CHARACTERIZATION OF APLF IN THE
NONHOMOLOGOUS END-JOINING PATHWAY

by

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A thesis submitted in conformity with the requirements
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Graduate Department of Medical Biophysics
University of Toronto

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Characterization of APLF in the Nonhomologous End-joining Pathway

Chloe Jean Macrae, Master of Science, 2008
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**ABSTRACT**

Nonhomologous end-joining (NHEJ) is a major DNA double-strand break (DSB) repair pathway. NHEJ is initiated through DSB recognition by the DNA end-binding heterodimer, Ku, while end-joining is accomplished by the XRCC4-DNA ligase IV (X4L4) complex. This thesis reports that APLF (Aprataxin and Polynucleotide kinase-Like Factor), an endo/exonuclease with a forkhead-associated (FHA) domain and two unique zinc fingers (ZF), interacts with both Ku and X4L4. The APLF-X4L4 interaction is FHA- and phospho-dependent, and is mediated by CK2 phosphorylation of XRCC4 in vitro. APLF binds Ku independently of the FHA and ZF domains, and complexes with Ku at DNA ends. APLF undergoes ionizing radiation induced ATM-dependent hyperphosphorylation and ATM phosphorylates APLF in vitro. Downregulation of APLF is associated with defective NHEJ and impaired DSB repair kinetics. These results suggest that APLF is an ATM target that is involved in NHEJ and facilitates DSB repair, likely via interactions with Ku and X4L4.
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<th>Symbol</th>
<th>Name</th>
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<td>γ-H2AX</td>
<td>Gamma-H2AX (histone variant H2AX phosphorylated at Ser-139)</td>
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<td>Lambda protein phosphatase enzyme</td>
</tr>
<tr>
<td>μCi</td>
<td>Microcurie (unit of radiation)</td>
</tr>
<tr>
<td>°C</td>
<td>Degree Celsius (unit of temperature)</td>
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<td>1X PIs</td>
<td>Complete protease inhibitor cocktail</td>
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<td>Downstream end of a DNA chain</td>
</tr>
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<td>5’</td>
<td>Upstream end of a DNA chain</td>
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<td>Ataxia with oculomotor apraxia type 1</td>
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<td>APLF</td>
<td>Aprataxin and polynucleotide kinase-like factor</td>
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<td>Aprataxin</td>
</tr>
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<td>Ataxia telangiectasia mutated kinase</td>
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<td>Adenosine triphosphate</td>
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<td>Base pairs (of DNA)</td>
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<td>Casein kinase 1</td>
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<tr>
<td>CK2</td>
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<td>Downstream end of a polypeptide chain (carboxy-termini)</td>
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<td>DAPI</td>
<td>4',6-diamidino-2-phenylindole fluorescent DNA stain</td>
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<td>DMEM</td>
<td>Dulbecco's modified Eagle's medium</td>
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<td>Dimethyl sulfoxide</td>
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<td>DNA-PK</td>
<td>DNA dependent protein kinase holoenzyme</td>
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<td>DNA-PKcs</td>
<td>DNA dependent protein kinase catalytic subunit</td>
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<td>DSB</td>
<td>DNA double-strand break</td>
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<td>DTT</td>
<td>Dithiothreitol redox reagent</td>
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<td>EDTA</td>
<td>Ethylene diamine tetracetic acid</td>
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<td>Ethylene glycol tetracetic acid</td>
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<td>EtBr</td>
<td>Ethidium bromide</td>
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<tr>
<td>FBS</td>
<td>Fetal bovine serum</td>
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<tr>
<td>FHA</td>
<td>Forkhead-associated domain</td>
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<tr>
<td>GST</td>
<td>Glutathione S-transferase protein tag</td>
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<tr>
<td>Gy</td>
<td>Gray (unit of radiation)</td>
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<tr>
<td>HIT</td>
<td>Histidine triad domain</td>
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<tr>
<td>HR</td>
<td>Homologous recombination</td>
</tr>
<tr>
<td>IR</td>
<td>Ionizing radiation</td>
</tr>
<tr>
<td>Ku</td>
<td>Heterodimer of Ku70 and Ku80 subunits</td>
</tr>
<tr>
<td>ng, μg, mg, g</td>
<td>Nano-, micro-, milli-, -gram (g) unit of mass</td>
</tr>
<tr>
<td>nL, μL, mL, L</td>
<td>Nano-, micro-, milli-, -litre (L) unit of volume</td>
</tr>
<tr>
<td>nm, μm, mm, m</td>
<td>Nano-, micro-, milli-, -metre (m) unit of length</td>
</tr>
<tr>
<td>nM, μM, mM, M</td>
<td>Nano-, micro-, milli-, -molar (M) unit of concentration</td>
</tr>
<tr>
<td>NHEJ</td>
<td>Nonhomologous end-joining</td>
</tr>
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<td>NT</td>
<td>Non-targeting (siRNA)</td>
</tr>
<tr>
<td>N-terminal</td>
<td>Upstream end of a polypeptide chain (amino-termini)</td>
</tr>
<tr>
<td>Acronym</td>
<td>Full Form</td>
</tr>
<tr>
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</tr>
<tr>
<td>PARP-1</td>
<td>Pol(ADP-ribose) polymerase 1</td>
</tr>
<tr>
<td>PBS</td>
<td>Phosphate-buffered saline</td>
</tr>
<tr>
<td>PCR</td>
<td>Polymerase chain reaction to amplify DNA</td>
</tr>
<tr>
<td>PFGE</td>
<td>Pulsed field gel electrophoresis</td>
</tr>
<tr>
<td>PNK</td>
<td>Polynucleotide kinase</td>
</tr>
<tr>
<td>PSI-BLAST</td>
<td>Position specific iterative-basic local alignment search tool</td>
</tr>
<tr>
<td>S.E.M.</td>
<td>Standard error of the mean</td>
</tr>
<tr>
<td>SDS-PAGE</td>
<td>Sodium dodecyl sulfate polyacrylamide gel electrophoresis</td>
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<tr>
<td>siRNA</td>
<td>Small interfering RNA duplexes</td>
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<tr>
<td>SSB</td>
<td>DNA single-strand break</td>
</tr>
<tr>
<td>TBE</td>
<td>Tris-borate-EDTA buffer</td>
</tr>
<tr>
<td>V(D)J</td>
<td>Variable (diversity) joining recombination</td>
</tr>
<tr>
<td>VP16</td>
<td>Etoposide, a chemical inhibitor of the topoisomerase II enzyme</td>
</tr>
<tr>
<td>WCE(s)</td>
<td>Whole cell extract(s)</td>
</tr>
<tr>
<td>WRN</td>
<td>Werner syndrome protein</td>
</tr>
<tr>
<td>X4L4</td>
<td>XRCC4-DNA ligase IV complex</td>
</tr>
<tr>
<td>XLF</td>
<td>XRCC4-like factor</td>
</tr>
<tr>
<td>XRCC1</td>
<td>X-ray repair cross complementing protein 1</td>
</tr>
<tr>
<td>XRCC4</td>
<td>X-ray repair cross complementing protein 4</td>
</tr>
<tr>
<td>ZF</td>
<td>Zinc finger</td>
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CHAPTER 1: INTRODUCTION
1.1 - Cellular responses to DNA double-strand breaks

