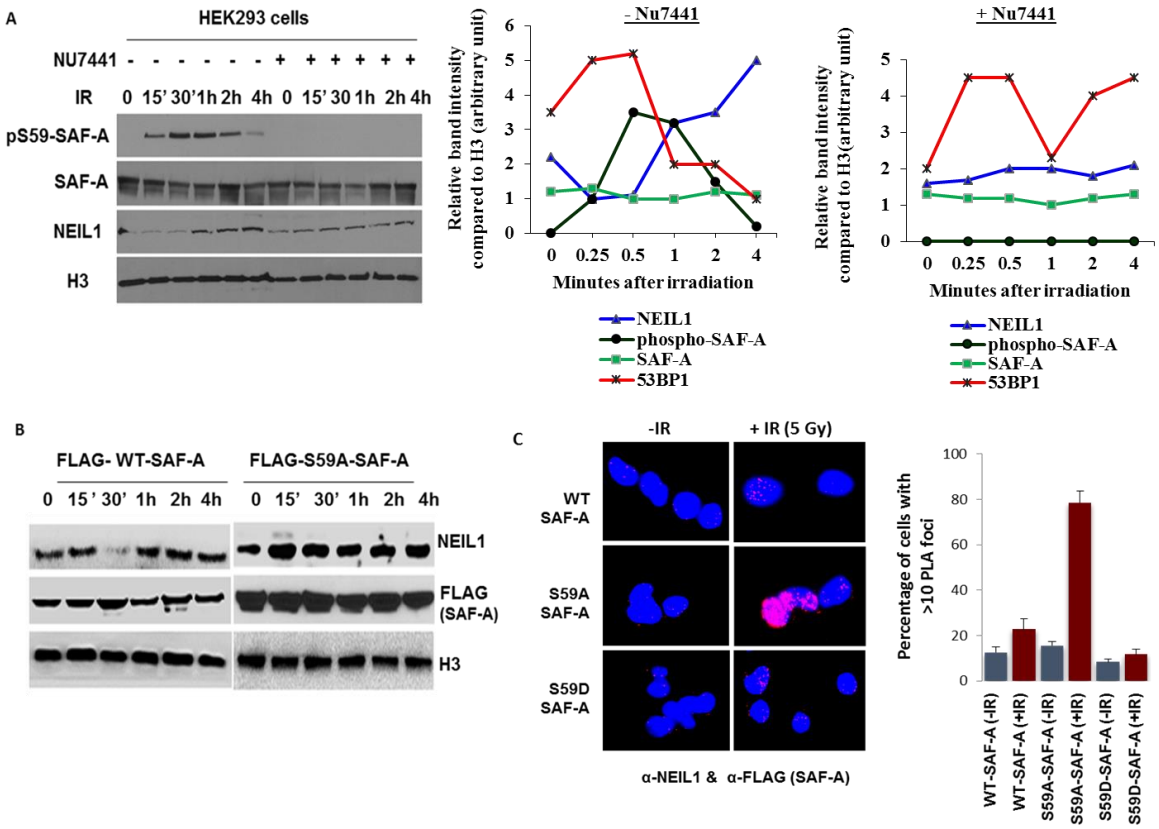

Figure 36. SAF-A phosphorylation at S59 reduces NEIL1 recruitment at the chromatin. (A) Analysis of levels of SAF-A, phospho-serine59 SAF-A, NEIL1, 53BP1 and H3 at the chromatin fraction of HEK293 cells with/without treatment with NU7441, at different time points after irradiation (0, 15m, 30m, 1h, 2h, 4h) (*unpublished data, Chunying Yang*). (B) Analysis of levels of NEIL1, SAF-A and H3 at the chromatin in endogenous SAF-A depleted HEK293 cells with transient expression of either FLAF-WT-SAF-A or FLAG-S59A-SAF-A. (C) PLA for FLAG-tag and NEIL1 to check interaction of FLAG-WT-SAF-A, FLAG-S59A-SAF-A or FLAG-S59D-SAF-A with NEIL1 in HEK293 cells at 30 m after irradiation; quantification of PLA foci is shown in the right panel.

5.2.2. KU FORMS DISTINCT REPAIR COMPLEX WITH SAF-A AND NEIL AFTER IRRADIATION

Our preliminary co-IP and *in vitro* co-elution studies showed that Ku interacts with both NEIL1 and SAF-A which increase after irradiation (Figure 34A-D, 35A). To further validate that Ku forms distinct protein complexes with both NEIL1 and SAF-A, size exclusion chromatographic analysis of control and irradiated U2OS cell nuclear extract was performed. The range of molecular weight for purification with HiPrep 16/60 Sephacryl S-300 HR column for globular proteins is 1×10^4 –1.5×10^6 with the largest complexes eluted first and the smallest molecules eluted at the end. Western blot analysis of the fractions from irradiated cells showed that SAF-A was mainly eluted in the early fractions # 23-29 with the peak at # 24 fraction, suggesting it is associated with large protein complexes (Figure 37). On the other hand, NEIL1 was eluted in fractions # 29-32, which could be smaller repair complexes. Ku80 was eluted in fractions # 23-35 with two distinct peaks in # 24 and # 31 which corresponds to SAF-A and NEIL1 respectively. In the fractions from untreated cells Ku eluted with SAF-A but not with NEIL1, confirming that Ku-NEIL1 interaction occurs only after irradiation and its physiological implication could be inhibition of BER activity at the chromatin, that has been previously observed via DG activity assays (Figure 35E, 35F).

