XRCC4-XLF COMPLEXES FACILITATE DNA DOUBLE-STRAND BREAK REPAIR IN CELLS BY BRIDGING BROKEN DNA ENDS

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ABSTRACT

XRCC4-XLF COMPLEXES FACILITATE DNA DOUBLE-STRAND BREAK REPAIR IN CELLS BY BRIDGING BROKEN DNA ENDS

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The classical non-homologous end-joining (c-NHEJ) pathway is largely responsible for repairing DNA double-strand breaks (DSBs) in mammalian cells. Absence of c-NHEJ causes genomic instability in mice and leads to deficiencies in V(D)J recombination. XLF, (the last bona fide c-NHEJ factor discovered) is known to stimulate ligation by the core ligation complex: XRCC4-Ligase 4. However, the precise mechanism by which XLF stimulates XRCC4-Ligase 4 mediated DNA ligation is not well understood. Recent structural studies have shown that XLF can interact with XRCC4 to form filaments of alternating XRCC4 and XLF dimers; these filaments mediate DNA end bridging *in vitro*, providing a potential mechanism by which XLF might stimulate ligation. Here, we show that disrupting the interaction between XRCC4 and XLF by XRCC4 mutation, thereby abolishing filament formation, affects V(D)J recombination in cells and hinders the ability of cells expressing these mutants to survive in response to zeocin, a radiomimetic drug. Furthermore, we characterize an XLF mutant (L115A) that does not interact with XRCC4, and thus does not form filaments or bridge DNA *in vitro.* However, this mutant is fully sufficient in stimulating ligation of either blunt or cohesive DNA ends by X4/Lig4 *in vitro.* This separation of function mutant fully complements the zeocin sensitive phenotype and V(D)J recombination deficits of some XLF deficient cell strains but not

others, suggesting a variable requirement for DNA bridging in different cell types. To determine whether lack of XRCC4/XLF bridging might be compensated for by other factors, candidate repair factors were disrupted in XLF or XRCC4 deficient cells. Loss of either ATM or the newly described XRCC4/XLF like factor, PAXX accentuates the cellular requirement for XLF. In the case of ATM/XLF loss, the increased cellular requirement can be attributed to its bridging function; however in case of PAXX/XLF loss the increased requirement for XLF is independent of bridging.

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KEY TO ABBREVIATIONS

53BP1	p53 binding protein 1
APLF	aprataxin PNK like factor
ATLD	ataxia talengiectasia-like disorder
ATM	ataxia talengiectasia mutated
ATR	ataxia talengiectasia and Rad3 related
BRCA	breast cancer susceptibility protein
BRCT	BRCA1 C-terminus
CFP	cyan Fluorescent Protein
CSR	class switch recombination
DDR	DNA damage response
DNA	deoxyribonucleic acid
DNA-PK	DNA dependent protein kinase
DNA-PKcs	DNA dependent protein kinase catalytic subunit
DSB	double strand break
EDTA	Ethylenediaminetetraacetic acid
EMSA	electrophoretic mobility shift assay
FCS	foetal calf serum
FHA	forkhead associated
G_1 phase	gap 1 phase
H2A.X	histone 2A variant X
$H4K20^{Me2}$	dimethylated histone variant four

HR	homologous recombination
HRP	horseradish peroxidase
IR	irradiation
MDC1	mediator of DNA damage checkpoint protein 1
MEM	minimal essential medium
MRE11	meiotic recombination homolog 11
MRN	MRE11, RAD50, NBS1
MTT	3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide
NBS	Nijmegen breakage syndrome
NHEJ	non-homologous end joining
NP40	nonidet P-40
PAGE	polyacrylamide gel electrophoresis
PAXX	paralog of XRCC4 and XLF
PBS	phosphate buffered saline
PNKP	polynucleotide kinase phosphatase
PTIP	Pax transactivation-domain interacting protein
PVDF	polyvinylidene fluoride
RAG	recombination activating gene
RAP	receptor associated protein 80
RFP	red fluorescent protein
RIF1	replication timing regulatory factor 1
RNF	ring finger protein
ROS	reactive oxygen species

RSS	recombination signal sequence
S phase	synthesis phase
SAXS	small angle X-ray scatter
SDS	sodium dodecyl sulphate
TdT	terminal de-oxy transferase
UV	ultra-violet
V(D)J	variable, diversity, joining
XLF	XRCC4 like factor
XRCC4	X-ray cross complementing protein 4

Chapter 1: Introduction and Background

1.1 DNA damage-sources and types

Cellular DNA contains vital information that governs proper functioning of cells in a living organism. Various exogenous and endogenous agents can subject DNA molecules to various forms of damage. Exogenous sources include primarily radiation in the form of UV, X-rays and γ-rays. UV radiation is associated with cross-linking of DNA bases causing alteration in the DNA structure in the form of pyrimidine dimers (1). Ionizing radiation on the other hand causes DNA strand breaks that often result in complex DNA ends (1). Other exogenous chemicals that can cause DNA damage include radiomimetic drugs, cancer therapeutic drugs like topoisomerase inhibitors that cause double-strand breaks (DSBs) and mutagenic chemicals that can act as intercalating agents and hence interfere with normal DNA function (1). Endogenous sources of DNA damage include reactive oxygen species (ROS) that are formed as a by-product of cellular metabolism. ROS can lead to oxidative base damage in the DNA molecule that can lead to mis-pairing of damaged DNA bases and hence disrupt cellular function (1). Surprisingly, certain cells in our body (B and T lymphocytes) intentionally introduce DNA strand breaks by enzymes during the physiological processes of V(D)J recombination and class switch recombination (CSR) that lead to the formation of functional immunoglobulin molecules. These processes are tightly regulated such that the breaks imparted on the DNA molecule are promptly fixed by the appropriate repair machinery in cells (1).

DNA damage can be of various types depending on the type and source of damaging agent. For example, the chemical structure of DNA can be altered by various oxidative, alkylating or hydrolytic agents that may cause mis-pairing of DNA bases, formation of

bulky chemical adducts, deamination, depurination and other such modifications (2). These kinds of lesions can be repaired by base-excision, nucleotide excision and mismatch repair mechanisms in cells. DNA strands can also be cross-linked by various inter-strand and intra-strand crosslinking agents that affect strand separation required for events like transcription and replication (reviewed in (2). Depending on the complexity of the situation, these kinds of lesions can be repaired by nucleotide excision and other crosslink repair pathways that include the Fanconi anemia pathway in co-operation with homologous recombination (HR) (3). In addition, the DNA strand backbone can be severed by high energy ionizing radiations leading to the formation of single and double stranded DNA breaks (1). DNA DSBs are considered to be the most lethal form of DNA damage since in this case sequence information is lost from both strands of the DNA, thus imparting a mechanistic challenge for cells to tether the two DNA ends, preserve the genetic information and ensure proper repair. This chapter focuses on the mechanism of DNA double strand break repair, specifically the non-homologous end joining pathway (NHEJ) discussed below.

1.2 DSB sensing and signaling

DNA DSBs can be recognized by two major protein complexes- the Ku heterodimer complex consisting of Ku70/86 subunits (4) and the MRN complex consisting of proteins MRE11, RAD50, NBS1 (5). Binding of Ku subunits to DNA ends initiates the NHEJ mechanism of repair (discussed below), whereas binding of MRN at double stranded DNA ends leads to the recruitment of ATM (ataxia telangiectasia mutated) kinase, a master regulator that initiates the DNA damage response (DDR) (2). One of the earliest phosphorylation events performed by ATM upon its recruitment to DNA ends is that of

the histone variant H2A.X to form γ-H2A.X (6). Phosphorylation of H2A.X imparts a negative charge to the molecule which enables formation of an open chromatin structure that in turn might enable the recruitment of appropriate repair factors to initiate repair (7). Indeed it has been shown that phosphorylation of H2A.X by ATM triggers recruitment of numerous signaling factors that initiate a cascade of phosphorylation, ubiquitylation and sumoylation events that ultimately result in efficient checkpoint activation and repair of breaks by either HR or NHEJ (8). One of the primary functions of phosphorylated H2A.X is to recruit the protein MDC1 (mediator of DNA damage checkpoint protein 1) which directly binds phosphorylated H2A.X (9). MDC1 upon being phosphorylated by the ATM kinase recruits an E3 ubiquitin ligase RNF8 (ring finger protein 8) which ubiquitylates H2A.X and γ-H2A.X and in turn recruits RNF168 (ring finger protein 168). RNF168 recognizes RNF8 ubiquitylation products and along with RNF8 triggers a cascade of ubiquitylation events that are critical for efficient recruitment of downstream effectors (5). Some of the crucial effector proteins recruited by these ubiquitylation events are 53BP1 (p53 binding protein 1) and BRCA1 (breast cancer 1)-RAP80 (receptor associated protein 80) (10). 53BP1 promotes repair of double strand breaks by the NHEJ pathway, while BRCA1 mediates repair of DSBs by HR (7). NHEJ is the predominant DSB repair pathway that proceeds with rapid kinetics and is active throughout the cell cycle, whereas HR proceeds with slower kinetics and is mostly active during the S-phase of the cell cycle (11) when a replicated sister chromatid serves as a template to ensure high fidelity repair of genetic material. NHEJ on the other hand is template independent and entails direct end to end ligation of DNA DSBs

without a requirement for sequence homology. For the purposes of this study, we will focus on the repair mechanism of NHEJ.

1.3 NHEJ mechanism

The events that comprise the NHEJ pathway can broadly be classified into three major categories: end recognition, end processing and end ligation. Each of these steps are performed by a large assembly of proteins, some of which possess distinct enzymatic functions, while others serve as molecular scaffolds to enable the recruitment of additional proteins involved in the ligation reaction (1). Earlier it was thought that recruitment of NHEJ proteins occurred in a step-wise fashion where the proteins that are involved in end recognition bind to DNA ends and help to recruit factors involved in end processing, which in turn would attract factors involved in the ligation reaction once the ends were processed. However, the evidence of direct interaction among proteins involved in break sensing and end ligation argues that a supra-molecular protein complex formation occurs at the break site, including proteins involved in break sensing, processing and ligation instead of the step-wise recruitment of factors involved in the above processes (12).

1.3.1 End Recognition

The Ku heterodimer complex consisting of Ku70 and Ku86 subunits is abundant in mammalian cells and possesses high affinity to double-stranded DNA ends. These attributes enable the Ku heterodimer complex to be one of the earliest proteins that bind to ds-DNA ends in a sequence independent manner (13). Binding of the Ku complex to DNA ends then recruits the DNA dependent protein kinase catalytic subunit (DNA-PKcs). DNA-PKcs upon binding its regulatory subunit Ku forms a holoenzyme complex,

called DNA-PK that possesses a serine/threonine kinase activity (14). The enzymatic activity of DNA-PK is required for NHEJ, as loss of enzymatic function affects the ability of cells to repair DNA breaks (15,16). Although numerous substrates for DNA-PK's enzymatic activity have been identified such as Ku (17), Artemis (18), XRCC4 (X-ray cross complementing protein 4) (19-21), XLF (XRCC4-like factor) (22) and DNA ligase IV (23); phosphorylation of none of these substrates individually seem to be essential for NHEJ *in vivo* (22,24,25). However, studies performed by our collaborator Dr. Mauro Modesti have shown that phosphorylation of XRCC4 and XLF as a complex by DNA-PK in the presence of DNA causes dissociation of XRCC4-XLF complexes *in vitro* and leads to a decrease in ligation of linear DNA substrates by T4 DNA ligase *in vitro* (unpublished data). The other more relevant physiological target of DNA-PKcs kinase activity is the protein itself (26). Auto-phosphorylation of 465 kDa DNA-PKcs occurs in more than 60 target residues and is functionally quite complex. The outcome of most of the auto-phosphorylation events seem to function in changing the conformation of the molecule as clusters rather than individual residues (27-31). Change in the conformation of DNA-PKcs serves in modulating the accessibility of DNA ends to various downstream end-processing factors or to alternative repair pathways. Whereas phosphorylation of certain clusters in the molecule renders DNA ends to be 'open' and accessible to other factors; phosphorylation in certain other clusters restricts the ends from being accessed by other factors or pathways (20-24). Additionally, auto-phosphorylation of DNA-PKcs is also responsible for inactivation of the kinase and its subsequent dissociation from DNA ends (32).

1.3.2 End Processing

Processing of DNA ends is necessary to make the ends compatible for ligation by the ligation apparatus. Various DNA damaging agents often result in complex DNA end structures like bulky chemical adducts and non-ligatable secondary structures (1). Hence, in order for these ends to be ligated, such structures need to be resolved to make the ends compatible for ligation. End processing factors mainly fall into the category of nucleases and polymerases. Artemis is the most prominent nuclease involved in the NHEJ pathway. Artemis possesses an endonucleolytic activity (33) that is instrumental in opening DNA hairpin structure during V(D)J recombination (see below) (34). APLF (Aprataxin and PNK-like FHA protein) is another protein that possesses a 3' exonuclease activity and a single-stranded DNA endonuclease activity that has been implicated in NHEJ. Other examples of end modifying proteins involved in NHEJ are aprataxin and PNKP (polynucleotide kinase phosphatase). Aprataxin functions in repairing DNA ends that have undergone incomplete ligation leading to the formation of abortive ligation intermediates like adenylated DNA ends or nicks. These adenylate groups are removed by the enzymatic action of aprataxin (35). PNKP is unique in having dual kinase and phosphatase activites. It therefore functions by phosphorylating 5'-OH and removing 3'-phosphate from modified DNA termini (36). APLF, aprataxin and PNKP contain a conserved FHA (Fork-head associated) domain enables their interaction with XRCC4, thus connecting end processing to end ligation (37). Polymerases are the other major category of DNA end modifying factors in NHEJ, specifically pol μ, pol λ and TdT (Terminal deoxy transferase). TdT is expressed only in developing lymphocytes and functions during V(D)J recombination. It has the ability to

randomly add nucleotides in a template-independent manner (38). Pol λ functions in a standard template-dependent manner and helps to fill in the gaps associated with DNA ends (38). Pol μ is another polymerase that can add nucleotides in a template dependent and independent manner. It is unique in its ability to polymerize across a discontinuous template strand from one DNA end to another in the presence of the ligation complex and Ku (38).

1.3.3 End Ligation

The final ligation step of NHEJ is performed by a complex of three proteins, XRCC4, XLF and DNA ligase IV. The stable conformation of XRCC4 is in the form of a homodimer. Each monomer contains an N-terminal globular head, a middle coiled-coil region and a disordered C-terminal region (39). XRCC4 interacts with DNA ligase IV via its coiled-coil region (40). The coiled coil region of the molecule is also known to interact with other XRCC4 dimers to form stable tetramers (41). The head domain of XRCC4 interacts with XLF (42). XLF is structurally related to XRCC4. It also contains a globular N-terminal head, a coiled coil tail and disordered C-terminal domain and exists in the form of a homodimer (43). DNA ligase IV contains an N-terminal catalytic domain and two C-terminal BRCT domains. The region between the two BRCT domains makes stable hydrophobic contacts with XRCC4. DNA ligase IV is unstable in cells in the absence of its binding partner XRCC4 (44). Thus, XRCC4 not only stabilizes DNA ligase IV but also promotes adenylation of ligase IV (an essential step for ligation) (20). DNA ligase4 is unique in its ability to ligate double-stranded DNA ends across a gap, which is further stimulated in the presence of Ku (45). XLF is known to stimulate ligation of non-cohesive DNA ends *in vitro* (46-48) and promotes re-adenylation of DNA ligase

IV (49). Recent structural studies have shown that XLF interacts with XRCC4 via its head domain to form filamentous structures of alternating dimers of XRCC4-XLF complexes (50-53). The functional significance of these filaments is elaborated below.