The development of cancer is a multi-step process during which cells acquire the necessary characteristics of a cancer cell through a series of genetic mutations. Genomic integrity is maintained through intense surveillance and repair, thereby reducing the likelihood of acquiring these oncogenic mutations [1]. For this reason, genomic instability is thought to be an enabling characteristic of tumorigenesis, as many genes involved in sensing, repairing and suppressing DNA damage are often compromised in tumour cells [1]. There are many types of DNA damage, but the DNA double-strand break (DSB) is widely considered the most mutagenic and toxic form of damage [2]. DSBs can arise in the cell from both exogenous and endogenous sources. For example, cellular metabolism produces dangerous reactive oxygen species that can induce DSBs, and DSBs can also arise during DNA replication from stalled replication forks occurring when an unrepaired DNA single-strand break (SSB) is encountered [3]. It has been estimated that, in human cells, approximately 1% of SSBs are converted to DSBs during each cell cycle, resulting in about 50 DSBs per cell per cycle [4]. It is believed that the number of endogenous DSBs arising from various sources can be as high as 100 per cell per day, and this number is increased if cells are exposed to exogenous genotoxins like ionizing radiation (IR) [2]. DSBs pose a major threat to cell survival and genome stability. If inaccurately repaired or if left unrepaired, DSBs can result in mutations, gross genetic aberrations or cell death [5]. Therefore, the recognition and repair of DSBs are of critical importance to living organisms. Cell cycle control is also a critical component of DSB signaling, as cell cycle arrest gives increased time for DNA repair, while cell death is initiated in the case of irreparable DNA damage in order to prevent the mutations from being transmitted to daughter cells [2]. However, efficiency and fidelity of DSB repair remains an effective prevention for genomic instability and oncogenesis.
In humans, nonhomologous end-joining (NHEJ) and homologous recombination (HR) are the
major pathways for DSB repair. HR depends on the use of a homologous DNA template, while
NHEJ re-joins the DSB in a sequence-independent manner [6]. The most important factor
determining the choice of repair pathway is likely the cell cycle stage. HR is confined to the S
and G2 phases of the cell, relying on the presence of the sister chromatid template, whereas
NHEJ is active throughout the entire cell cycle [6]. Figure 1.1 summarizes the integrated network
of pathways involved in cellular responses to DSBs.