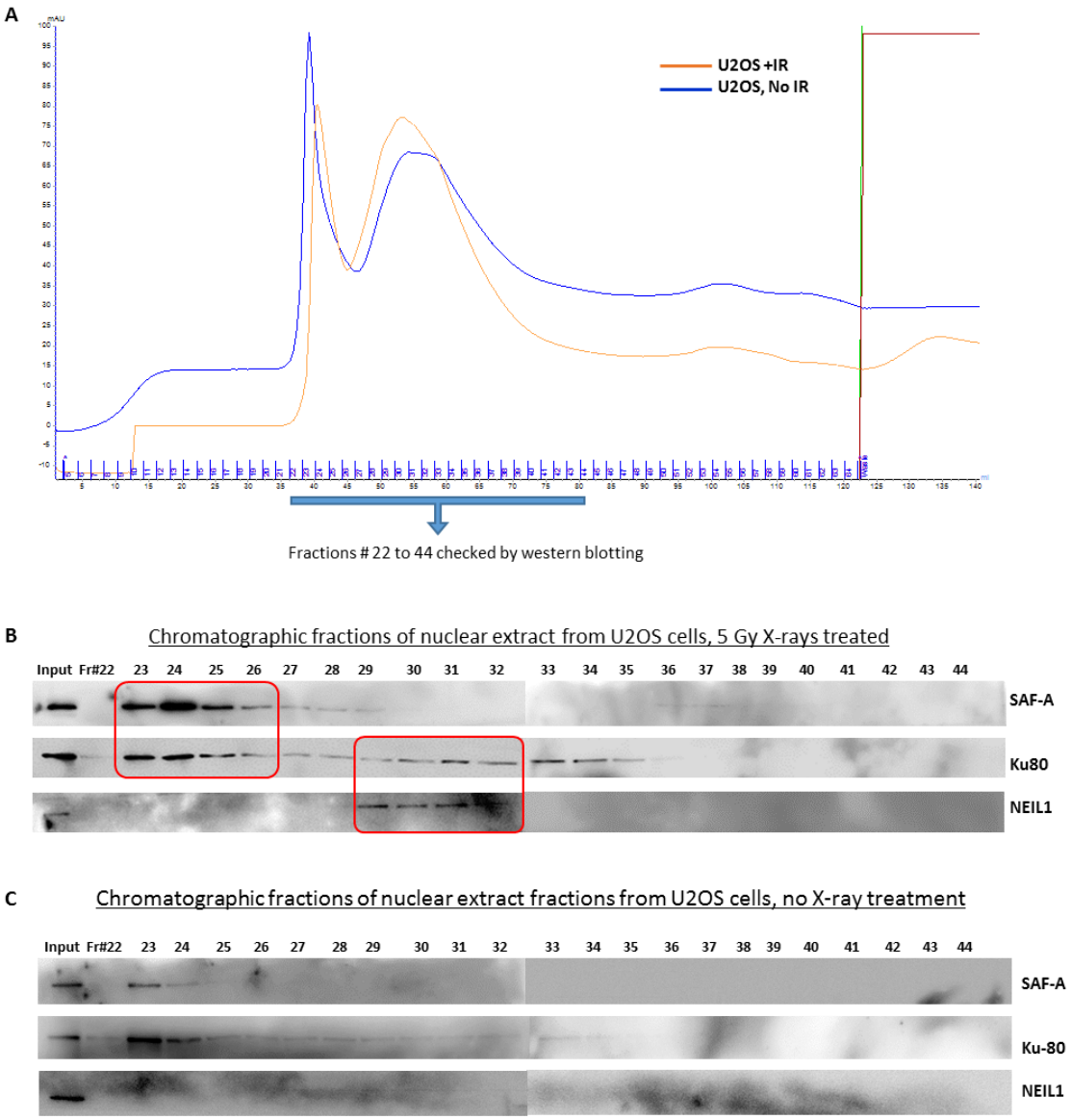

Figure 37. Gel filtration chromatographic analysis of control and irradiated U2OS cell nuclear extract. (A) Chromatogram for purification of U2OS cell nuclear extract, 5 Gy X-ray treated and without any treatment. 2 mg dialyzed and 0.2 micron filtered nuclear extracts were passed through HiPrep 16/60 Sephacryl S-300 HR with ÄKTA pure chromatography system (GE Healthcare Life Sciences). Fraction number 22 through 44 analyzed through western blotting, (B) western blot analysis of chromatographic fractions of nuclear extract from U2OS cells, 5 Gy X-rays treated, (C) western blot analysis of chromatographic fractions of nuclear extract from U2OS cells without any X-ray treatment; co-elution of SAF-A and NEIL1 with Ku80 have been highlighted.

5.2.3. PHOSPHORYLATION OF SAF-A AT SER59 IS CRITICAL FOR RADIOSENSITIZATION OF U2OS CELLS AFTER X-RAY INDUCED DAMAGE

Since SAF-A through its phosphorylation plays a critical role in coordination of NHEJ and BER at IR-induced damage clusters, it was checked if its depletion results in accumulation of unrepaired DSBs and radiosensitization of human cells. To monitor repair kinetics of DSBs induced by IR γH2AX foci kinetics assay was performed in post-irradiated control HEK293 cells, those with depletion of endogenous SAF-A with 3'UTR siRNA and expressing either WT SAF-A or non-phosphorylable mutant S59A SAF-A. γH2AX foci accumulated at 15 mins after irradiation which gradually reduced at 4 hrs in control HEK293 cells, however there were significant number of residual γH2AX foci in SAF-A depleted cells (Figure 38A). Depletion of endogenous SAF-A could be complemented by transient expression of WT SAF-A but not S59A SAF-A. This suggests SAF-A phosphorylation is essential for efficient DSB repair.