1.4 Tying up loose ends- an emphasis on DNA end bridging

As mentioned above, DSBs are considered the most toxic form of DNA lesion because both strands of the DNA become discontinuous thus posing a mechanistic challenge for the ends to be tethered close to each other to facilitate ligation. Paradoxically, cellular enzymes during the physiological processes of V(D)J recombination and CSR intentionally impart programmed DSBs in the DNA (1). Thus, it is not surprising that cells have evolved mechanisms to bridge or scaffold DNA ends, once DSBs are encountered. Numerous protein factors have been implicated to serve this function; some of these are outlined below:

1.4.1 XRCC4-XLF filaments

XRCC4 and XLF have no known enzymatic function of their own, but are known to stimulate ligation by DNA ligase IV *in vitro*. Biochemical studies have shown that both XRCC4 and XLF promote adenylation of DNA ligase IV (20,49), which provides a reasonable explanation for enhancing ligase activity. XRCC4 additionally serves as a scaffold to stabilize DNA ligase IV (44). In human cells, the molar ratio of XRCC4 dimers to ligase IV monomers is 3:1, thus suggesting that XRCC4 dimers exist independently of DNA ligase IV (54). XRCC4 and XLF share considerable structural similarity and can function together as a complex. In fact, XLF was identified as a binding partner of XRCC4 (42). Based on the interacting interfaces of XRCC4 and XLF homodimers, a model was proposed whereby XRCC4 dimers interact with XLF dimers

and XLF dimers in turn interact with XRCC4 dimers, thus leading to the formation of a filament of alternating dimers of XRCC4 and XLF (43). Subsequently, four independent laboratories have isolated crystal structures in support of this model (43-46). Moreover, small angle X-ray scattering (SAXS) analysis, carried out under more physiological conditions, has also supported this model (55). These filaments have been shown to co-operatively bind DNA and bridge linear DNA fragments *in vitro* (50). Moreover, images from scanning force microscopy reveal that these filaments form bundles of multiple filaments in the presence of DNA resulting in large nucleo-protein complexes with DNA molecules positioned end-to-end (43). These observations suggest that XRCC4-XLF filaments promote alignment of multiple DNA molecules. The data presented in this study aim to characterize the function of these filaments in cells.

1.4.2 DNA-PK

DNA-PK is an enzyme complex of three subunits: the regulatory subunits are composed of Ku70 and Ku86 heterodimer and the catalytic subunit is called DNA-PKcs. Both Ku and DNA-PKcs have been shown to support bridging of linear DNA fragments *in vitro* (56). Ku stimulates intermolecular ligation of blunt DNA substrates, indicating that it plays a role in aligning blunt DNA ends for ligation (57). Electron microscopy reveals that incubation of linear DNA fragments with DNA-PKcs results in circularization of DNA structure, indicating that DNA-PKcs upon binding DNA tends to bring the ends close to each other (56). An attractive model for the mode of DNA-PK action is that each double-stranded DNA end would be bound by Ku and DNA-PKcs complex and interaction between the complexes on both ends would promote synapsis. In support of this model, studies have shown that DNA-PKcs can phosphorylate itself across a gap in *trans* (58),

thus indicating that the complex on one DNA end communicates with the complex formed on the other end, thus leading to synapsis.

1.4.3 MRN Complex

The MRN complex composed of MRE11, RAD50, NBS1 is one of the earliest protein complexes that binds DNA ends. Within the complex, MRE11 is the only protein that possesses an enzymatic function. It exhibits a 3'-exonuclease activity and a single-stranded endonuclease activity that has been implicated in DNA end resection during HR (5). In addition to the nuclease activity of MRE11, the complex is also responsible for the activation of ATM kinase (59) which activates downstream checkpoint proteins. Hypomorphic mutations in MRE11 and NBS1 cause ataxiatelangiectasia-like disorder (ATLD) and Nijmegen breakage syndrome (NBS) in humans leading to growth retardation, immunodeficiency and lymphoid malignancies. Cells from these patients exhibit defects in cell cycle checkpoint, genomic instability and irradiation (IR) hypersensitivity (60). Scanning force microscopic studies reveal that the MRE11-RAD50 complex is mostly present in the form of a heterotetramer of M2R2 conformation, with two MRE11 molecules forming a globular head domain and two RAD50 molecules forming two extended arms on either side of the head (61). The head domains stably bind DNA, while the 'arms' have been shown to be flexible and dynamic in nature. With increasing concentrations of the protein complex, tethering of multiple DNA molecules was observed to be mediated by the long, flexible arms (54). Mutational analyses have shown that disruption of dimerization between MRE11 monomers preserved its nuclease function and interaction with RAD50/NBS1, but decreased its affinity for DNA, suggesting that the dimerization serves an architectural function that promotes DNA

binding and possibly synapsis (62). These mutations also affect genotoxin induced repair in yeast cells, suggesting a functional role for the dimerization and synapsis mediated by the protein (55).

1.4.4 H2A.X

H2A.X is a variant of histone protein H2A and a substrate for ATM kinase activity. H2A.X is rapidly phosphorylated on its C-terminal SQE motif by ATM or DNA-PK upon DSB induction and this phosphorylation event occurs over a span of a megabase region flanking the DSB (63). These phosphorylated residues then serve as an 'anchor' for the recruitment of multiple downstream proteins that can be visualized in the form of microscopic foci upon staining in living cells (7). It was thus hypothesized that this 'anchoring' function immobilizes DNA ends and protects them from progressing into chromosomal breaks. Consistently, loss of H2A.X in cells leads to increased persistence of chromosomal breaks leading to increased translocation events in activated B-cells (64). H2A.X does not seem to be directly involved in the joining function of DSBs, as DSBs formed during the process of V(D)J recombination are efficiently resolved even in the absence of H2A.X. Interestingly, absence of H2A.X decreases the efficiency of CSR which requires synapsis of distally located DNA DSBs. Since, synapsis of DNA breaks is achieved by the RAG endonuclease complex during V(D)J recombination (discussed below), it is speculated that H2A.X may function in bridging DNA ends during class switch recombination.

1.4.5 53BP1

53BP1 is a 200 kDa multidomain protein. It contains a large N-terminal region spanning more than half of the sequence that contains multiple S/TQ sites that are

phosphorylated by ATM and ATR upon DNA damage (reviewed in (65). Phosphorylation of these residues is critical for the interaction with downstream proteins Rif1 and PTIP. The central part of the protein contains a nuclear localization signal and tandem tudor domains that bind dimethylated histone ($H4K20^{Me2}$) and ubiquitylated H2A.X. Thus, this part of the protein is required for the formation of nuclear foci upon induction of DSBs. N-terminal to the tudor domain is an oligomerization domain that contributes to chromatin binding. Finally, the C-terminal region contains a pair of BRCT domains that are required for repair in the heterochromatin region (reviewed in (65)). One of the major functions of 53BP1 is to inhibit DNA end resection thereby inhibiting HR thus indirectly promoting NHEJ. The blocking of end resection occurs by the recruitment of downstream protein Rif1, however the exact mechanism of how Rif1 blocks end resection is not clearly understood (66,67). The ability of 53BP1 to promote NHEJ has also been studied in the context of dysfunctional telomeres. It has been shown that in uncapped telomeres, when the shelterin complex that maintains telomeric ends is disrupted, 53BP1 is recruited to the telomeric ends and mediates toxic NHEJ leading to fusion of two telomeres (68). The fact that 53BP1 is not a core NHEJ factor but seems to promote NHEJ suggests that it has an accessory function in supporting NHEJ perhaps by scaffolding DNA breaks. In support of this notion, it has been shown that cells lacking 53BP1 show increased translocation events in activated B-cells, suggesting that loss of 53BP1 affects appropriate joining in these cells (64). 53BP1 is critical for the process of CSR, which requires joining of two distally, positioned DSBs in specialized ‘switch’ regions by NHEJ. Studies have shown that in the absence of 53BP1 there is enhanced intra-switch region joining as opposed to inter-switch region joining,

suggesting that 53BP1 might play a role in synapsing distant switch region breaks to promote inter-switch recombination (69) . Moreover, 53BP1 has been shown to facilitate long range DNA joining during V(D)J recombination in T-lymphocytes, which was proposed to be mediated by oligomerization of multiple 53BP1 molecules localized at the junctions of V, D or J segment separated by a certain distance, thus leading to synapsis (70).

1.5 Clinical Significance of NHEJ

NHEJ is essential for the resolution of DSBs introduced during V(D)J recombination, a process that generates functional immunoglobulins and T-cell receptors (TCRs) in lymphocytes (71). Immunoglobulins can directly bind soluble antigens to elicit a humoral response, while TCRs bind to a processed form of antigen and elicits a more systemic immune response. Hence, one can imagine that impairment of NHEJ can lead to severe immunodeficiency due to impairment in formation of functional immunoglobulins and TCRs (72). Moreover, persistence of unrepaired DSBs (due to malfunction of NHEJ) can also lead to an increased frequency of chromosomal translocations, which in turn, can give rise to various types of cancers, especially lymphomas and leukemias (1). V(D)J recombination is a somatic recombination process that occurs in developing lymphocytes to generate a large variety of functional immunoglobulins (Igs) and TCRs that recognize diverse antigens. The process involves joining of a particular V (variable), D (diversity) and J (joining) gene segment from a pool of numerous V, D and J segments arranged on a chromosome (reviewed in (1). The process of V(D)J recombination is initiated by binding of RAG (Recombination activating gene) proteins (RAG1 and RAG2) to specific sequences on the chromosome called recombination

signal sequences (RSS), which flank the various V, D and J coding exons (reviewed in (73). In the first step, RAG proteins bind to two RSSs, one flanking the V region exon and the other flanking the J region (in case of Ig light chain rearrangement). Binding of RAGs to RSSs induces a conformational change that brings the V and J regions of the chromosome in close proximity (73). The RAG complex then introduces DSBs at the junctions of the signal and coding sequences, thus generating blunt signal ends and covalently sealed hair-pinned coding ends (73). These ends can then be processed and joined by NHEJ pathway to produce signal joints and coding joints respectively (reviewed in (74). Human patients harboring hypomorphic mutations in core NHEJ proteins like ligase IV, XRCC4, DNA-PKcs exhibit severe immunodeficiency and growth retardation (72). DNA-PKcs mutations are also associated with severe neurological disorders (75). XLF null human patients have been described; they exhibit immunodeficiency, growth retardation, microcephaly (76) and a failure in self-renewal capacity of haematopoietic stem cell progenitors (77). Human patients with Ku mutations have not been reported to date. Patients harboring mutations in Artemis also exhibit immunodeficiency and lymphoid malignancies in some cases (reviewed in (72). Therefore, NHEJ is not only important for the preservation of genomic stability but also for the development of a functional immune system.

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Chapter 2: The interaction between XRCC4 and XLF is important for DNA double-strand break repair in cell

Part A: XRCC4 interacts with XLF to facilitate DNA double-strand break repair and promote V(D)J recombination in cells.

2A.1 Introduction

XRCC4 and XLF are proteins thought to be involved primarily in the ligation step of NHEJ. XRCC4 directly interacts with DNA ligase IV and stabilizes its expression in cells (44). XRCC4 also makes direct hydrophobic contacts via its head domain with XLF (43). XLF and XRCC4 share common structural features. Both proteins stably exist as homodimers. They both bind DNA in a sequence independent but length dependent fashion and exhibit co-operative binding to increasing lengths of linear DNA molecules. Both XRCC4 and XLF promote re-adenylation of DNA ligase IV, thereby stimulating the ligation reaction (20,43,49,78). Closer examination of the interaction interface between XRCC4 and XLF homodimers revealed that these dimers can tandemly interact to form a filament like structure consisting of repeating units of XRCC4-XLF heterotetramer. Crystal structures from four independent laboratories support this model (50-53). Computational modeling has shown that these filaments can interact with each other to form filament bundles (50). *In vitro* studies have shown that these complexes can bridge linear DNA fragments and disruption of these complexes by disrupting the interaction interface between XRCC4 and XLF by mutation, abolished bridging *in vitro* (50). This study aims to characterize the function of these filament complexes in cells, by disrupting the interaction of XRCC4 with XLF. A panel of eight variant constructs of XRCC4 (table 1) were made by our collaborator (Dr. Mauro Modesti) and tested for their ability to interact with XLF; all 8 variants failed to interact with XLF *in vitro* (43). Here we

examine the effect of these variants in joining DNA substrates in cells in response to enzyme induced DSBs or irradiation induced DSBs.

2A.2 Materials and Methods

2A.2.1 V(D)J recombination assays Extrachromosomal VDJ recombination assays utilizing the signal joint substrate (pJH201) outlined in figure 1 and coding joint substrate (pJH290)(79) were performed in XR1 cells. Briefly, cells plated at 20 to 40% confluency in 60 mm diameter dishes were transiently transfected with 1 µg substrate , 4 µg each of RAG1 and RAG2 constructs, and 4 µg of the indicated expression construct or empty vector using the fugene6 transfection reagent according to the manufacturers' instructions. 48 hrs after transfection, substrate plasmids were isolated by alkaline lysis and subjected to DpnI restriction enzyme digestion for 1 hr. DpnI-digested DNA was transformed into competent DH5α cells (Invitrogen) according to the manufacturers' instructions. Transformed cells were spread onto LB agar plates containing 100 µg/ml ampicillin only or with 100 µg/ml ampicillin and 22 µg/ml chloramphenicol. The percentage of recombination was calculated as the number of colonies resistant to ampicillin and chloramphenicol divided by the number of colonies resistant to ampicillin.
The VDJ substrates encoding fluorescent proteins (outlined in figure 3) were utilized in 293 cells. Briefly, extrachromosomal fluorescent VDJ assays were performed on cells plated at 20-40% confluency into 24-well plates overnight. Cells were transfected with 0.125 µg substrate, 0.25 µg RAG1 and 0.25 µg RAG2 per well using polyethylenimine (PEI, 1 µg/ml, Polysciences) at 2 µl/1 µg DNA. In experiments with additional expression plasmids, 0.25 µg of the expression plasmid or vector control was included. Cells were harvested 72 hr after transfection and analyzed for CFP and RFP expression

by flow cytometry. The percentage of recombination was calculated as the percentage of live cells expressing CFP (formed upon successful recombination) divided by the percentage expressing RFP (produced constitutively irrespective of recombination).

2A.2.2 Zeocin sensitivity assay Clonogenic survival assays were performed for XR1 and AA8 cells. Briefly, a hundred cells were plated for each transfectant into complete medium containing the indicated dose of zeocin in 60 mm diameter tissue culture dishes. After 7 to 10 days, cell colonies were stained with 1% (w/v) crystal violet in ethanol to measure relative survival. MTT (Sigma) staining was performed to assess cell viability for 293 cells. 30,000 to 50,000 cells were plated in each well of a 24-well plate, containing medium with varying concentrations of zeocin. After 5 to 7 days of zeocin treatment, cells were treated with 1 mg/ml MTT solution for 1 hr. Medium containing MTT was then removed and formazan crystals thus produced were solubilized in acidic isopropanol. Absorbance was read at 570 nm to determine relative survival.

2A.2.3 Irradiation sensitivity assay 4000 cells from each of the XRCC4 wild type and mutant clones were harvested and treated with varied doses of ionizing radiation in serum free media, using a ^{60}Co source. Immediately after irradiation, cells were plated back into 100 cm^2 dishes containing αMEM supplemented with 10% FCS. After seven days, colonies were fixed in methanol and stained with crystal violet to establish relative survival.

2A.2.4 Pull-down assay 10 µg pEF vector constructs expressing either wild type or mutant forms of C-terminal His-tagged XRCC4 or XLF were transfected into 293 cells. 48 hrs after transfection cells were harvested and washed with PBS. Pellets were lysed

with 1 ml lysis buffer (50 mM Tris-pH7.5, 120 mM sodium chloride, 0.5% NP40, 1 mM sodium fluoride, 1 mM sodium orthovanadate, protease inhibitor cocktail), and rocked for 30 minutes on ice. Lysates were centrifuged and the supernatant used for pull down assays. 50µl Ni-NTA Agarose beads (Qiagen; Valencia, CA) were added to 1 ml cell lysate containing 10 mM imidazole and rocked for 3 hrs at 4^{o}C. Beads were collected by centrifugation and washed three times in the same buffer containing 50 mM imidazole. After washing, beads were re-suspended in 30 µl 4X SDS-PAGE buffer and analyzed by immunoblotting.