Figure 1.1 – Key components of cellular responses to DSBs. Processes of cell cycle
control, DSB repair, and cell death all co-ordinate to prevent genomic instability.

1.2 The nonhomologous end-joining pathway

In humans, NHEJ is the predominant repair mechanism for DSBs induced by DNA
damaging agents, such as IR [7, 8], and also plays a key role in the processing and repair of the
developmentally regulated DSBs that occur during V(D)J recombination, the process by which T
cell receptor and antibody diversity is established [9]. One of the core components of the NHEJ
pathway includes the DNA-dependent serine/threonine protein kinase catalytic subunit (DNA-PKcs) and the DNA end-binding heterodimer Ku70/Ku80 (Ku), which collectively form the active DNA-dependent protein kinase (DNA-PK) holoenzyme [10]. Another core component includes the XRCC4-DNA ligase IV (X4L4) complex, which catalyzes DNA end-joining [8]. XRCC4 is a nuclear phosphoprotein that lacks intrinsic catalytic activity but has been shown to tightly bind, stabilize and modestly stimulate DNA ligase IV activity [11-15]. The complexity of the NHEJ pathway has been further illuminated by the recent discovery of an additional NHEJ core component, XRCC4-like factor (XLF, also known as Cernunnos), which co-associates with the X4L4 complex and promotes NHEJ in response to IR-induced DNA damage [16, 17]. The precise role of XLF remains undetermined, although it appears to be involved in the stimulation of X4L4 ligation activity [18-20]. Mammalian cells deficient in these core NHEJ components share phenotypes that include radiosensitivity, impaired V(D)J recombination, and predisposition to malignancy, underscoring their critical role in DSB repair [8]. Whereas genetic studies have firmly established the importance of NHEJ for IR resistance and DSB repair, the mechanistic details of NHEJ and its regulation remain less clear, and consequently, there exist many models for the NHEJ pathway.

One current model for the NHEJ-mediated repair of DSBs suggests a sequential order of assembly, which is a useful way to envision the process. In this simplified model for NHEJ, the first step involves recognition of the DSB by the DNA end-binding Ku heterodimer, which forms a ring structure with a central hole that accommodates duplex DNA and is lined with positively charged amino acids that interact with the negatively charged DNA backbone in a sequence-independent manner [21]. Ku binds to the free DNA ends, protecting them from nucleolytic degradation, and recruits DNA-PKcs, as these components come together to form the DNA-PK
holoenzyme, leading to the activation of its protein kinase activity. The binding of DNA-PKcs results in an inward translocation of Ku along the DNA, and synopsis of the DNA ends by DNA-PK, a process of DNA end-bridging during which the broken ends of DNA are tethered and maintained in close proximity to promote intermolecular ligation [21]. During synopsis, the broken ends of DNA remain accessible to DNA end processors, that act to modify the damaged termini to make them amenable to re-ligation [22]. DNA end processing is followed by the recruitment of the X4L4 complex that catalyzes DNA end joining [8]. Although XRCC4 possesses weak intrinsic DNA-binding activity, its ability to bind to DNA does not account for the stimulatory effect on the ligation of DSBs by XRCC4-DNA ligase IV \textit{in vitro} [14, 23]. These results suggest that the XRCC4-DNA ligase IV complex needs to be physically recruited to DSBs by other NHEJ components, and studies have demonstrated a role for Ku and DNA-PKcs in this regard [24, 25]. Following repair of the DSB, the repair factors dissociate [26]. Figure 1.2 summarizes the basic sequence of events in the NHEJ pathway. Of course, the NHEJ pathway is significantly more complicated than as presented in this figure. There are many variations on this basic sequence of events, which are also known as ‘NHEJ subpathways’ and they vary in different ways from this classical NHEJ pathway, depending on the type of DSB or damaged termini being repaired [27-34]. However, these subpathways remain poorly defined, and the canonical NHEJ pathway as outlined in Figure 1.2 remains a good framework for understanding the basic process involved in DSB end-joining.
Following formation of a DSB by IR, the Ku heterodimer binds to the exposed broken ends. As Ku translocates inwards along the DNA, DNA-PKcs binds to Ku on the newly exposed ends, coming together to form the DNA-PK holoenzyme. DNA-PK co-ordinates synapsis, a process whereby the two broken ends of DNA are tethered in close proximity to prevent diffusion. The broken ends are protected from degradation, but remain accessible to the various DNA end processors that associate to repair the damaged DNA ends. At this stage the X4L4 complex is recruited and DNA end-joining by DNA ligase IV occurs. After the DSB is repaired, the NHEJ components dissociate.
1.3 DNA end processing