To check if depletion of SAF-A enhanced radiosensitzation of HEK293 cells clonogenic survival assay was performed. There was significant reduction of clonogenic survival of SAF-A depleted HEK293 cells compared to control cells. Moreover, transient expression of WT SAF-A in endogenous SAF-A depleted cells improved clongenic survival while S59 SAF-A further reduced clonogenic survival (Figure 38B). This confirms that SAF-A phosphorylation is critical for survival of human cells after irradiation.

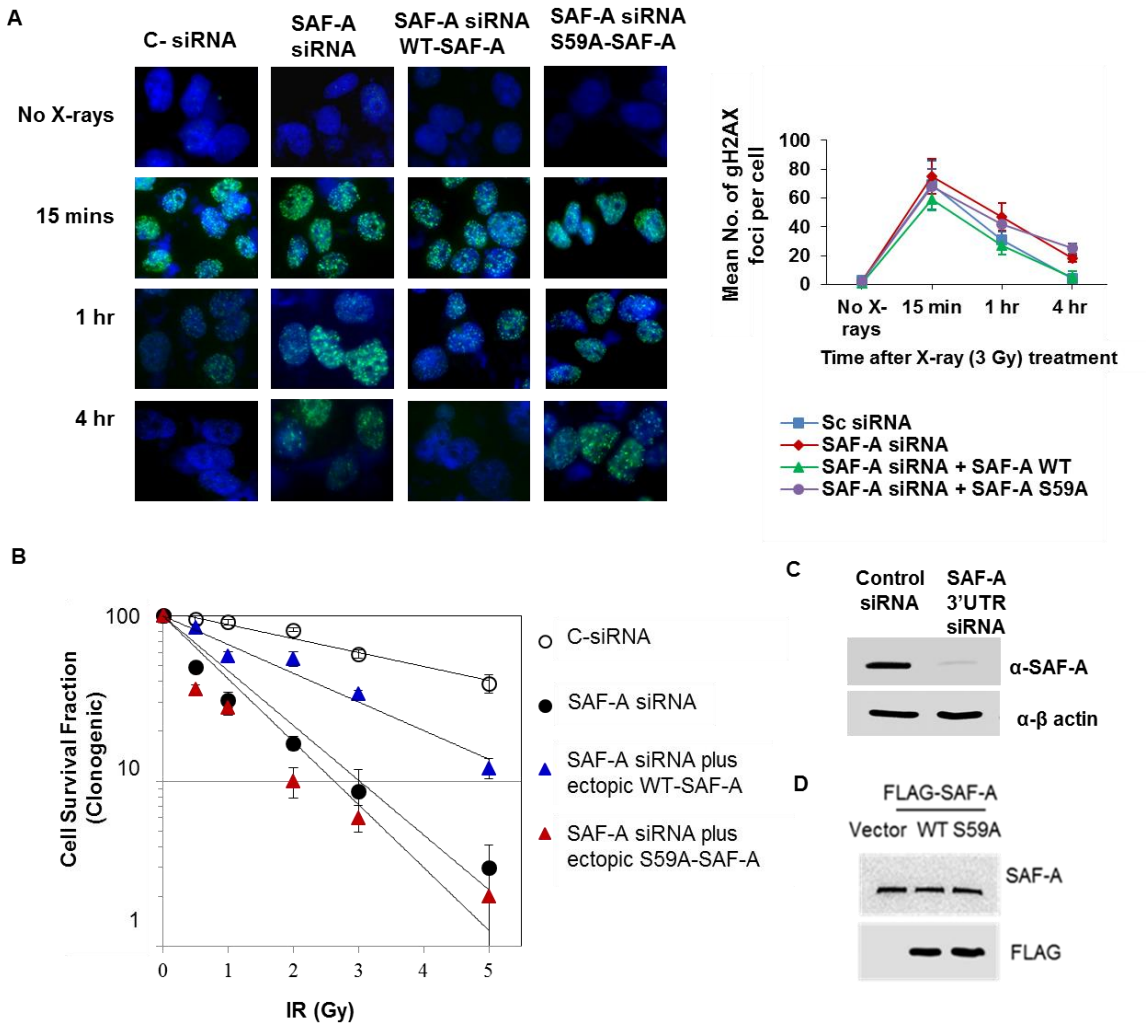

Figure 38. SAF-A phosphorylation is required for repair of IR-induced genome damage (A) γH2AX foci kinetics assay, (B) Clonogenic assay, performed with HEK293 cells transfected with 3'UTR-specific SAF-A siRNA alone or in combination with plasmids for FLAG- WT/S59A-SAF-A and irradiated at 48 h, (C) SAF-A 3'UTR siRNA, (D) FLAG-WT-SAF-A and FLAG-S59A-SAF-A expression compared to endogenous SAF-A.

5.3. DISCUSSION

While numerous studies have focused on the repair of IR-induced DSBs by the NHEJ pathway, bi-stranded, non-DSB lesion clusters, which include oxidized bases and AP sites that are generated at a much higher level than DSBs, have received little attention. It is likely that the deleterious effects of radiation are primarily caused by clustered damage

rather than overt DSBs alone [13]. Earlier studies suggested that overt DSBs in irradiated cells are re-joined first, followed by repair of clustered non-DSB damage at a slower rate [239]. However, how such distinct repair processes are coordinated has not been investigated. As demonstrated in this study and elsewhere [221], non-DSB damage, primarily repaired via the BER/SSBR pathway, also contributes to the radiosensitivity of tumor cells. Because BER generates intermediate SSBs, repair of IR-induced bi-stranded damage clusters could create additional DSBs and lead to loss of genomic sequences. Furthermore, these additional strand breaks in the vicinity of an overt DSB could cause large deletions.