2A.2.5 Immunoblotting Antibodies utilized in this study include a polyclonal rabbit anti-XRCC4 reagent [Abcam; Cambridge, MA], and polyclonal anti-XLF reagent [Abcam; Cambridge, MA]. Whole cell extracts were obtained by re-suspending cell pellets in solubilization buffer containing 50 mM hepes (pH7.5), 150 mM NaCl, 0.1% triton X-100, 5 mM manganese chloride, 50 mM sodium fluoride, 2 mg/ml DNAse 1 and protease inhibitor cocktail. 25 µg of each cell extract were electrophoresed on an 8% SDS-PAGE gel and transferred to PVDF membranes. Membranes were probed with either rabbit polyclonal antibody to XRCC4 or XLF. Anti rabbit HRP was used as secondary antibody and membranes were exposed to chemiluminescent substrate to visualize XRCC4 or XLF.

2A.3 Results

2A.3.1 XRCC4 variants that do not interact with XLF restore signal, but not coding end joining in XRCC4 deficient CHO (XR1) cells. To examine if the ability of XRCC4 to interact with XLF and thereby form filaments is important for V(D)J recombination an extrachromosomal V(D)J recombination assay was performed in XR1 cell strain. As

shown in figure 1B, wt XRCC4 and all eight variants restore similar levels of signal joint recombination. In contrast, significantly reduced levels of coding joints are supported by XRCC4 variants that do not interact with XLF, when compared to wt XRCC4. Moreover, examination of recombined junctional sequences from wt and variant samples showed no significant differences in terms of the number of nucleotide deleted or added at the ligated junction (table 2). These data suggest that the interaction of XRCC4 with XLF may be important for coding joint formation, but is surprisingly dispensable for generation of signal joints in XR1 cells. An explanation for the impairment of coding but not signal joint formation could be due to inefficient synapsis of coding ends. Signal ends are known to be held close to each other in a post cleavage complex by the RAG endonuclease, whereas coding ends are not (80). Thus, synapsis of signal ends may not be absolutely required for signal end ligation, whereas, coding ends require synapsis to align them close to each other prior to ligation. It is thus possible that the XRCC4 variants that fail to interact with XLF and form filaments are unable to tether the coding ends, thus impairing ligation. Whereas, wt XRCC4-XLF filaments are able to bridge coding ends, hence facilitating ligation.

2A.3.2 XRCC4 variants that do not interact with XLF fail to restore signal and coding end joining in XRCC4 deficient 293 cells. Given that human XRCC4 variants that do not interact with XLF show impaired coding end joining in hamster XR1 cells, we tested two of these variants in a human cell line (293 cells) from which endogenous XRCC4 expression was disrupted by CRISPR/Cas9 mediated gene targeting. The deficiency of these variants in interacting with XLF in 293 cells was further confirmed by pull down assays from cell extracts (figure 2). Interestingly, both XRCC4 variants that

failed to interact with XLF also failed to support signal and coding end resolution in 293 cells compared to wild type complemented cells, although coding joint formation was more severely impaired than signal joints (figure 3). This again suggests that the interaction of XRCC4 with XLF is important for V(D)J recombination, however different cell types show varying degrees of dependence for XRCC4-XLF interaction and potential filaments formed by XRCC4-XLF complexes.

2A.3.3 XRCC4 variants that do not interact with XLF only partially restore radioresistance in XRCC4 deficient XR1 cells. Results from the V(D)J assay demonstrated that XRCC4 variants that do not interact with XLF have a deficiency in V(D)J recombination. In order to determine if disrupting the interaction between XRCC4 and XLF affects DSB repair induced by irradiation or the radiomimetic drug zeocin, constructs expressing wt XRCC4, XRCC4 variants K65/99E, K72/90/99E, or the appropriate vector control were transfected into XR1 cells and independent cell strains expressing wt or variant XRCC4 were obtained. These stable cell strains, were then treated with varying doses of ionizing radiation or zeocin. As shown in figure 4, cells expressing wt XRCC4 are substantially less radiosensitive than cells lacking XRCC4 (vect). However, cells expressing variant forms of XRCC4 were more radiosensitive than wt. This suggests that XRCC4's ability to interact with XLF in cells is functionally important for DSBR in XR1 cells.

2A.3.4 XRCC4 variants that do not interact with XLF fail to restore zeocin resistance in XRCC4 deficient 293 cells. Since hamster CHO cells fail to reverse radiosensitivity in the presence of XRCC4 variants K65/99E and K72/90/99E, we examined if human XRCC4 deficient 293 cells would show the same phenotype. Stable

cell strains expressing wild type and variant forms of XRCC4 were generated and treated with graded doses of the radiomimetic drug zeocin. As expected, XRCC4 variant K65/99E that does not bind XLF was deficient in reversing zeocin sensitivity unlike wt XRCC4 (figure 5). This further demonstrates that the interaction between XRCC4 and XLF and thereby potential filament formation is important for human cell survival in response to a radiomimetic drug induced DNA damage possibly by facilitating NHEJ.

2A.3.5 Overexpression of XRCC4 variants in wild type CHO cells (AA8) exert a dominant negative effect on DSBR Given that XRCC4 mutant cell strains are radiosensitive compared to wt strains, it was next considered if these variants would dominantly inhibit NHEJ in NHEJ proficient wild type CHO cells. To assess this possibility, wt and mutant XRCC4 were stably overexpressed in the wt CHO cell strain AA8 and tested for zeocin (a DSB inducing agent) sensitivity. As shown in figure 6, overexpression of XRCC4 variants that cannot interact with XLF markedly sensitize AA8 cells as compared to cells expressing wt XRCC4, consistent with the hypothesis that XRCC4 variants exert a dominant negative effect on DSBR. One explanation for the dominant negative effect could be, that the XRCC4 variants that fail to interact with XLF might sequester endogenous ligase IV molecules, thus disrupting the endogenous XLF-XRCC4-Ligase IV complex. This would indicate that a fully functional XLF-XRCC4-Ligase IV complex is required for effective NHEJ in cells.

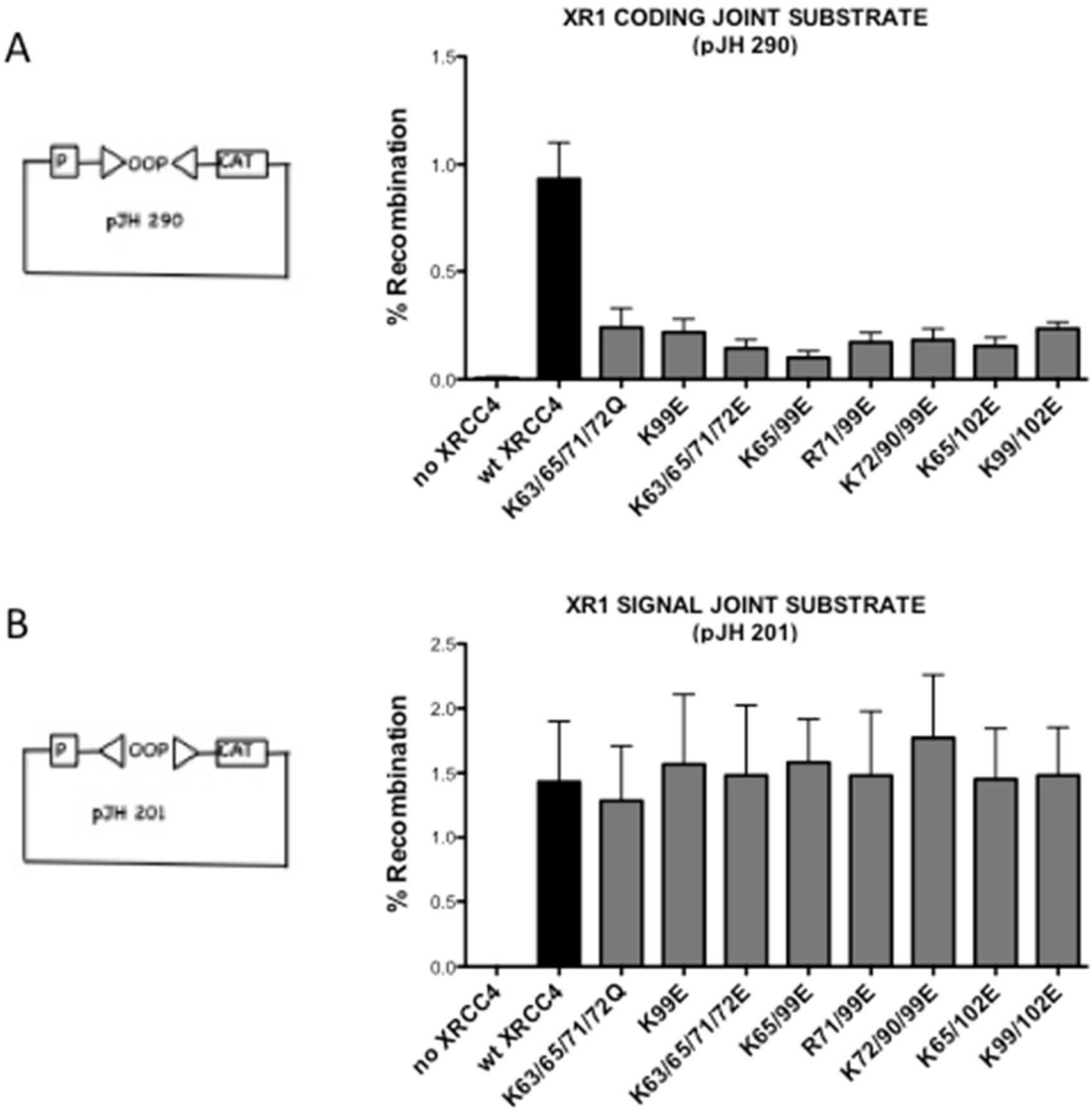
2A.3.6 XRCC4 variants that do not interact with XLF do not affect NHEJ in mouse ES cells. Human XRCC4 variant proteins K65/99E and K72/90/99E that do not interact with XLF show a more severe phenotype in human cells compared to hamster cells. To further determine if this phenotype varies with different species or different cell types,

mouse ES cells were obtained from our collaborator (Dr. Jeremy Stark) where endogenous XRCC4 expression was disrupted by gene targeting (81). These cells were used to perform extrachromosomal V(D)J recombination assays as before. Surprisingly, neither variant showed a joining deficient phenotype in these cells (figure 7). Furthermore, stable mouse ES cell strains expressing wild type or variant form of XRCC4 were fully capable of reversing zeocin sensitivity unlike human and hamster cells (figure 7). This observation implies that different species or cell types have varying degrees of dependence for stable XRCC4-XLF complexes and potential filament formation in facilitating DNA repair and that ES cells are refractory to loss of XRCC4-XLF complexes.

TABLE 1- List of XRCC4 variants that fail to interact with XLF

XRCC4	BINDING		
	XLF	LIG IV	DNA
WILD TYPE	Y	Y	Y
MUT 3 (K63Q,K65Q,R71Q,K72Q)	N	Y	Y
MUT 22 (K99E)	N	Y	Y
MUT 29 (K63E,K65E,R71E,K72E)	N	Y	Y
MUT 46(K65E, K99E)	N	Y	Y
MUT 48 (K72E,K90E,K99E)	N	Y	Y
MUT 47 (R71E,K99E)	N	Y	Y
MUT 49 (K65E,K102E)	N	Y	Y
MUT 61 (K99E,K102E)	N	Y	Y
MUT 62 (K65E,R71E,K72E)	N	Y	Y

FIGURE 1- XRCC4 variants that do not interact with XLF restore signal, but not coding end joining in XRCC4 deficient CHO (XR1) cells.



Recombination percentage of coding joint substrate (A) and signal joint substrate (B) in XR1 cells transiently expressing Rag1, Rag2, wt and variant forms of XRCC4. Note that the decrease in coding joining by XRCC4 variants is significant ($P<0.05$) as opposed to signal joining. Left panel shows schematic of coding joint (A) and signal joint (B)

FIGURE 1 (cont'd)

substrate utilized for V(D)J assays. The triangles represent recombination signal sequences recognized by RAG proteins. CAT is the gene for chloramphenicol acetyl transferase and 'P' signifies its promoter. OOP is a transcriptional terminator. Appropriate recombination events lead to deletion of OOP and expression of chloramphenicol acetyl transferase thus leading to formation of chloramphenicol resistant colonies.

TABLE 2- Coding junction sequence characteristics.

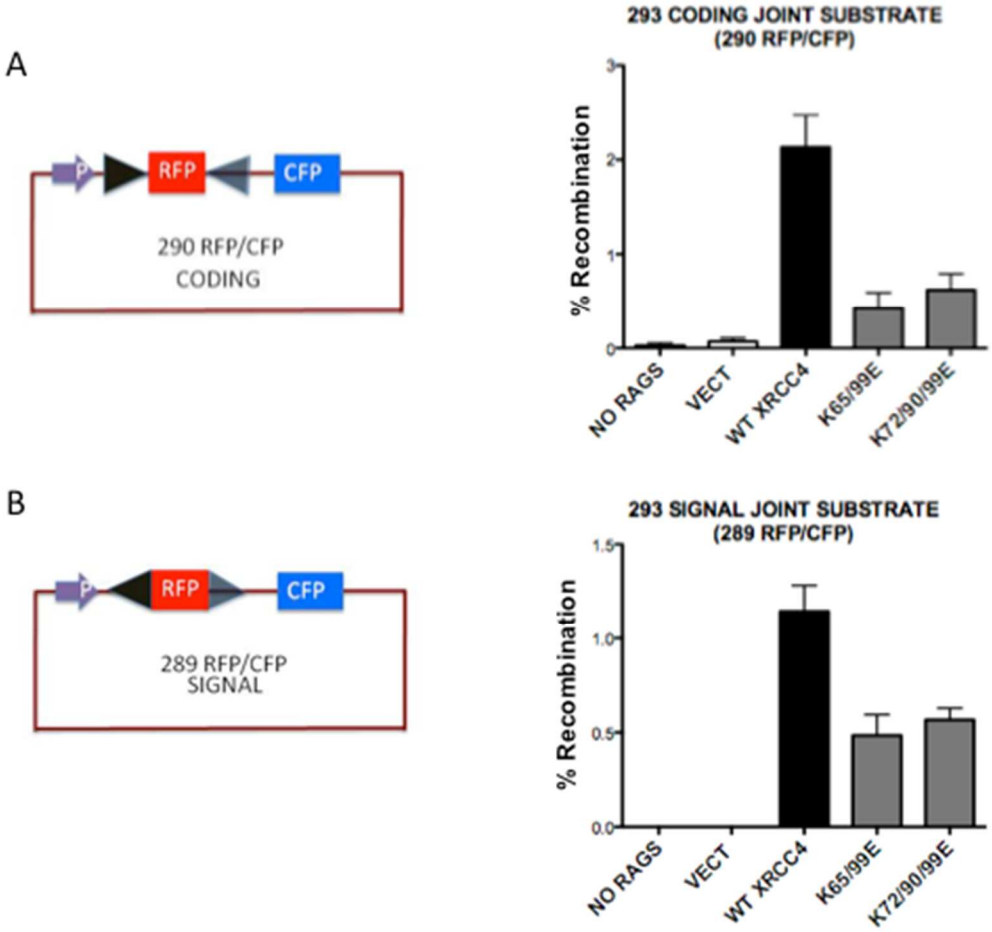
Table shows sequence characteristics from isolated coding joint sequences. Note that the number of nucleotides inserted or deleted at the junctions does not differ significantly between wt and variants. This indicates that end-processing in not affected in presence of XRCC4 variants.

SAMPLE	TOTAL NUMBER OF SEQUENCES	AVERAGE NO. OF DELETIONS	AVERAGE NO. OF ADDITIONS	% utilizing SHORT SEQUENCE HOMOLOGY
XRCC4 WT	46	3.7	0.06	45
XRCC4 (K65/99E)	35	4	0.28	45
XRCC4 (K72/90/99E)	46	4.69	0.04	32

FIGURE 2- XRCC4 variants K65/99E and K72/90/99E fail to interact with XLF in human 293 cell extracts.

Top panel shows relative expression levels of XRCC4 and XLF in 293 cells transiently transfected with wt and variant forms of His-tagged XRCC4 plasmid constructs. Bottom panel shows relative amounts of XRCC4 and XLF recovered from Ni-NTA coated agarose beads after incubation with above lysates. Note that XLF is retained in the beads in presence of wt XRCC4 but not XRCC4 variants.

FIGURE 3- XRCC4 variants that do not interact with XLF fail to restore signal and coding end joining in XRCC4 deficient 293 cells.