In addition to the core components of the NHEJ pathway, there exists a host of accessory proteins that act to facilitate NHEJ, many of which assist in the DNA end processing stage prior to end-joining. DNA end processing is an essential step in NHEJ and is almost invariably required prior to DNA ligation, given that many forms of DNA damage, including IR, often produce DSBs with modifications that are not amenable to simple re-ligation. For example, it is not uncommon for DSBs to contain damaged backbone sugars or DNA bases, gaps, incompatible overhangs, or end-blocking groups such as phosphoglycolate, 3’ phosphate, or 5’ hydroxyl modifications [2]. Therefore, a variety of different enzymatic activities are required to process specific types of DSBs. Three DNA polymerases of the Pol X family have been implicated in NHEJ end processing including the template-independent terminal deoxytransferase (TdT), Pol μ, and Pol λ polymerases, which are all similarly recruited by NHEJ factors to fill in gaps and overhangs at DSBs [35-37]. Human tyrosyl-DNA phosphodiesterase (hTdp1) can remove protein moieties and 3’-phosphoglycolate groups from double-stranded DNA [38-40]. The Werner syndrome protein (WRN) possesses both helicase and exonuclease activities and interacts with Ku to process DNA ends [41-43]. Artemis is a 5’-3’ exonuclease that complexes with and is phosphorylated by DNA-PKcs to acquire endonucleolytic activity on 5’ and 3’ overhangs and hairpin structures, thereby acting to process DSBs in the NHEJ and V(D)J pathways [44]. ATM is a kinase involved in DNA damage signaling and cell cycle checkpoint responses [45], and Artemis also functions in an ATM-dependent NHEJ subpathway to repair a small subset (~10%) of IR-induced DSBs that are normally repaired with slow kinetics and are otherwise irreparable by other DNA repair enzymes, and consequently, disruption of this pathway results in substantial radiosensitivity [29, 30]. The bifunctional enzyme polynucleotide kinase (PNK) possesses both
5’ DNA kinase and 3’ DNA phosphatase activities, is able to repair the unligatable 5’-hydroxyl and 3’-phosphate DNA termini that are often produced by IR [46-48], and functions in both DNA SSB and DSB repair pathways [49-51]. Aprataxin (APTX) is a member of the histidine-triad (HIT) domain family of nucleotide hydrolases/transferases [52, 53], and is defective in autosomal recessive spinocerebellar ataxia AOA1 (ataxia with oculomotor apraxia type 1) [53], and is also implicated in both SSB and DSB repair pathways [54]. APTX can bind to DNA and can resolve abortive DNA ligation intermediates by catalyzing the nucleophilic release of adenylate groups from 5’ termini, rendering them amenable to re-ligation [54-56].