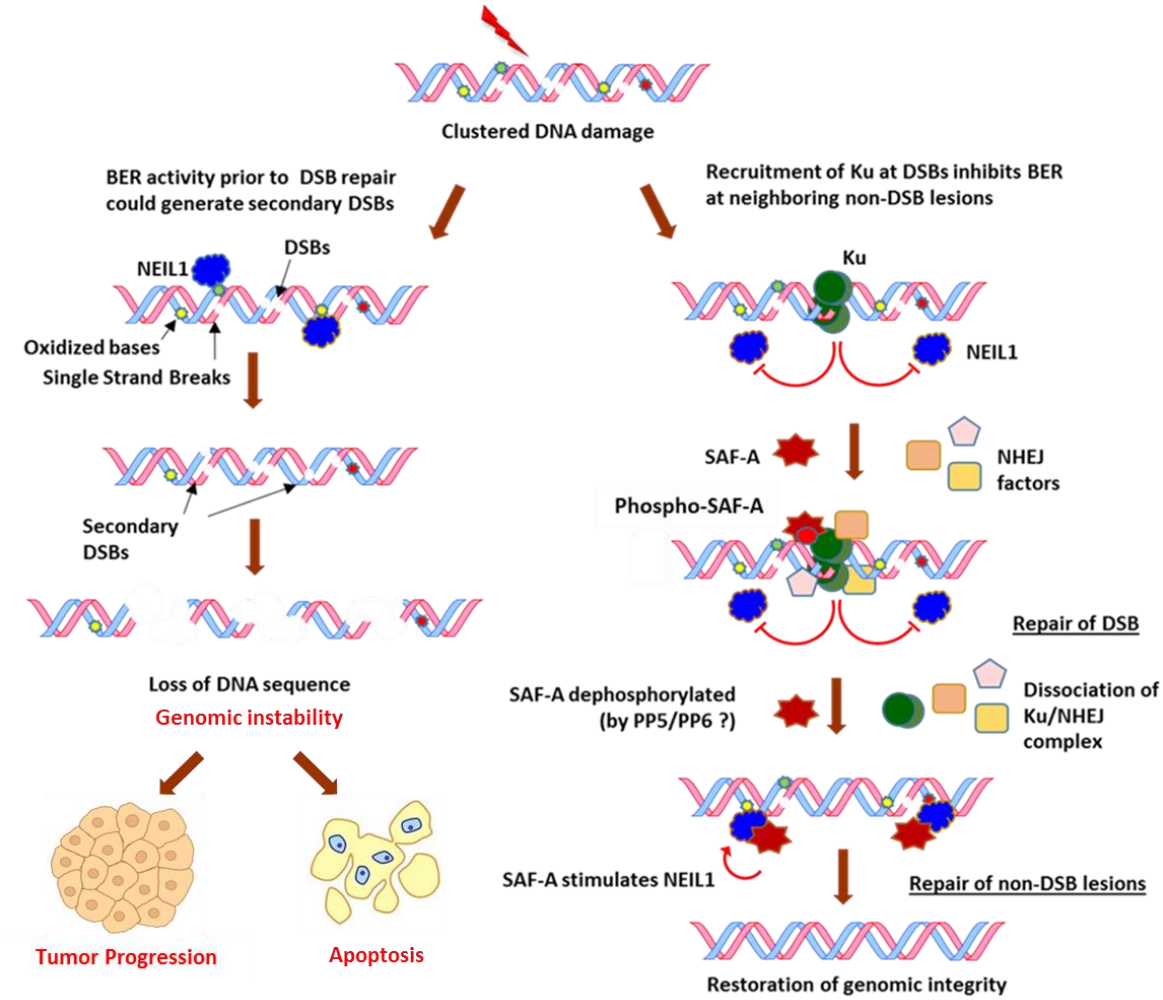

Figure 39. Model of temporal regulation of IR-induced clustered damage in human genome. Repair of DSBs in the IR-induced damage cluster is initiated by Ku recruited at DSB, which then assembles the NHEJ complex after recruiting and activating DNA-PKcs. Ku also inhibits BER of oxidized bases and AP sites. Early phosphorylation of SAF-A at Ser59 by DNA-PK correlates with transient dissociation NEIL1 from chromatin to prevent BER initiation. Residual DGs in chromatin are inhibited by Ku while NHEJ occurs. After completion of DSBR SAF-A is dephosphorylated, relieving Ku inhibition of BER, and restoring NEIL1 levels in chromatin. Ku and SAF-A thus act as a molecular switch for NHEJ → BER transition.

Earlier studies suggested that NHEJ alone is not sufficient to handle radiation-induced damage clusters in mammalian cells [239]. Okayasu and his colleagues [240] showed by measuring chromosome fragmentation and γH2AX foci formation that NHEJ inadequately repairs clustered damage. Recently, it was reported that high-energy IR could kill more cells than low-energy IR at the same dose level because of inefficient Ku-dependent NHEJ repair [241], which was later confirmed in NHEJ-deficient mice.