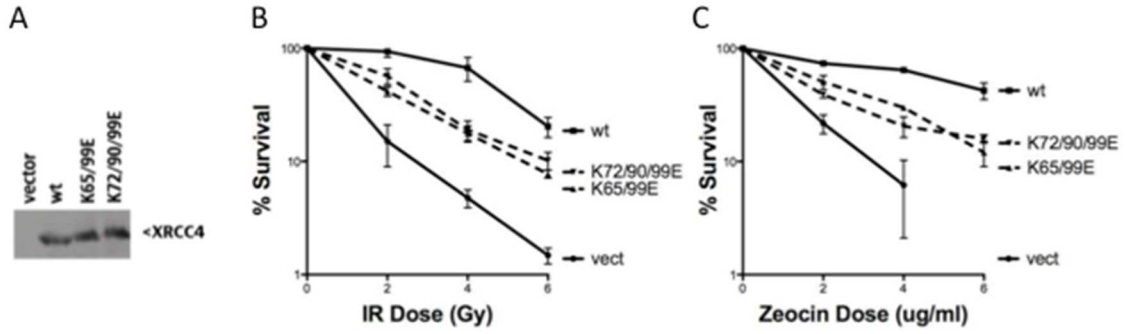


Recombination percentage of coding joint substrate (A) and signal joint substrate (B) in XRCC4 deficient 293 cells transiently expressing Rag1, Rag2, wt and mutant forms of XRCC4. Note that the decrease in coding joining by XRCC4 variants is statistically significant (p=0.0022) according to two-tailed Mann-Whitney test. Similarly, there is significant decrease in signal joining by XRCC4 variants K65/99E (p= 0.0152) and K72/90/99E (p=0.0022) according to two-tailed Mann-Whitney test. Left panel shows schematic of coding joint (A) and signal joint (B) substrate utilized for V(D)J assays. The

FIGURE 3 (cont'd)

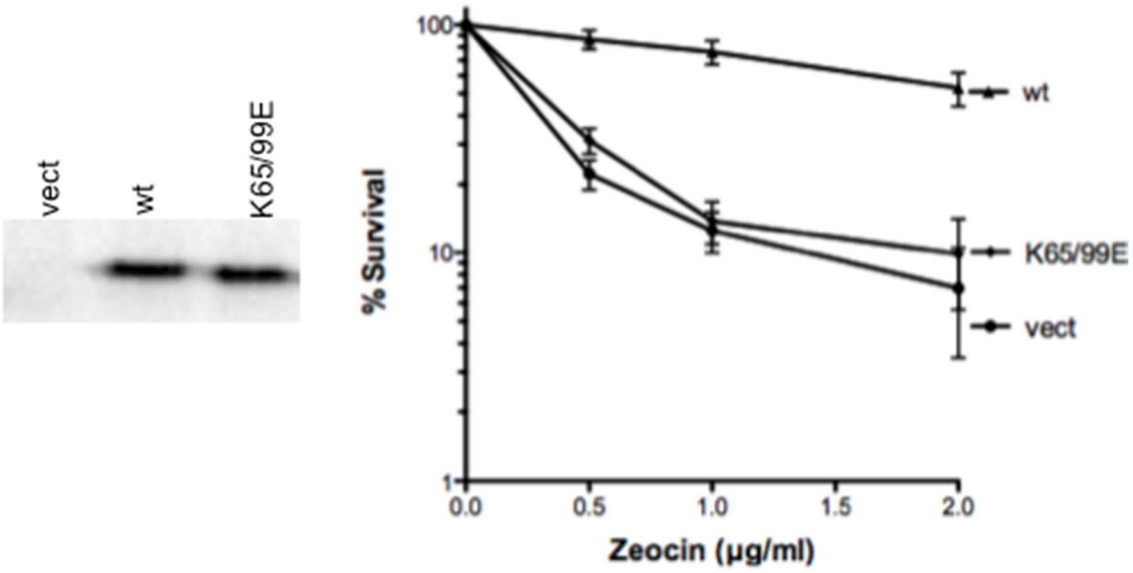
triangles represent recombination signal sequences recognized by RAG proteins. Appropriate recombination events lead to deletion of RFP and expression of CFP which can be analyzed by flow cytometry.

FIGURE 4- XRCC4 variants that do not interact with XLF partially restore radiosensitivity and zeocin sensitivity in XR1 cells.



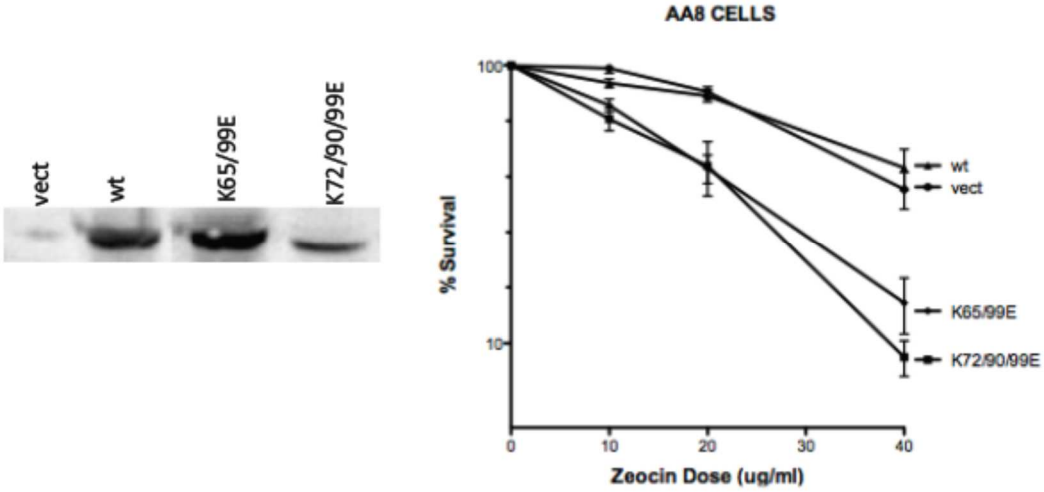
Left panel (A) shows stable expression of wt and variant forms of XRCC4 in XR1 cells. (B) and (C) show irradiation sensitivity and zeocin sensitivity respectively of cell lines described in (A). Error bars indicate SEM of four independent experiments

FIGURE 5- XRCC4 variants that do not interact with XLF fail to restore zeocin resistance in XRCC4 deficient 293 cells.



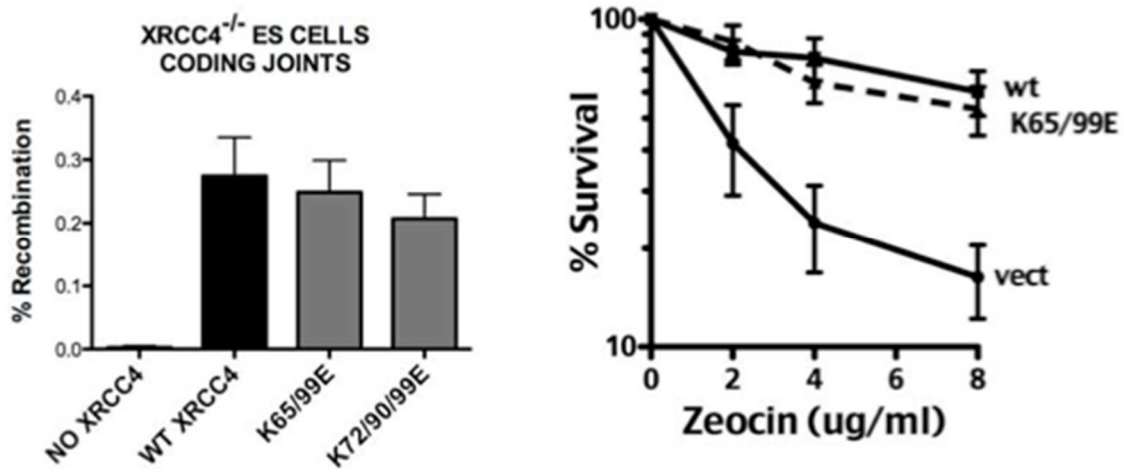
Left panel shows XRCC4 deficient 293 cells stably expressing wt and variant forms of XRCC4. Right panel shows zeocin kill curve assays on above-mentioned cells. Note that cells expressing mutant XRCC4 are markedy sensitive to the radiomimetic drug, zeocin. Error bars indicate SEM of at least three independent experiments.

FIGURE 6- Overexpression of XRCC4 variants in wt CHO cells (AA8) exert a dominant negative effect on DSBR.



Left panel shows wt CHO (AA8) cells stably over-expressing wt and mutant forms of XRCC4. Right panel shows zeocin kill curve assays on above-mentioned cells. Error bars indicate SEM of at least four independent experiments.

FIGURE 7- XRCC4 variants that do not interact with XLF do not affect NHEJ in mouse ES cells.



Left panel shows recombination percentage of coding joint substrate in XRCC4 deficient ES cells expressing wt and mutant XRCC4. Error bars indicate SEM of five independent experiments. Right panel shows zeocin kill curve assays of XRCC4 deficient ES cells stably expressing wt and mutant forms of XRCC4. Error bars indicate SEM of three independent experiments.

2A.4 Discussion

The process of NHEJ entails meticulous co-ordination among several protein factors that either recognize the DNA lesion or process the lesion to make it compatible for ligation by the ligation apparatus comprising of XRCC4, XLF and DNA ligase IV. XRCC4, which is part of the ligation complex, interacts with numerous other NHEJ proteins like DNA-PK, PNKP, APLF, XLF and DNA ligase IV (37). Polynucleotide kinase (PNKP) functions in processing DNA ends as it contains 5' DNA kinase and 3' phosphatase activities thus enabling it to repair 5'-OH and 3'-phosphate on DNA ends. APLF is known to act as a scaffolding protein, which helps to recruit XRCC4 and XLF to the chromatin following DNA damage. Thus, XRCC4's interaction with DNA-PK, PNKP and APLF links the ligation apparatus to earlier steps in NHEJ like DNA break sensing and processing. XRCC4 has long been known to be important to stabilize DNA ligase IV in cells and also stimulate its adenylation (20). Not much was known about the importance of XRCC4's interaction with the more recent NHEJ ligation factor, XLF in cells. *In vitro* studies have shown that complexes formed by XRCC4-XLF interaction promote bridging of linear DNA fragments (50). Our VDJ studies underscore the importance of the XRCC4-XLF interaction with respect to joining DNA breaks induced by RAG endonuclease mediated enzymatic cleavage. Thus, these complexes and potential filaments enable joining of not only simple, blunt ended signal ends or partially cohesive coding ends, but also complex DNA ends that are produced by γ-irradiation. Moreover, different cell types seem to have varying requirement for functional filament complexes. The basis for this difference in phenotype in different cell types is unclear. It is possible that the deficiency in bridging by XRCC4-XLF complexes in some cells may

be compensated functionally by additional bridging proteins in those cells. Thus, it will be interesting to understand what other cellular factors may compensate for the loss of filament function in various cell types.

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Part B: XLF interacts with XRCC4 to promote DNA double-strand break repair and facilitate joining of non-cohesive DNA ends in cells

2B.1 Introduction

XLF is the most recently discovered NHEJ factor identified as an XRCC4 interacting protein in yeast two-hybrid screens (42) and as a missing factor in patients exhibiting severe immunodeficiency (82). *XLF* gene was found to be mutated in certain patients exhibiting immune deficiencies, microcephaly and growth retardation (76). Cells derived from these patients showed hypersensitivity to irradiation, defective V(D)J recombination of extrachromosomal DNA substrates and impaired DNA end ligation. Surprisingly, XLF deficient mice exhibit relatively normal V(D)J recombination and lymphocyte development (83). However, XLF deficient mouse ES cells and mouse embryonic fibroblasts (MEFs) show hypersensitivity to IR and defects in extrachromosomal V(D)J recombination (83,84). On the other hand, XLF deficient pro-B cells perform wt levels of V(D)J recombination. These observations suggested that XLF deficiency is compensated by other factors in lymphocytes in the context of V(D)J joining in mice. Finally, in the context of end joining, XLF has been shown to stimulate the ligation reaction *in vitro*, particularly the joining of non-cohesive DNA ends (46,47). However, the mechanism of how this is achieved is unclear. XLF promotes readenylation of ligase IV and inter-molecular ligation of linear DNA substrates as opposed to intra-molecular ligation (49,78), suggesting that it helps in aligning multiple DNA fragments for ligation. Here we define two XLF mutants that do not interact with XRCC4 and presumably fail to form filaments. Both mutants fail to support bridging of

DNA fragments *in vitro*, however, one of them supports XRCC4-ligase4 mediated ligation, while the other does not. Using these mutants we find that all cell types are dependent on XLF's ability to stimulate ligation but XLF's ability to bridge DNA ends is dispensable for some cell types but not others. Finally, we find that bridging by XLF-XRCC4 complexes promote joining of non-cohesive DNA ends in cells.

2B.2 Materials and Methods

Our collaborator (Dr. Mauro Modesti) performed bridging and ligation assays outlined below and provided us with XLF mutant constructs.

2B.2.1 Ligation assays Reactions (10 μl) contained 100 ng of linearized pUC19 plasmid (digested with XbaI for cohesive end ligation or with SmaI for blunt end ligation), 2 mM $MgCl_2$, 1 mM ATP, 75 mM KCl, 10 mM HEPES pH 8.0, 0.5 mM EDTA, 5% glycerol and the indicated final concentrations of proteins. T4 DNA ligase (New England Biolabs) was used at a final concentration of 8 units/μl. After a 30 min incubation at room temperature, the samples were deproteinized by addition of pronase (1.25 μg/μl final concentration) and Sarkosyl (1.25 % final concentration) and incubated at 55°C for 30 min. The reaction mixtures were fractionated by agarose gel electrophoresis using Tris-Borate-EDTA buffer and stained with ethidium bromide. Gel images were acquired as indicated for the EMSA assays.

2B.2.2 Bridging Assay This assay was performed as described in (50)

2B.2.3 Pull down assays, V(D)J recombination assays and zeocin assays These assays were performed as described in chapter 2, part A.

2B.2.4 Immunoblot Analysis This assay was performed as described in chapter 2, part A. Additional antibodies used in this study are rabbit anti ligase IV polyclonal antibody (Proteintech) and mouse anti actin antibody (Santa Cruz Biotechnology).

2B.3 Results

2B.3.1 XLF variants L115A and L115D fail to interact with XRCC4 and Ligase IV in cell extracts. Structural studies have indicated that XLF residue L115 is critical for its interaction with XRCC4, as it inserts into the hydrophobic head domain of XRCC4 forming a 'Leu-lock' (51). Consistently, *in vitro* studies have shown that mutating the codon corresponding to XLF L115 to alanine disrupts its interaction with XRCC4 (43). Furthermore, Malivert et al. have shown that mutating the codon corrsponding to L115 to aspartate also disrupts its interaction with XRCC4 *in vitro* (85). Thus, we used both variants L115A and L115D to examine if they interact with XRCC4 *in vivo*. We overexpressed C-terminal His-tagged wt and mutant XLF in 293 cells and performed Ni-NTA pull down assays. As expected, wt XLF interacted with both XRCC4 and ligase IV, whereas L115A and L115D failed to interact with both XRCC4 and ligase IV in cells (figure 8). Thus XLF variants behave similarly *in vitro* and in cells.

2B.3.2 XLF variants L115A and L115D fail to bridge linear DNA fragments *in vitro*. Bridging of linear DNA fragments by wt XRCC4 and XLF complexes has been reported before. Moreover, XRCC4 mutants that fail to interact with XLF are deficient in bridging DNA ends *in vitro* (50). Our collaborator adopted a reciprocal approach to test if XLF variants L115A and L115D were also deficient in bridging DNA ends *in vitro*. Briefly, a 1 kb biotinylated DNA fragment was pulled down upon streptavidin beads and the presence of a 0.5 kb DNA fragment in the pull-down fraction was monitored by gel

electrophoresis. As expected, both L115A and L115D failed to support DNA end bridging *in vitro,* consistent with the results derived from XRCC4 mutants (figure 9).