1.4 The role of PNK and APTX in DNA repair

Both PNK and APTX function as end processors in DNA repair. PNK possesses three domains, including an amino-terminal forkhead-associated (FHA) domain, which is a specific phosphothreonine-binding module [57], a 5’ DNA kinase domain, and a 3’ DNA phosphatase domain [58]. Stable down-regulation of PNK in human cells results in hypersensitivity to various DNA damaging agents including IR, camptothecin, and hydrogen peroxide, and increases the frequency of spontaneous mutation [59], demonstrating the importance of PNK for DNA repair. PNK is recruited to DSBs through interactions with XRCC4, a core component of the NHEJ pathway. Studies examining the XRCC4-PNK interaction have revealed that the association is dependent on the serine/threonine protein kinase, CK2, whereby CK2-mediated phosphorylation of XRCC4 at threonine residue 233 directs interactions with PNK via its amino-terminal FHA domain [51]. Interestingly, PNK is recruited to SSBs through interactions with XRCC1, which is a SSB repair scaffold protein analogous to XRCC4 in DSB repair. Studies examining the XRCC1-PNK interaction have revealed that the association is also mediated by CK2.
phosphorylation of XRCC1, whereby CK2-mediated phosphorylation of XRCC1 at a cluster of sites directs interactions with PNK via its amino-terminal FHA domain [60]. APTX possesses three domains, including an amino-terminal FHA domain, a central HIT domain, and a carboxy-terminal C2H2-type zinc finger motif [53]. APTX is required for full cellular resistance to oxidative DNA damage and methyl methanesulfonate treatment [61, 62], demonstrating the importance of APTX for DNA repair. Like PNK, the amino-terminal FHA domain of APTX binds to CK2-phosphorylated XRCC1 and XRCC4 [63], implicating APTX in SSB repair and DSB repair, respectively. Therefore, the amino-terminal FHA domains of the DNA end processing proteins PNK and APTX are functionally similar and interact with CK2-phosphorylated XRCC1 and XRCC4, which are analogous SSB and DSB scaffold proteins.

1.5 A unique subclass of FHA domains

Interestingly, the FHA domain of PNK comprises a divergent member of this domain family and is not readily identified as an FHA domain by common domain recognition algorithms [51, 52]. Only two additional members of the PNK FHA subgroup have been identified, including APTX and a third protein encoded by open reading frame 13 of chromosome 2 (C2orf13) [51], which we, and others, have termed APLF for Aprataxin and Polynucleotide kinase-Like Factor [64], but is also referred to as PALF (Polynucleotide kinase and Aprataxin-Like FHA protein) [65], or Xip1 (XRCC1-interacting protein 1) [66] in the literature. Sequence alignment information for the PNK, APTX, APLF, and other selected FHA domains was used previously to construct a phylogenetic tree that uses sequence relatedness to assess evolutionary distance, thereby demonstrating that the FHA domains of PNK, APTX, and APLF are closely related FHA domains that comprise a unique FHA domain subfamily [51].
Consistent with the observation that the PNK FHA domain is a divergent FHA domain, it was demonstrated that the PNK FHA domain also demonstrates a unique mode of phosphopeptide recognition among FHA domains, exhibiting preferences for sequences N-terminal to the phosphothreonine, while other characterized FHA domains typically show specificity for the C-terminal sequences [51, 57, 58]. The PNK FHA domain binding targets on XRCC1 and XRCC4 suggest a preference for acidic residues in the vicinity of the phosphothreonine [51, 60], which is likely due to positively charged lysine and arginine residues in the binding pocket of the PNK FHA domain that may be responsible for the recognition of acidic residues [58]. Interestingly, as demonstrated in Figure 1.4, sequence alignment of the PNK, APTX, and APLF FHA domain sequences alongside the consensus sequence for all FHA domains reveals the presence of positively charged lysine and arginine residues that are unique to PNK, APTX, and APLF.

Given that the FHA domains of PNK and APTX are functionally similar and interact with CK2-phosphorylated XRCC1 and XRCC4 to participate in DNA repair, and that the FHA domains of PNK, APTX, and APLF are uniquely similar and comprise a newly-identified FHA domain subclass, we hypothesized that APLF, like PNK and APTX, participates in DNA repair.
1.6 APLF

APLF appears to be an evolutionarily conserved protein and homologues have been identified in mammals, rodents, chicken, and sea urchin (Figure 1.5), although APLF is not present in unicellular eukaryotes, including yeast. Initially, the only domain identified in APLF using standard domain recognition algorithms was the amino-terminal FHA domain (Figure 1.5). However, the carboxy-terminal region of APLF contained two motifs that appeared to be well-conserved across species (Figures 1.5). For this reason, additional predictive structural analysis of these regions was performed.