The mechanisms by which BER and NHEJ crosstalk at the damage clusters to prevent larger loss of DNA sequences is not known. However, this study demonstrates coordinated, sequential NHEJ repair of overt DSBs that precedes repair of surrounding oxidized bases in irradiated cells. The sequential NHEJ→BER model (Figure 39) is supported by three key observations: (1) Ku immunocomplexes in human cells contain BER proteins including DGs and APE1, all of which are directly inhibited by Ku via binary interaction. This observation is consistent with prior reports of Alt-NHEJ suppression by DNA-PK/Ku [242-244]. (2) BER inhibition by Ku is alleviated by SAF-A during NHEJ, but not by the phosphorylated protein [225, 228]. It is thus likely that after NHEJ completion, SAF-A acts as a molecular switch for the NHEJ→BER transition. (3) Consistent with this model, SAF-A regulates transient dissociation of chromatin-bound NEIL1 at 15-60 min after irradiation, presumably to prevent BER initiation and allow overt DSBs sealing via NHEJ. Prevention of the dissociation of chromatin-bound NEIL1 by DNA PK inhibition, SAF-A depletion or ectopic non-phosphorylatable S59A mutant, support this scenario. Furthermore, release of chromatin-bound NEIL1 correlates well with

the kinetics of SAF-A phosphorylation and NHEJ of overt DSBs after IR [217, 245], suggesting a tightly regulated pathway. However, while NEIL1 level in chromatin fraction is restored 1 h after IR, SAF-A dephosphorylation requires 2-4 h, suggesting the recovery may involve additional factors or mechanisms.

Once overt DSBs are repaired via NHEJ, repair of non-DSB lesions via BER/SSBR may be initiated, which involves PARP-1 and XRCC1 [217]. Additional DSBs generated during the repair of bi-stranded damage clusters [220-224] are likely repaired exclusively by Alt-EJ because the presence of PARP-1 inhibits Ku recruitment and thus NHEJ [102, 217]. Consequently, the hierarchy of NHEJ→BER prevents greater loss of genomic sequences that would otherwise occur with simultaneous BER and NHEJ activity.

CHAPTER VI: GENERAL CONCLUSIONS AND FUTURE DIRECTIONS

6.1. GENERAL CONCLUSIONS

Distinct DNA repair pathways involved in repair of IR-induced DSBs and non-DSB lesions have been individually studied through various *in cell* and *in vitro* approaches with specific repair substrates that has given in-depth knowledge on the protein repertoire and mechanism of each pathway. However, cross-talk among different repair pathways have been poorly addressed so far, which was an important aspect of this study. We envisaged Alt-EJ of DSBs as a two-step SSBR where key SSBR factors XRCC1/PARP1 could dynamically interact with early DSBR protein MRN. This is due to activation of both SSBR and DSBR machineries in irradiated cells that are recruited at the clustered DNA damage. Thus, although NHEJ is the major DSB repair pathway, Alt-EJ could significantly enhance in irradiated cells, which utilizes random microhomology sequences near the DNA ends for DSB repair, thus inducing extended deletions. Evidences for comparatively low but robust Alt-EJ presented in this study and by other groups suggest Alt-EJ's contribution to DSB repair is significant in irradiated cells, particularly where NHEJ fails due to extended ssDNA or chemically blocked termini that are difficult to process. Moreover, Alt-EJ has been shown to be major pathway in BRCA1/2 deficient breast or ovarian cancer cells where extensively resected ends cannot be repaired through either NHEJ or defective HR [112, 113]. Most solid tumors have hypoxic microenvironment that also perturbs DNA repair pathways through downregulation of Rad51 and decreasing HR [246], causing microsatellite and chromosomal instability [247], presumably through Alt-EJ. Thus, Alt-EJ can not only confer resistance to cancer cells but instigate cancer progression through enhancing genomic instability, one of its hallmarks [248]. The current study elucidated activation of XRCC1 repair complexes, through CK2-mediated phosphorylation, as the underlying mechanism for enhancement of Alt-EJ in irradiated cells. Although, several

other factors of Alt-EJ are yet to be known, recircularization of linearized plasmids by XRCC1-IP *in vitro* suggest that XRCC1 forms a core component of Alt-EJ pathway that either directly or indirectly interacts with most Alt-EJ factors. Thus, further proteomic analysis of XRCC1-IP can reveal other players of the pathway. Moreover, this study also emphasized role of XRCC1's inter-BRCT disordered domain which is heavily phosphorylated and provides scaffold for interaction with multiple proteins including Mre11. Disordered domains are difficult to study since they cannot be crystallized owing to their inherent flexibility, however, they are critical for dynamic protein-protein interactions. Thus, the current findings warrant further structural investigation on XRCC1's flexible domains.