2B.3.3 XLF variant L115A supports inter-molecular ligation by DNA ligase IV, while L115D does not A well defined function of XLF is its ability to enhance inter-molecular ligation of linear DNA substrates (thus forming concatemers) over intra-molecular ligation of a linear DNA molecule (forming circular monomer) when in complex with XRCC4-ligase IV (78). This result suggested that XLF has the ability to synapse DNA ends, thus enabling ligation of multiple DNA fragments. Thus, we hypothesized that filaments formed by XRCC4-XLF complexes would promote formation of inter-molecular ligation products. To examine this possibility, our collaborator performed intermolecular ligation assays using linearized plasmid as a substrate and purified XRCC4, XLF and T4 ligase proteins. As expected, wt XRCC4-XLF complexes can form intermolecular ligation products while L115A and L115D mutants are unable to bridge DNA fragments and hence unable to perform intermolecular ligation (figure 10A). Surprisingly, in the same reaction, when T4 ligase is replaced by the canonical NHEJ ligase- DNA ligase IV, L115A is capable of supporting formation of concatemers, unlike L115D (figure 10B). From these observations we concluded that XLF L115A behaves as a separation of function mutant that fails to bridge DNA ends but promotes ligation by DNA ligase IV. On the other hand, XLF L115D being a more disruptive mutant, fails to bridge DNA ends and also fails to stimulate ligation by the XRCC4-ligase4 complex.

2B.3.4 XLF variant L115A variably supports V(D)J joining in different cell strains, while L115D does not support V(D)J joining Given that XRCC4 mutants that do not

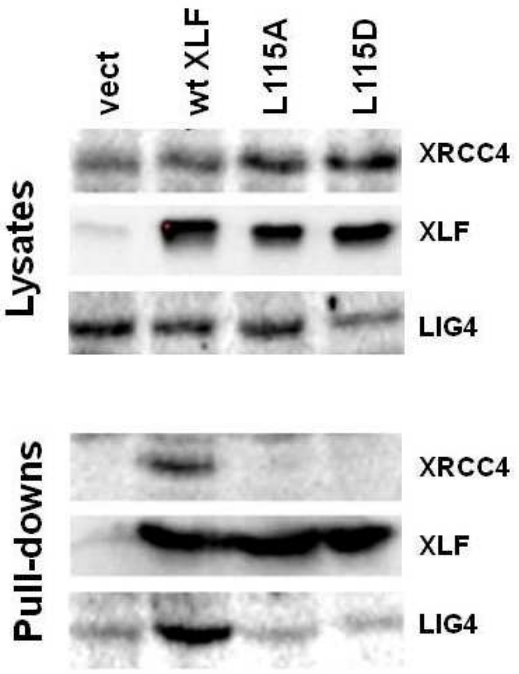
interact with XLF fail to support joining of extrachromosomal V(D)J substrates in most cell types, we examined if XLF mutants would behave the same way. pJH201 and pJH290 substrates described before were used to assess signal and coding joint formation in $XLF^{-/-}$ ES cells, while the fluorescent protein encoding V(D)J substrates (290/RFP/CFP, 289/RFP/CFP) were used to monitor V(D)J recombination in XLF deficient 293 cells. As expected, XLF L115D failed to support joining of V(D)J substrates in all cases. XLF L115A was fully capable of forming signal and coding joints in XLF deficient ES cells, similar to the effect observed by XRCC4 mutants in XRCC4 deficient ES cells (Chapter2, partA). However, XLF L115A could only partially restore coding and signal joining in XLF deficient human 293 cells (figure 11). This result suggests that different cell types have variable degrees of requirement for XLF's function in DNA bridging for the resolution of V(D)J recombination intermediates.

2B.3.5 XLF variant L115D fails to reverse zeocin sensitivity in all cell types, while L115A has variably reverses zeocin sensitivity in different cell types. Since XRCC4 mutants that do not interact with XLF show zeocin sensitivity in most cell types, we determined if XLF mutants that do not interact with XRCC4 would show the same phenotype. We obtained a panel of XLF deficient human and mouse cell lines, and generated stable strains expressing wt or mutant forms of XLF (figure 12A). The host cell lines used are mouse XLF deficient ES cells and pre-B cells (obtained from Dr. F.W. Alt); human XLF deficient HCT116 (obtained from Dr. Eric Hendrickson), 293 (generated for these studies by CRISPR/Cas9 targetting) and 2BN cells (obtained from Dr. Mauro Modesti). 2BN cells are fibroblasts derived from a patient who lacked expression of full length XLF. In all of the above cell strains wt XLF robustly reversed

zeocin sensitivity compared to vector controls. XLF mutant L115D was deficient in reversing zeocin sensitivity. L115A moderately reversed zeocin sensitivity in some cell strains but not in others (figure 12B-F). We concluded that XLF's ability to bridge DNA ends by complex formation with XRCC4 is differentially required in different cell types. In contrast, XLF's ability to stimulate the ligase complex is required in all cell types.

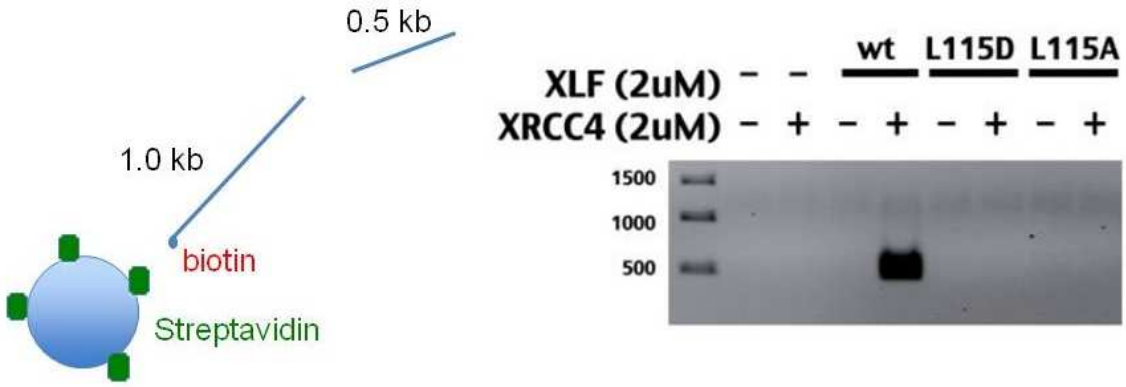
2B.3.6 XLF interacts with XRCC4 to promote joining of non-cohesive DNA ends in cells. Previous reports have shown that XLF promotes alignment and joining of non-cohesive DNA ends by the NHEJ ligation complex *in vitro* (46,47,86). This finding led us to determine if the ability of XLF to bridge DNA was required for joining incompatible DNA ends. Indeed, *in vitro* studies have shown that XLF L115A fails to support joining of mismatched DNA ends (43,48), thus supporting the idea that formation of XRCC4-XLF complexes promote joining of mismatched DNA ends. We next investigated if XLF L115A would also fail to support joining of non-cohesive DNA ends in cells. To this end, we generated coding joining substrates with perfectly matched and completely mismatched 3' overhangs. XLF L115A was markedly deficient in joining mismatched DNA ends as opposed to matched DNA ends (figure 13). Notably, there is reduced overall joining efficiency of the modified coding joint substrates, because the RAG endonuclease complex is uniquely sensitive to the coding end sequence surrounding the conserved heptamer of the recombination signal sequence (87). Therefore, comparison between L115A and wt XLF is possible only among each individual substrate and not between the substrates.

FIGURE 8- XLF variants L115A and L115D fail to interact with XRCC4 and Ligase IV in cell extracts.



Immunoblot analyses of lysates from 293 cells transiently transfected with His-tagged wt and mutant forms of XLF probed with antibodies for XRCC4, XLF or Ligase4 (top). Immunoblot analyses of pulldown fractions recovered from Ni-NTA agarose beads 3hrs after incubation of cell lysates with beads and subsequent washing. Immunoblot was probed with antibodies to XRCC4, XLF or Ligase4 (bottom).

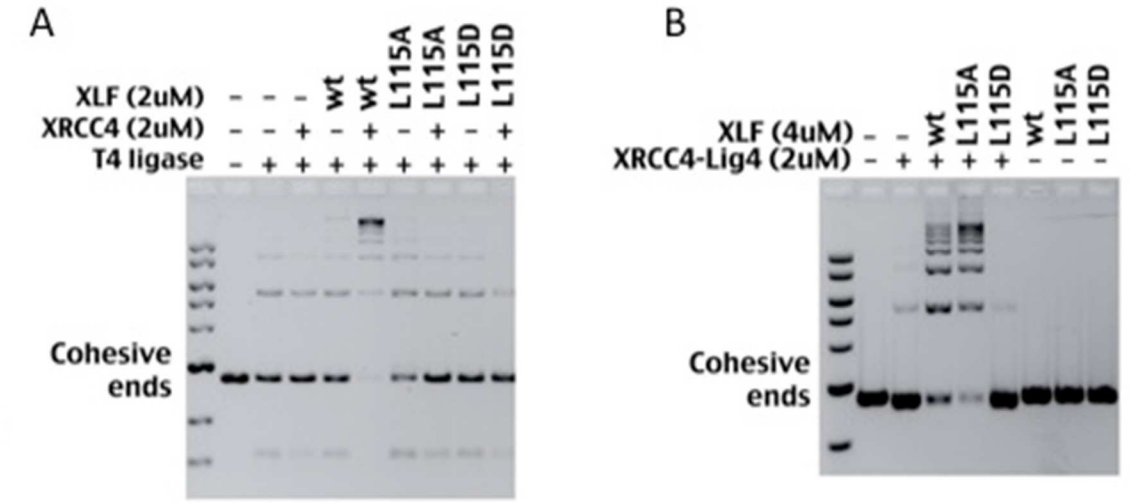
FIGURE 9- XLF variants L115A and L115D fail to bridge linear DNA fragments *in vitro*.



Left panel shows a schematic representation of the DNA bridging assay. Right panel depicts agarose gel showing recovery of DNA fragments bound to streptavidin beads by ethidium bromide staining.

**Our collaborator Dr. Mauro Modesti's lab performed this experiment.

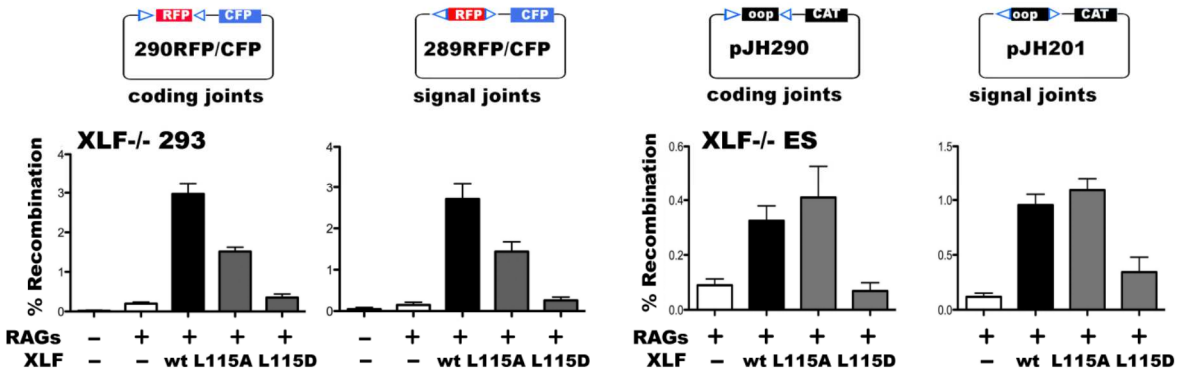
FIGURE 10- XLF mutant L115A fails to support inter-molecular ligation by T4 ligase but completely supports ligation by DNA ligase4.



Ethidium bromide staining of agarose gels showing ligation products obtained from *in vitro* ligation reactions as described in Materials and Methods. In (A), T4 DNA ligase is utilized. In (B) XRCC4/Lig complexes are utilized.

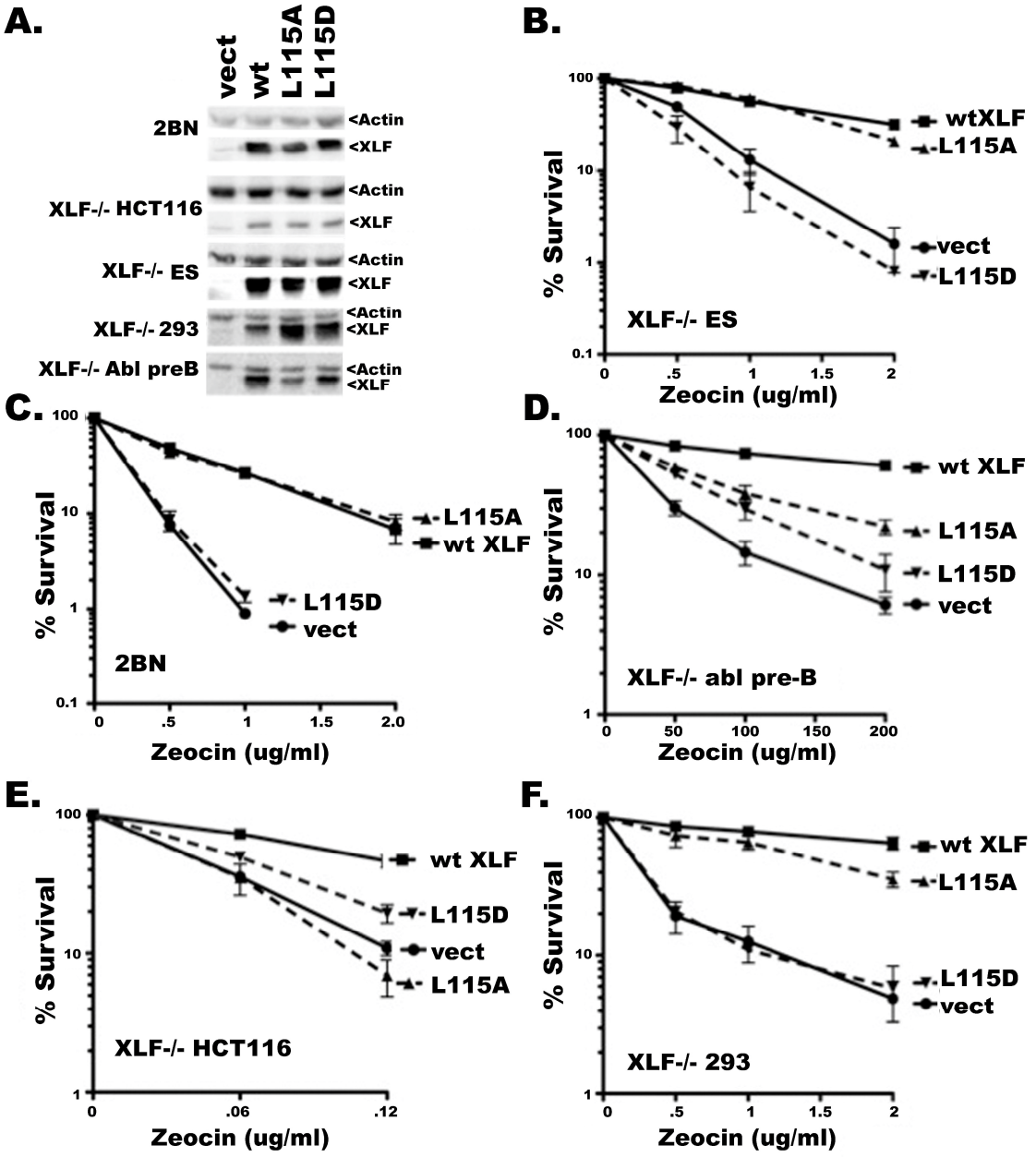
**Our collaborator Dr. Mauro Modesti's lab performed this experiment.

FIGURE 11- XLF mutant L115A variably supports V(D)J joining in different cell strains, but L115D does not support V(D)J joining.