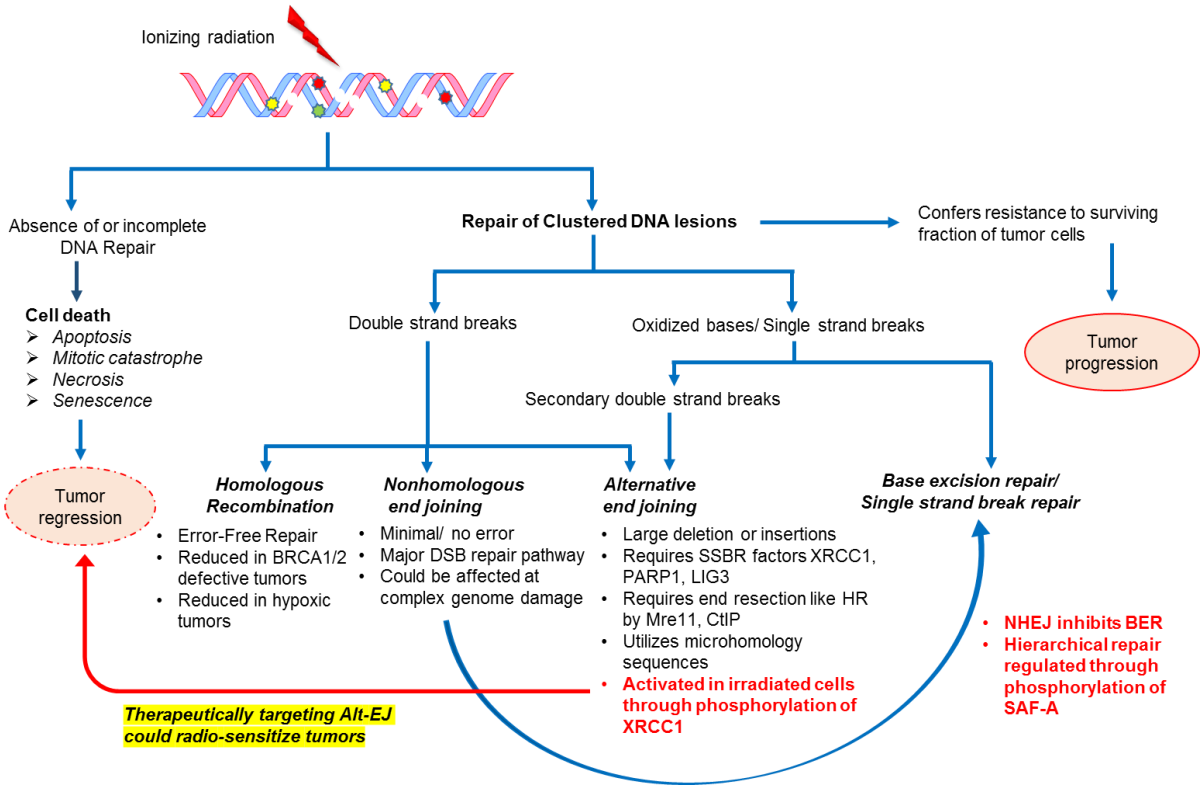

Figure 40. New insights on the repair mechanisms at ionizing radiation-induced DNA damage – contribution of Alt-EJ and NHEJ → BER hierarchical repair.

Identification of hierarchical repair at IR-induced damage coordinated by a non-canonical DNA/RNA binding protein SAF-A underscores not only cross-talk between NHEJ and BER pathways, but non-canonical role of RNA binding proteins in DNA damage response and repair. Several other groups have implicated role of RNA-binding proteins in DSB repair, such as regulation of DNA end resection by hnRNPUL [226]and stimulation of NHEJ by NONO [249]. Moreover, small RNAs have been reported to facilitate DSB repair through directing chromatin modification and recruiting repair protein [250], while transcript RNA could itself provide template for homologous recombination [251]. Thus, repair of IR-induced DNA lesions is an immensely complex phenomenon with role of myriad of proteins with distinct enzymatic activity, non-enzymatic scaffolding proteins, regulation through post-translational modifications, chromatin remodeler, etc.

6.1. FUTURE DIRECTIONS

Characterization of XRCC1's role in forming distinct IR-activated Alt-EJ proficient repair complex, brought into light several new questions regarding regulation of Alt-EJ. The first question is that if XRCC1 acts as a dimer for Alt-EJ, analogous to Ku70/Ku80/DNA-PKcs heterodimer in NHEJ. DSB repair requires to transiently hold and stabilize the two termini of the broken DNA which is facilitated by dimeric NHEJ complex. Interestingly, XRCC1, through BRCT-domain-mediated interactions, can form homodimer and heterodimer with LIG3 which also stabilizes DNA ends [165, 252]. Moreover, XRCC1 through its scaffolding action recruit multiple DNA repair factors such as DNA polymerases, DNA end processing enzymes and DNA end resection enzymes forming a complete arsenal for DNA repair, similar to Ku/DNA-PKcs complex. Phosphorylation of XRCC1 by DNA-PK at Ser371, located in its BRCT1 domain was reported to affect its dimerization [165]. This suggests that XRCC1 could possibly act as a dimer during Alt-EJ, which is prevented by the predominant NHEJ through phosphorylation of XRCC1 at

Ser371 by DNA-PK. However, it was found that the IP of XRCC1-S371D, the phospho-mimic which supposedly contains monomeric XRCC1 could recircularize pNS as good as XRCC1-WT IP. This warrants further *in cell* validation of XRCC1 dimerization and its implication in Alt-EJ. For this, we have developed a PLA-based dimerization assays and found evidences for XRCC1-dimers which was enhanced in DNA-PK inhibited cells (data not shown). We will carry out *in situ* dimerization assays with XRCC1-S371D and XRCC1-S371A mutants, in future. Moreover, it is also questionable whether phosphomimic mutant really mimic transient phosphorylation. Thus further investigation is necessary to reveal the physiological consequence of DNA-PK mediated phosphorylation of XRCC1.

Another question is that which DNA polymerases are required during Alt-EJ. Role of Polθ has been extensively studied in Alt-EJ (SD-MMEJ) by multiple groups [90-92], while Polβ and Polλ have also been reported to carry out some form of Alt-EJ [96]. We found XRCC1 interacts with both Polθ and Polλ after irradiation through co-IP and PLA studies (data not shown), however the specific residues for interaction are yet to be characterized.

Very little is understood regarding how the overhanging ssDNA strands on either end of the DSB anneal and stabilize during Alt-EJ. In a recent review Sfeir et. al. have implicated role of an annealing helicase, SMARCAL1 that prevents replication fork collapse and provides telomere integrity [253, 254], in providing stability at the DSB until the repair is complete [255]. Thus, SMARCAL1 could be another piece in the puzzle whose characterization is worth investigation. Apart from this there could several chromatin-associated factors that could aid Alt-EJ through chromatin remodeling and providing the DNA end resection factors and other proteins access to the DSB, p400 ATPase being one of such factors recently reported in Alt-EJ [256].

Although radio-therapy remains an important core component of the cancer treatment regimen, more recently, it has been suggested that its combination with other

therapeutic approaches such as small molecule inhibitors or immunotherapy could drastically improve prognosis [257]. Moreover, DNA repair capacity of cancer cells has been lately shown to predict their susceptibility towards immune-checkpoint blockade drugs, which is a major breakthrough in cancer therapy in recent times [258]. In this study deficiency in mismatch-repair (MMR) pathway was found to be clinically beneficial for targeting colorectal tumors with anti-programmed death 1 (PD-1) immune checkpoint inhibitor, pembrolizumab. Genetic defects in MMR enhances mutational burden of the tumors, resulting in expression of abnormal proteins in tumor cells that could help in identification of the tumors as foreign bodies by immune cells and destroy them. Since alternative end joining is a heavily error-prone DNA repair process, it would be curious to investigate if enhanced Alt-EJ in tumor cells could be similarly utilized to target them through immune-therapy.

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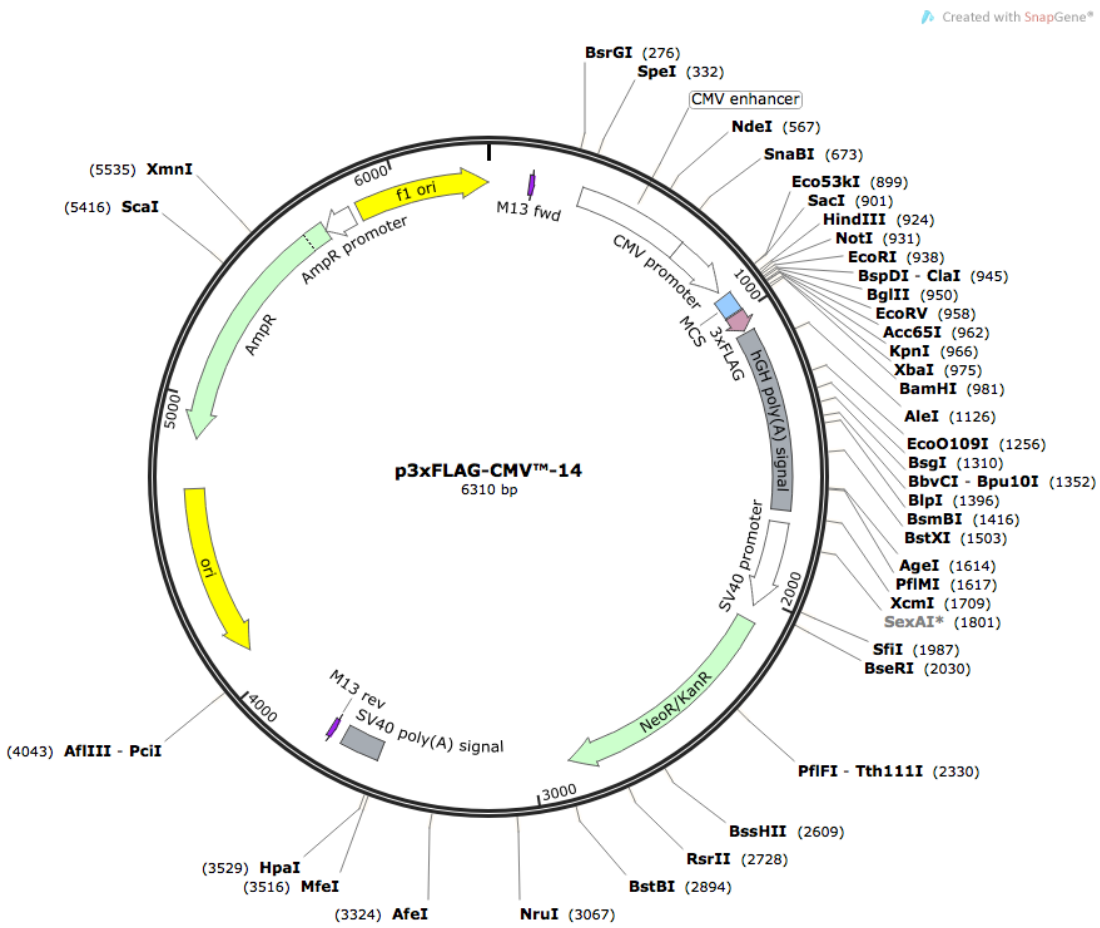
APPENDIX I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MCS
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hGH poly(A) signal
SV40 promoter
NeoR/KanR
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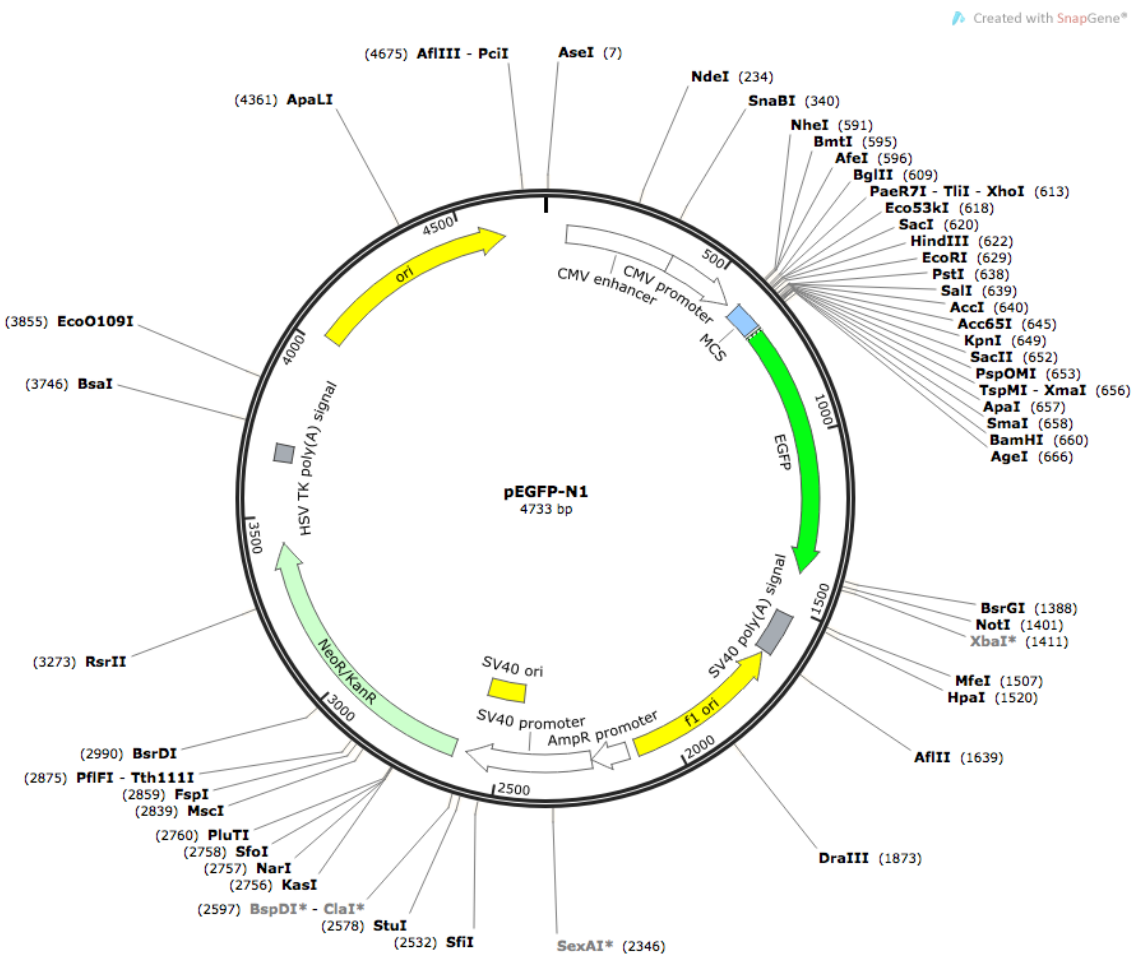
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VITA