Fluorescent substrates (depicted top panel) were utilized to detect coding and signal joints in VDJ assays in 293 cells whereas substrates pJH290 and pJH201 (depicted top panel) were utilized to detect coding and signal joints in VDJ assays in ES cells. Bottom panels show percent recombination of episomal fluorescent coding and signal joint substrates in XLF-deficient 293 cells transiently expressing full-length Rag1, Rag2, wild type and mutant forms of XLF. Note that the decrease in percent recombination observed by L115A and L115D are statistically significant (wt Vs L115A p=0.021; wt Vs L115D p=0.0002) according to two-tailed Mann-Whitney test. Error bars indicate SEM from five independent experiments. Bottom panel shows percentage recombination of pJH290 (left) and pJH201 (right) in XLF deficient mouse ES cells transiently expressing full length Rag1, Rag 2, wt and mutant forms of XLF. Note that the decrease in recombination percentage observed by L115D compared to wt is statistically significant (p=0.0019- coding joint assay; p=0.0047- signal joint) according to two-tailed Mann-Whitney test. Error bars indicate SEM from four independent experiments.

FIGURE 12- XLF mutant L115D fails to reverse zeocin sensitivity in all cell types, while L115A has variable degrees of zeocin resistance in different cell types.

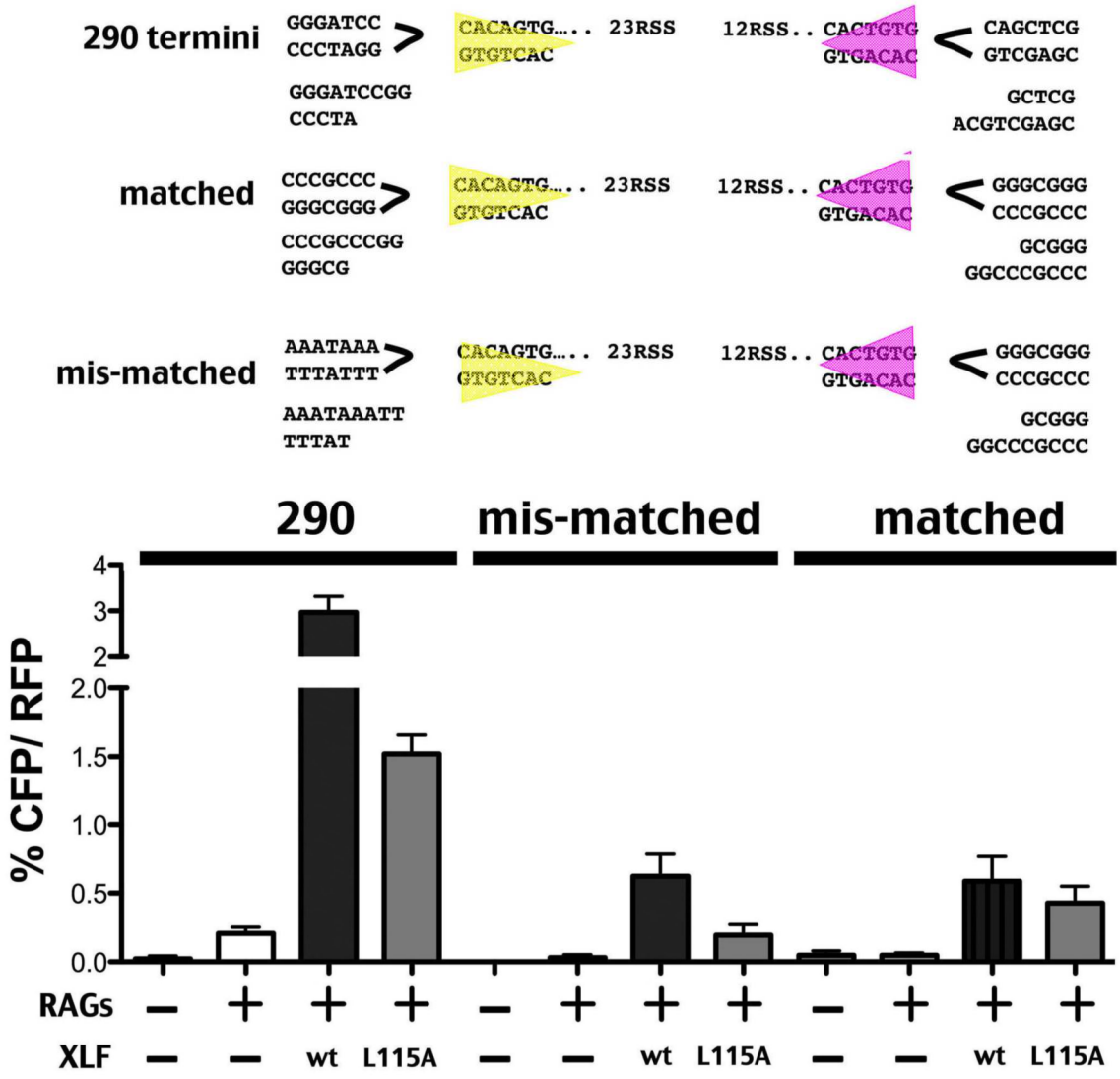


(A) Immunoblot showing stable expression of actin, wild type or mutant XLF in various XLF-deficient cell strains, as indicated. (B to F) Zeocin sensitivity of the indicated cell

FIGURE 12 (cont'd)

strains stably expressing equivalent levels of wild type or mutant XLF. Error bars indicate SEM from at least three independent experiments.

FIGURE 13- XLF interacts with XRCC4 to promote joining of non-cohesive DNA ends in cells.



Schematic (top) showing altered coding end sequences to generate perfectly matched or mismatched overhangs in the fluorescent coding joint substrate depicted in Figure 11. The colored triangles represent recombination signal sequence; the part of sequence adjoining the triangles represent coding end sequences that are hair-pinned upon RAG mediated cleavage. Opening of hairpins by artemis, generates overhangs

FIGURE 13 (cont'd)

that are either matched or mismatched as indicated. Comparison of recombination rate of episomal substrates with matched and mismatched coding ends in XLF-deficient 293 cells transiently expressing Rag1, Rag2, wild type and mutant forms of XLF (bottom). Error bars indicate SEM from four independent experiments. Note that the decrease in recombination rate observed by XLF mutant L115A compared to wild type is statistically significant for 290 ($p=0.0021$) and mismatched ($p=0.0404$) termini according to two-tailed Mann-Whitney test. There is no significant difference observed between recombination rate of wt XLF and XLF mutant L115A in presence of matched termini.

2B.4 Discussion

Previous studies have shown that XLF stimulates the activity of the XRCC4-LigaseIV complex *in vitro*, but the mechanism by which it stimulates the ligation complex was largely unknown. One study reported that XLF promotes re-adenylation of ligase IV complexed to XRCC4, thus facilitating the ligation reaction (49). Investigators had speculated that binding of XLF to XRCC4-Ligase IV complex imparts a conformational change that makes the ligase4 complex more poised to carry out ligation. The observation that XLF promotes joining of multiple linear DNA substrates to form concatemers suggests that XLF helps in synapsing linear DNA molecules thus facilitating ligation (78). Our studies provide an additional mechanism by which XLF interacts with XRCC4-Ligase IV complex and presumably forms filaments to promote bridging of broken DNA ends. The XLF mutant L115A serves as a potential separation of function mutant that preserves the ability to stimulate the ligation reaction by XRCC4-Ligase IV complex but is deficient in DNA end bridging. This mutant enables us to distinguish between the relative importance of bridging versus ligation stimulation. Indeed, L115A seems to be deficient in the resolution of V(D)J intermediates in certain cells and survival of various cell types in response to DNA DSB inducing agents implying that the bridging function of XLF is important for cell survival. Given that XLF supports synapsis of linear DNA substrates, it is conceivable that this synaptic function could promote joining of non-compatible DNA ends by tethering the ends even in the absence of base-pair annealing. Consistently, an XLF mutant that is deficient in bridging (L115A) is also deficient in joining mismatched DNA ends. XLF's ability to promote ligation of non-cohesive DNA ends might have implications in recombinant DNA

technology. The property of XRCC4-XLF filaments to support bridging is important in tethering broken DNA ends, thus preventing events like chromosomal translocations and serves as a mechanism for preventing genomic instability. Indeed, mouse cells that lack XLF show increased genomic instability compared to wt cells (84). Therefore, XLF is an important NHEJ factor that helps to maintain genomic stability in cells.

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Chapter 3: Bridging of DNA ends by XRCC4-XLF complexes is synergistic with ATM but independent of PAXX

3.1 Introduction

XLF deficiency is tolerated variably in different cell types. XLF deficient mouse embryonic stem cells and mouse embryonic fibroblasts are sensitive to irradiation and deficient in V(D)J joining of extrachromosomal substrates. On the other hand, XLF deficient pro-B cells are proficient in V(D)J joining of both extrachromosomal and integrated DNA substrates (83). Studies have shown that ATM, H2A.X, 53BP1 or DNA-PKcs have redundant functions with XLF in mice (88-90). Deficiency of XLF along with either ATM, 53BP1 or DNA-PKcs results in severe growth retardation in mice and marked impairment in V(D)J joining of chromosomally integrated substrates in lymphocytes, as compared to either factor alone. $XLF^{-/-}H2A.X^{-/-}$ mice are embryonic lethal; B-cells obtained from conditional knock-out mice show marked impairment in V(D)J joining upon deleting H2A.X along with XLF (88). Given that all of the redundant protein factors described have been implicated in bridging DNA ends (64,70,91), we hypothesized that the redundancy with XLF can be attributed to DNA bridging. Alternatively, since all of the factors mentioned above also participate in the DNA damage response, it is possible that XLF has redundant functions in DNA damage signaling. To test our hypothesis, we utilize our bridging deficient XLF L115A mutant to examine if it is functionally redundant with ATM. Our data suggest that the bridging function of XLF is complementary to ATM.

Recently a novel protein called PAXX (Paralog of XRCC4 and XLF) that resembles XRCC4 and XLF in structure has been described (92-94). We determined, if XLF and PAXX have complementary functions in cells. We find that XLF and PAXX have

overlapping function in the context of reversing zeocin sensitivity and V(D)J recombination, but the bridging function of XLF is independent of PAXX.

3.2 Materials and Methods

3.2.1 Generation of XLF$^{-/-}$ATM$^{-/-}$ and XLF$^{-/-}$PAXX$^{-/-}$ 293 cell strains Cas9-targeted gene disruption was performed using methods similar to those reported by Mali *et al.* (95). Briefly, gRNAs specific for XLF, ATM, XRCC4 or PAXX were synthesized as 455 bp fragments (Integrated DNA Technologies). The synthesized fragments were cloned into pCR2.1 using a TOPO TA cloning kit according to the manufacturers' instructions (Life Technologies). Cells were transfected with 1 μg gRNA plasmid and 1 μg Cas9 expression plasmid (Addgene). In some cases, cells were co-transfected with 0.2 μg of pcDNA6 (Life Technologies) or pSuper-Puro to confer blasticidin or puromycin resistance. Western blotting was used to identify clones with deletions in each of these factors. The 19 mers specific for each factor synthesized into the 455 bp fragments are as follows:

ATM: TCTTTCTGTGAGAAAATAC

XRCC4: CCTGCAGAAAGAAAATGAA

XLF: GGCCTGTTGATGCAGCCAT

PAXX-1: CTGGCCTTTGACCTCTCCA

PAXX-2: TGCTTCACGCCGGACAGCCT

3.2.2 V(D)J recombination and zeocin sensitivity assays As described in chapter1, part A.

3.2.3 Immunoblot Analysis As described in chapter1, part A. Additional antibodies used for this part of the study are goat anti-C9ORF142 (Santa Cruz Biotechnology) for

PAXX, rabbit anti-ATM (Serotec). The DNA-PKcs antibody (42-27) was the generous gift of Tim Carter.

3.2.4 I-Sce joining assay 293 cells were plated at 20 to 40% confluency onto 24-well plates overnight. Cells were transfected with 0.125 μg substrate, 0.25 μg of expression plasmid encoding either I-SceI or I-SceI-Trex2 fusion protein as indicated and 0.25 μg of XLF or XRCC4 expression construct as indicated using PEI as discussed above. 72 hr post transfection, cells were harvested and analyzed by flow cytometry. Percentage of cells expressing RFP and CFP were determined using FlowJo software. Joining efficiency was calculated as the percentage of live cells expressing CFP over the percentage of live cells expressing RFP.

3.3 Results

3.3.1 Loss of ATM accentuates the zeocin sensitivity of XLF L115A in 293 cells. Given that an XLF mutant (L115A) that does not bridge DNA demonstrates mild zeocin sensitivity in 293 cells (shown in Chapter 2, part B), we examined if loss of additional bridging factors, like ATM would exacerbate this phenotype. To determine this, $XLF^{-/-}$ $ATM^{-/-}$ double deficient 293 cells were generated (figure 14A) by CRISPR/Cas9; wt and mutant forms of XLF were stably expressed in these cells (figure 14B) and treated with graded doses of zeocin. In contrast to what was observed in $XLF^{-/-}$ 293 cells, three independent clones of XLF L115A exhibited severe zeocin sensitivity compared to wt cells in $XLF^{-/-}ATM^{-/-}$ double deficient cells (figure 14C). This shows that loss of ATM exacerbated the zeocin sensitivity of XLF L115A, thus indicating that loss of bridging by XLF might be compensated for by ATM.

3.3.2 XLF L115A is deficient in joining tandem DNA DSBs in the absence of ATM. Since XLF L115A is capable of stimulating ligation by DNA ligase IV but deficient in bridging, we determined if it would affect the efficiency of joining tandem DNA breaks introduced by the enzyme I-Sce1 on an episomal DNA substrate. Furthermore, we wanted to examine if the distal end joining efficiency by L115A would be altered in the absence of ATM. To test this question, we introduced a plasmid substrate that has two consecutive I-Sce1 sites separated by an RFP cassette and followed by a CFP cassette (figure 15A), a plasmid expressing I-Sce1 endonuclease, mutant and wt XLF into cells. Thus, joining of the two I-Sce1 mediated breaks would result in CFP expression. We observed that XLF L115A does not significantly affect joining of consecutive I-Sce1 mediated breaks in XLF deficient cells but markedly reduces the joining efficiency of distal DNA ends in $XLF^{-/-}ATM^{-/-}$ double deficient cells (figure 15B). The inefficiency of XLF L115A in joining clustered tandem double strand breaks in the absence of ATM further supports the hypothesis that the bridging function of XLF is redundant with ATM.

3.3.3 Loss of ATM accentuates the V(D)J joining deficiency of XLF L115A in 293 cells. Another instance of joining consecutive DNA double strand breaks is the process of V(D)J recombination. We next investigated if the deficiency in joining coding and signal ends by XLF L115A would be worsened in the absence of ATM. Similar V(D)J recombination assays were performed in $XLF^{-/-}ATM^{-/-}$ double deficient 293 cells. The efficiency of coding and signal joining by L115A is further reduced in $XLF^{-/-}ATM^{-/-}$ double deficient cells compared to $XLF^{-/-}$ cells (figure 16).

3.3.4 Loss of ATM accentuates the V(D)J joining deficiency of XRCC4 variants K65/99E and K72/90/99E. The above observations suggest that bridging by XRCC4-

XLF complexes is redundant with ATM. To further test this hypothesis, a reciprocal approach was adopted to determine if XRCC4 mutants (K65/99E, K72/90/99E) that fail to bind XLF also show an exacerbated phenotype in the absence of ATM. To this end, we generated XRCC4$^{-/-}$ATM$^{-/-}$ double deficient 293 cells and studied the effect of XRCC4 mutants in joining V(D)J substrates. The deficiency in signal and coding joint formation by XRCC4 mutants is further exacerbated in the absence of ATM, thus suggesting that potential bridging by XRCC4-XLF complexes is complementary with ATM (figure 17).

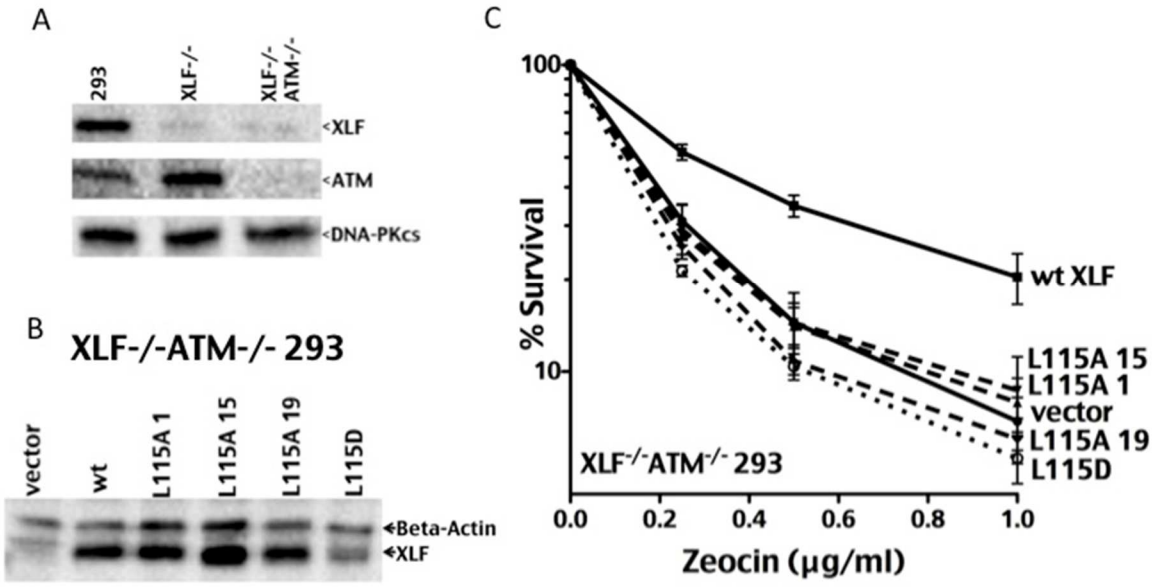
3.3.5 PAXX and XLF functionally overlap in the context of zeocin sensitivity and joining of V(D)J substrates. Very recent studies have identified a novel protein that belongs to the XRCC4-XLF family of proteins called PAXX. The overall structure of this protein is similar to XRCC4 and XLF in that it also exists as a dimer in cells and contains a globular N-terminal head domain and a coiled-coil C-terminal tail domain. In order to understand if PAXX shares similar functional characteristics as XLF, XLF$^{-/-}$ PAXX$^{-/-}$ double deficient 293 and HCT116 cells were generated (figure 18A) and treated with graded doses of zeocin. We observed that cells deficient in both XLF and PAXX are markedly more sensitive than XLF deficient cells (figure 18B). This result suggests that XLF and PAXX have overlapping functions in cells.

We next examined if PAXX and XLF functionally overlap with respect to joining of V(D)J substrates. To address this question, we performed V(D)J assays in XLF$^{-/-}$PAXX$^{-/-}$ double deficient 293 cells and found that these cells were more deficient in joining V(D)J substrates than XLF single deficient cells (figure 19A). Moreover, genetic

complementation of PAXX and XLF in the double deficient cells restored wt levels of V(D)J joining (figure 19B).

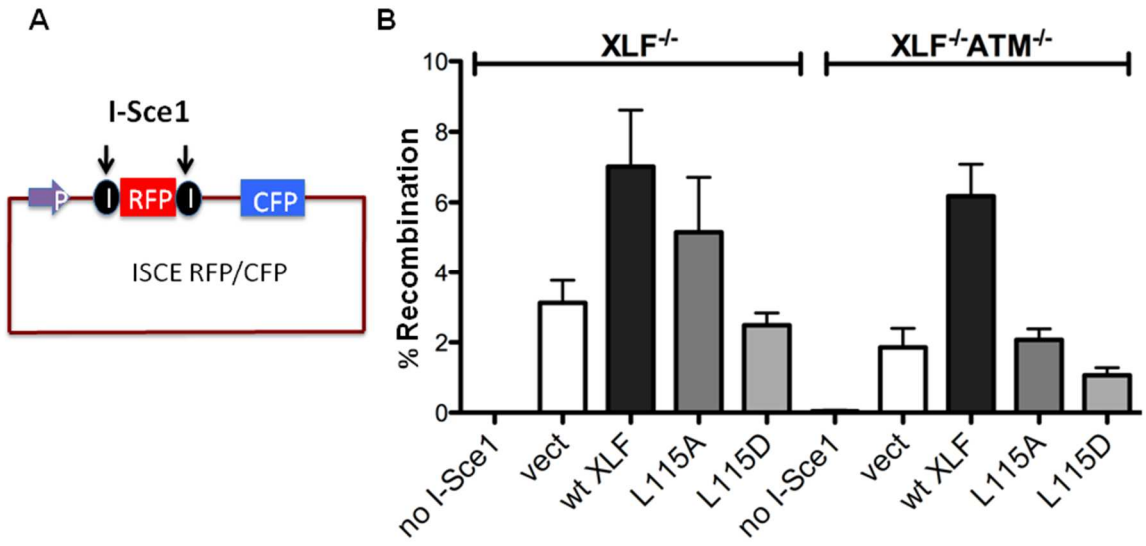
3.3.6 Functional overlap between XLF and PAXX is independent of XLF's bridging function. To understand if the functional overlap between XLF and PAXX can be attributed to the bridging function of XLF, we tested the L115A mutant form of XLF (that fails to bridge DNA ends) for its ability to perform V(D)J recombination in $XLF^{-/-}PAXX^{-/-}$ 293 cells. We observed that loss of PAXX did not further exacerbate the joining deficient phenotype of L115A (figure 20). This suggests that the bridging function of XLF is independent of PAXX.

FIGURE 14- Loss of ATM accentuates the zeocin sensitivity of XLF L115A in 293 cells.



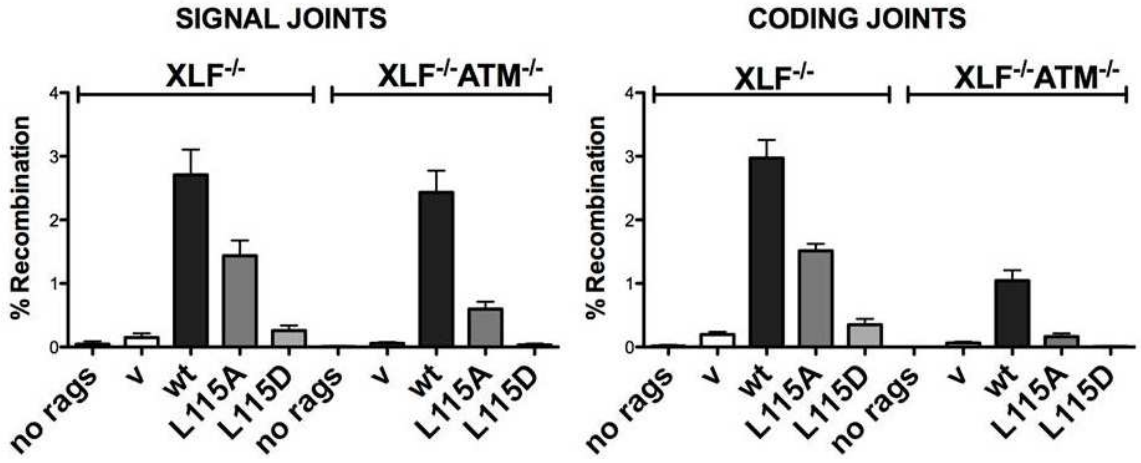
(A) Immunoblot showing expression levels of XLF and ATM in wt 293 cells, $XLF^{-/-}$ and $XLF^{-/-}ATM^{-/-}$ 293 cells. DNA-PKcs expression serves as the loading control. (B) Immunoblot showing stable expression levels of wt and mutant forms of XLF in $XLF^{-/-}$ $ATM^{-/-}$ 293 cells. Beta-actin expression serves as the loading control. (C) Zeocin kill curve assays of cell strains shown in (B). Error bars indicate SEM of three independent experiments.

FIGURE 15- XLF L115A is deficient in joining tandem DNA double strand breaks in the absence of ATM.



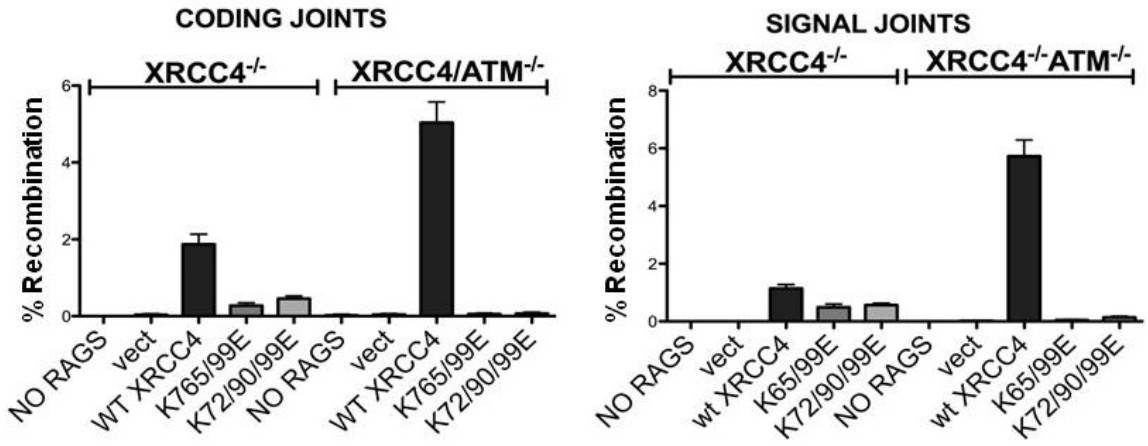
(A) Schematic of substrate used to assess joining of distal DNA ends. I indicates I-Sce1 site and P represents promoter followed by RFP and CFP cassettes. (B) Percentage joining of distal DNA ends in the presence of wt and variant forms of XLF in XLF deficient and XLF/ATM double deficient 293 cells. Error bars indicate SEM of three independent experiments.

FIGURE 16- Loss of ATM accentuates the V(D)J joining deficiency of XLF L115A in 293 cells.



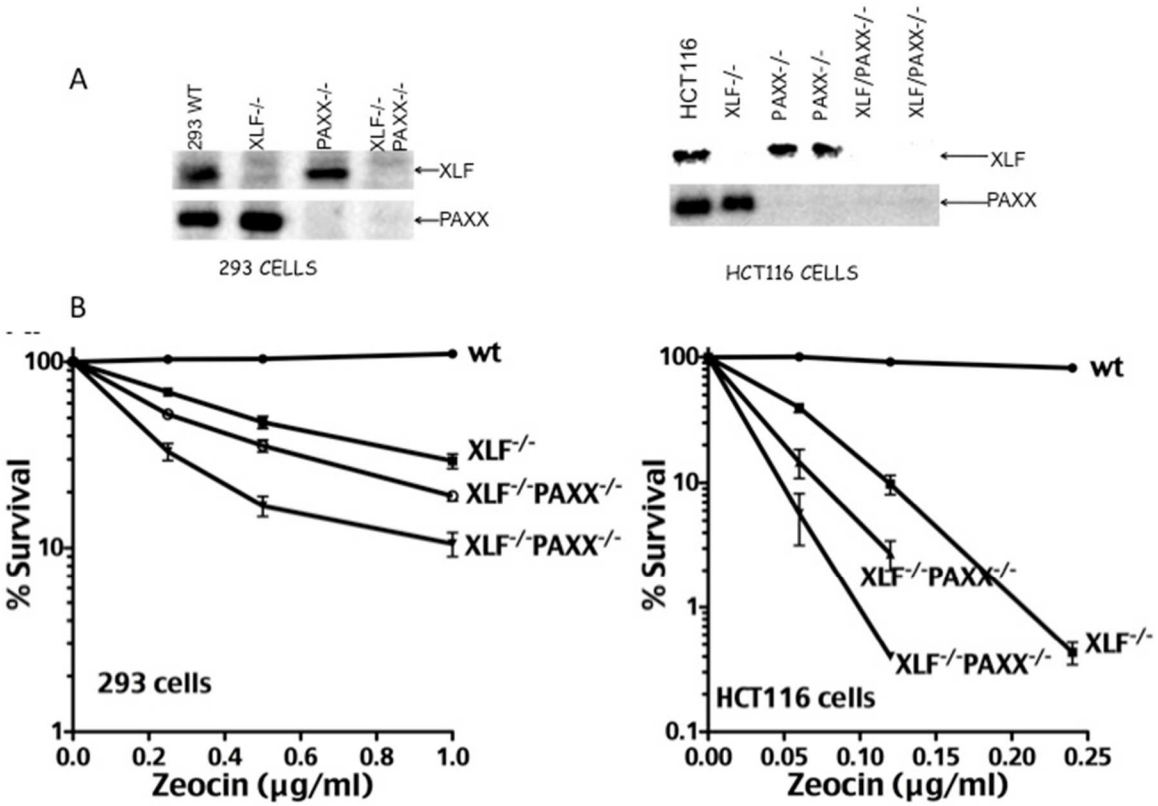
Recombination percentage of episomal coding and signal joining substrates comparing XLF deficient and XLF/ATM doubly-deficient 293 cells transiently expressing Rag1, Rag2, wild type and variant forms of XLF as indicated. Error bars indicate SEM from at least 3 independent experiments. Note that XLF L115A shows significantly lower coding end joining rate in XLF deficient (p=0.0001) and XLF-/-ATM-/- (p=0.0022) 293 cells. Similarly, XLF L115A performs significantly reduced signal joining in XLF deficient (p=0.0210) and XLF-/-ATM-/- (p=0.0022) cells.

FIGURE 17- Loss of ATM accentuates the V(D)J joining deficiency of XRCC4 mutants K65/99E and K72/90/99E.



Percentage recombination of episomal coding joint and signal joint substrates in XRCC4 deficient and XRCC4/ATM double deficient 293 cells transiently expressing Rag1, Rag2, wild type and mutant forms of XRCC4. Note that the decrease in coding joint formation by XRCC4 mutants in XRCC4/ATM double deficient cells is significantly low ($p= 0.0048$) compared to wild type XRCC4 according to two-tailed Mann-Whitney test. Similarly, the decrease in signal joint formation by XRCC4 mutants K65/99E and K72/90/99E are significantly low ($p= 0.0048$ and 0.0050 respectively) compared to wild type XRCC4 according to two-tailed Mann-Whitney test in XRCC4/ATM double deficient 293 cells.

FIGURE 18- PAXX and XLF functionally overlap in the context of zeocin sensitivity.



(A) Immunoblots showing expression levels of XLF and PAXX in $XLF^{-/-}$ and $XLF^{-/-}PAXX^{-/-}$ 293 (left) and HCT116 (right) cells. (B) Zeocin kill curve assays with $XLF^{-/-}$ $PAXX^{-/-}$ 293 (left) and HCT116 (right) cells. Error bars represent SEM from three independent experiments.

FIGURE 19- PAXX and XLF functionally overlap in the context of joining V(D)J substrates.