Arijit Dutta was born to Mrs. Rita Dutta and Mr. Tapan Kumar Dutta on December 6, 1986 in Hiranpur, Jharkhand, India. Arijit received both his primary and secondary education under the curricula of Central Board of Secondary Education (CBSE), India. He passed his All India Secondary School Examination (AISSE, 10th grade) in the year 2002 and All India Senior School Certificate Examination (AISSCE, 12th grade) in the year 2004 from Dayanand Anglo Vedic (DAV) Model School, Durgapur. During his early education he received several awards including National Children Scientist Award, 2004 by SANKALP, an all-India organization for integrated participatory development. Arijit then attended his undergraduate studies in Microbiology (major) at Durgapur College of Commerce and Science, Rajbandh, under University of Burdwan and earned his Bachelor of Science (B.Sc.) degree in 2007 with 2nd university rank. After that he got selected for a prestigious Integrated M.Sc.-Ph.D. program in Plant Molecular Biology and Biotechnology at Bose Institute, one of the earliest premier research institute in India, founded by Acharya Jagadish Chandra Bose. He was awarded M.Sc. degree with 1st rank, jointly by Bose Institute and University of Calcutta, in 2009. In the meantime Arijit passed in National Eligibility Test 2009 organized by Council of Scientific and Industrial Research (CSIR-NET), with 105 all-India rank and qualifying for CSIR Junior Research Fellowship. Arijit also received an all-India rank of 314 in Graduate Aptitude Test in Engineering (GATE)-Life Sciences, 2009 scoring 96.75 percentile. He worked as a junior research fellow at Indian Institute of Chemical Biology (IICB), Kolkata, a CSIR research institute at the lab of Dr. Siddhartha Roy for short time, before joining Biochemistry and Molecular Biology Graduate Program at the University of Texas Medical Branch in Galveston (UTMB) in the Fall of 2011.

Arijit's graduate studies at UTMB has been very successful with several academic scholarships and travel awards, both from the department and the Graduate School of

Biomedical Sciences, some of which are Barbara Bowman Memorial Award (2014), Shirley Patricia Parker Scholarship (2014, 2015), and Mason Guest Scholar Award (2015). Arijit also presented his research work at multiple distinguished national symposiums such as American Society for Biochemistry and Molecular Biology (ASBMB, 2014) and Environmental Mutagenesis and Genomics Society (EMGS, 2013 & 2015) supported by respective travel awards. He won a best poster award at Structural Biology and Molecular Biophysics Symposium, 2015, UTMB and recently delivered a platform presentation at 46th EMGS meeting at New Orleans, LA, with EMGS travel award.

Arijit Dutta can be contacted at through his parents at MA-4, Rabindra Pally, B-Block, Durgapur-713201, West Bengal, India.

This dissertation was typed by: Arijit Dutta