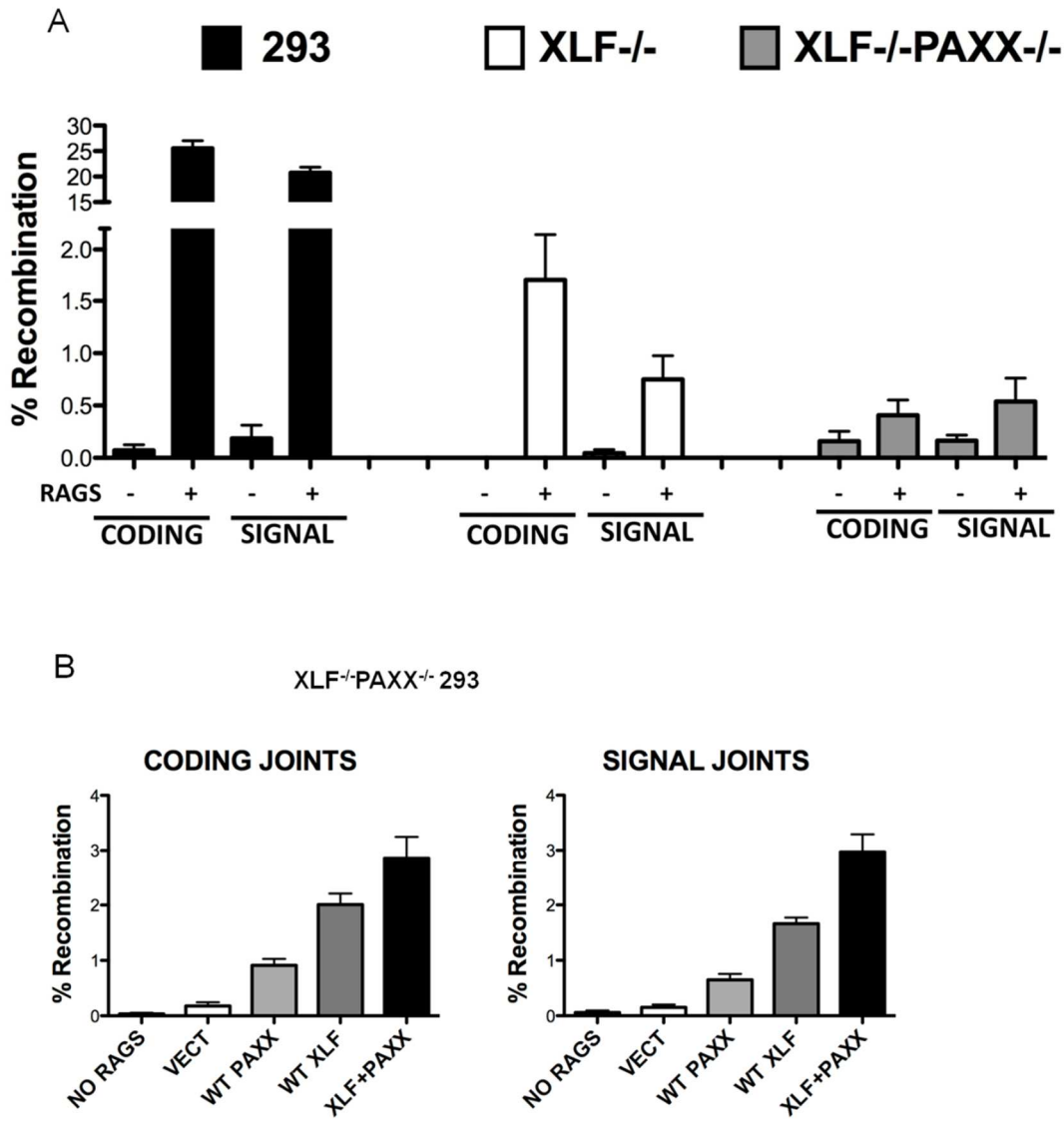
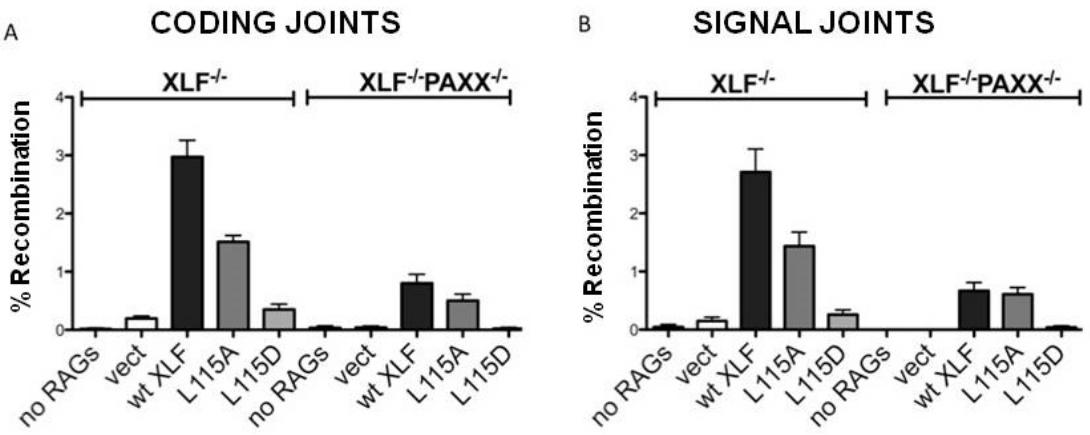


FIGURE 19 (cont'd)

(A) Percentage recombination of episomal coding (290/RFP/CFP) and signal (289RFP/CFP) joining substrates in wt 293, $XLF^{-/-}$ and $XLF^{-/-}PAXX^{-/-}$ 293 cells. Error bars indicate SEM of three independent experiments. Note that the decrease in recombination percentage in XLF deficient and XLF/PAXX double deficient cells is significantly low ($p<0.05$). (B) Percentage recombination of coding and signal joining substrates in $XLF^{-/-}PAXX^{-/-}$ 293 cells transiently expressing substrates, Rags, XLF and/or PAXX. Error bars indicate SEM of three independent experiments.

FIGURE 20- Functional overlap between XLF and PAXX is independent of XLF's bridging function.



Recombination percentage of coding joint substrate (A) and signal joint (B) substrate in XLF deficient and XLF/PAXX double deficient 293 cells transiently expressing Rag1, Rag2, wt and mutant forms of XLF. Error bars represent SEM from three independent experiments. Note that there is no significant difference in the recombination percentage observed in presence of wt XLF and L115A in XLF/PAXX double deficient cells.

3.4 Discussion

Data presented in this study support the idea that XLF and ATM function redundantly with each other, with respect to DNA end bridging. However, the exact mechanism of how XLF or ATM can substitute for each other's bridging function remains to be answered. ATM functions upstream of H2A.X and 53BP1, both of which have been implicated in DNA bridging. ATM phosphorylates H2A.X and brings about a more 'global' change around the DSB that impact recruitment of additional bridging factors. On the other hand, XLF is recruited to the DSB via Ku binding, which is more localized. It is thus likely that the bridging function of ATM-H2A.X-53BP1 axis functions in parallel to XRCC4-XLF filament mediated bridging.

We also show that XLF and PAXX have overlapping functions in the context of reversing zeocin sensitivity and in V(D)J joining; however, this functional overlap is independent of the bridging function of XLF. Studies have shown that PAXX stimulates joining of non-cohesive DNA ends (94) and helps in stable recruitment of NHEJ proteins to chromatin fraction (92). It will thus be interesting to understand if these functions of PAXX overlap with XLF.

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Chapter 4: Summary and Future Directions

The process of NHEJ is important not only for the maintenance of genomic stability but also for the process of V(D)J recombination that is essential for the development of lymphocytes (71). The V(D)J recombination process entails impartment of programmed DSBs by the RAG endonuclease complex (RAG1 and RAG2) in specific regions of the DNA called recombination signal sequences (RSS) (73). These breaks are exclusively repaired by the process of NHEJ (71). This study focuses on the ligation apparatus of NHEJ. The final ligation reaction is catalyzed by DNA ligase IV in association with XRCC4 and XLF. Crystallographic studies have shown that XRCC4 and XLF interact directly with each other to form filament like structures of repeating alternating units of XRCC4 and XLF homodimers (50-52,54). Structural studies have also shown that each individual filament can stack up against each other to form bundles of filaments (52), however existence of filament bundles in cells remain to be determined. Scanning force microscopy has shown that formation of such filament bundles is facilitated in the presence of DNA (50). *In vitro* DNA binding studies have shown that XRCC4 and XLF together bind DNA to form super-shifted protein-DNA complexes that migrate as a diffused smear in an agarose gel possibly representing heterogeneous collection of large nucleo-protein complexes. Disruption of the interaction between XRCC4 and XLF by mutation, disrupted formation of these super-shifted species suggesting that XRCC4-XLF bind DNA as a complex to form high-order molecular weight structures (50). Furthermore, XRCC4 and XLF together have been shown to mediate bridging of linear DNA fragments *in vitro*, which is abolished when the interaction between XRCC4 and XLF is disrupted by mutation (50). Very recent elegant super-resolution microscopic studies have revealed that these filaments can indeed be visualized in cells upon

induction of DNA damage (96). Furthermore, these filaments colocalize with Ku at the site of a DSB. Data presented in this study aim to characterize the function of these filaments in cells, particularly the function of DNA bridging by these filaments in cells. To this end, we utilized XRCC4 and XLF mutants that are deficient in interacting with each other (and thereby fail to form filaments) and studied the effects of these mutants in various cell-based assays such as V(D)J recombination assays and radiation/radiomimetic drug sensitivity assays.

We utilized XRCC4 mutants that fail to interact with XLF and fail to bridge DNA ends *in vitro* to examine their effect in cells. We first performed V(D)J assays in XRCC4 deficient hamster cells (XR1) expressing XRCC4 mutants that do not interact with XLF and found that formation of coding joints is markedly affected in these cells in comparison to signal joints. The marked reduction in coding joints by XRCC4 mutants could be due to impaired end-processing of hair-pinned coding ends or due to a deficiency in ligation. Sequencing of coding junctions isolated from these cells expressing XRCC4 mutants revealed that the amount of nucleotide added or deleted at the junctions were similar to that of cells expressing wt XRCC4, thus indicating that end-processing was not affected. The relatively normal levels of signal joining with XRCC4 mutants hinted that the ligation reaction was not affected. Thus we hypothesized that the deficiency in joining was due to impairment of DNA end bridging by XRCC4 mutants that fail to form filaments. Signal ends are held together by RAG proteins in a post-cleavage complex (80), thus possibly alleviating the requirement of bridging by XRCC4-XLF complexes. Consistently, XRCC4 deficient 293 cells show a greater deficiency in coding joining by XRCC4 mutants compared to signal end joining. Interestingly, XRCC4

deficient ES cells are unaffected for coding and signal joining by XRCC4 mutants. Thus, we conclude that different cell types have varying requirement for bridging by XRCC4-XLF complexes.

We next performed radiation sensitivity assays and found that hamster XR1 cells (deficient in endogenous XRCC4) expressing mutant XRCC4 show a moderate sensitivity to irradiation and the radiomimetic drug zeocin in absence of functional filaments; whereas human XRCC4$^{-/-}$ 293 cells show greater sensitivity to zeocin. As expected from V(D)J joining efficiency, XRCC4$^{-/-}$ ES cells are similarly zeocin resistant to wt cells. We hypothesize that the sensitivity to irradiation in the absence of filaments is possibly due to lack of efficient DNA bridging.

We also adopted a reciprocal approach in which we utilized XLF deficient cell strains and studied the effect of XLF mutants that do not interact with XRCC4 for V(D)J joining. Interestingly, we identified a separation of function XLF mutant (L115A) that was unable to bridge DNA ends but surprisingly capable of stimulating the activity of DNA ligase IV similar to wt XLF. Similar to our observation with XRCC4 mutants, we find that XLF L115A, which is solely deficient in bridging DNA ends has a moderate deficiency in coding and signal end joining in 293 cells but is capable of V(D)J joining in XLF deficient ES cells. Moreover, zeocin sensitivity assays reveal that L115A is moderately sensitive in 293 cells but completely reverses zeocin sensitivity in ES and 2BN cells. Surprisingly XLF deficient HCT116 cells are markedly sensitive to zeocin in presence of L115A, indicating that these cells are more dependent on XLF mediated DNA bridging. Reports have shown that HCT116 cells have a markedly reduced expression level of ATM (97), a protein factor that is itself implicated in maintaining DNA end stability (91) and is

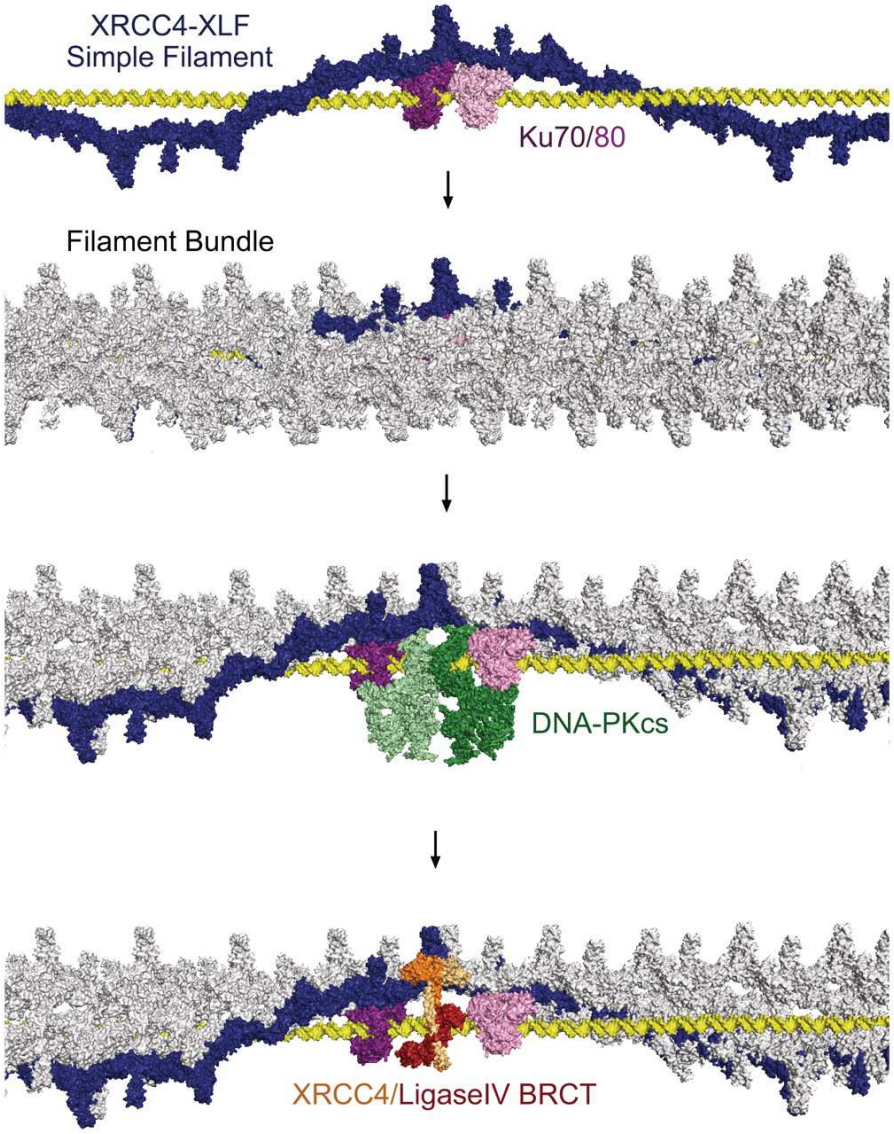
required for efficient recruitment of additional bridging factors like MRN and 53BP1. Thus, it is possible that decrease in ATM expression level sensitizes HCT116 cells to loss of bridging by XLF. Moreover, XLF and ATM has been shown to have redundant functions in V(D)J joining (88).

The above observation led us to hypothesize that the functional redundancy between XLF and ATM could be attributed to the bridging function of XLF (in complex with XRCC4). To test this hypothesis we examined if loss of ATM in addition to XLF in 293 cells resulted in further exacerbation of L115A phenotype. We found that the zeocin sensitive and V(D)J joining phenotype of L115A in XLF deficient 293 cells is indeed further exacerbated in the absence of ATM. This indicated that the bridging function of XLF and ATM are redundant.

In conclusion, our studies reveal a novel DNA end bridging function for XRCC4, XLF and DNA ligase IV, in addition to its well defined DNA end ligation function during NHEJ. It is thus conceivable that these proteins can be recruited earlier during NHEJ to stabilize and bridge the DNA ends prior to ligation, contrary to the earlier models of NHEJ, where the proteins performing the ligation reaction were thought to be recruited at the final step. Studies from the Chen laboratory has shown that XLF is recruited to DNA break site by Ku (98,99), indicating that XLF (perhaps in complex with XRCC4) can be recruited by Ku earlier during NHEJ. Moreover, live cell imaging technique has also shown that XRCC4-XLF complexes co-localize with Ku bound DNA supporting the idea that Ku recruits the filaments to the DNA break site upon induction of a DSB (96). Thus a new model of NHEJ emerges (figure 21), whereby, a DSB is recognized by Ku, which recruits XRCC4-XLF filaments and DNA-PKcs independently. The presence of

DNA-PKcs further promotes protein-protein interactions to stabilize the entire complex (98). Phosphorylation of XRCC4-XLF complexes by DNA-PKcs remodels the filaments to dissociate and allows processing of DNA ends followed by ligation (100).

FIGURE 21- Model for bridging by XRCC4-XLF filaments



For future research, it will be interesting to study the effect of these bridging deficient mutants in mice, especially in the context of V(D)J recombination and overall genomic

stability in an ATM deficient background. Furthermore, since the process of class switch recombination (CSR) requires synapsis of distal switch region DNA breaks, it will be interesting to study if these mutants affect the efficiency of CSR.

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