Figure 1.4 – APLF is an evolutionarily conserved protein. Alignment of human APLF and selected homologues by MultAlin [67]. Invariant residues are identified by red text, while residues with greater than 50% consensus are identified by blue text. The FHA domain is highlighted by the yellow box and two additional well-conserved regions are highlighted by green boxes.

The carboxy-terminal APLF amino acid sequence was analyzed using the QuickPhyre Fold Recognition Algorithm [68], which searches for remotely homologous protein structures using the primary amino acid sequence. This algorithm predicted that the two conserved regions within the APLF carboxy-terminus had structural homology to the classical C2H2, C2HC and CCCH
type zinc fingers. The amino acid sequence of the two APLF zinc fingers appears most similar to the classical C2H2 type of zinc finger, which is a peptide domain that can bind DNA, RNA or protein substrates [69]. However, unlike previously characterized C2H2 type zinc fingers that have a CX2-CX12-HX2-H consensus, the APLF zinc fingers have a CX5-CX6-HX5-H consensus and contain a shortened inter-cysteine-histidine loop (CX6 vs. CX12-H), which is thought to be important in determining binding specificity [69]. Figure 1.6 identifies the well-conserved regions in APLF that are predicted to be unique zinc fingers with the CX5-CX6-HX5-H consensus.

**Figure 1.5 — APLF may contain a novel type of zinc finger.** Alignment of human APLF and selected homologues by MultAlin [67]. Invariant residues are identified by red text, while residues with greater than 50% consensus are identified by blue text. The region identified by the QuickPhyre Fold Recognition Algorithm [68] as potential zinc fingers are denoted as ZF1 and ZF2 and are highlighted by the green boxes. They both broadly conform to the CX5-CX6-HX5-H consensus. The zinc coordinating cysteine and histidine residues important for zinc finger function are denoted by black arrows.

Therefore, the CX5-CX6-HX5-H consensus may represent a novel type of zinc finger. These unique zinc fingers had not been previously described in the literature, and we sought to determine whether or not this consensus appeared in any other protein sequence. A PSI-BLAST search was performed with the carboxy-terminal region of APLF, and we identified other proteins containing similar motifs, which are outlined in Figure 1.7.
Figure 1.6 – APLF may contain a novel type of zinc finger also found in various DNA repair proteins. A PSI-BLAST search was performed with the carboxy-terminal region of APLF and homologous sequences from several different species were identified containing similar zinc finger motifs with the CX5CX5HX5H consensus sequence. Many of the proteins identified have identified or predicted functions in various aspects of DNA repair. An alignment of the APLF zinc finger and of the similar zinc fingers identified using MultAlin [67] is shown. Invariant residues are identified by red text.

Interestingly, the proteins containing the unique CX5CX5HX5H type zinc finger corresponded to proteins with putative roles in DNA damage signaling and repair, including homologues of Ku70, uracil DNA glycosylase, Rad53, DNA ligase III, and tyrosyl-DNA phosphodiesterase (Figure 1.7). Of note, only APLF and one additional protein of unknown function (CG6171-PA) in Drosophila melanogaster contained tandem zinc fingers, while the remainder of the proteins contained only a single motif.

1.7 Study aims and summary

Given that APLF is the third FHA-containing protein belonging to the divergent FHA domain subgroup also comprised of PNK and APTX [51], and that both PNK and APTX are implicated in SSB repair and DSB repair [49, 50, 54], we undertook a detailed characterization of the role of APLF in DNA repair. Because PNK and APTX have both been shown to interact
with XRCC4 [51, 63], which is a core component of the NHEJ pathway, we focused our research on the characterization of APLF in the context of DSB repair and the NHEJ pathway. The results presented in this thesis demonstrate that APLF physically interacts with the X4L4 complex in a phospho-dependent manner and that, like PNK and APTX, CK2-mediated threonine phosphorylation of XRCC4 at residue 233 directs interactions with the FHA domain of APLF. Surprisingly, we also show that, unlike PNK and APTX, APLF interacts with Ku, independently of the APLF FHA or zinc finger domains, and that APLF can complex with Ku at DNA ends. We show that depletion of APLF from human cells is associated with both defective NHEJ and impaired DSB repair kinetics following IR. We also show that APLF undergoes ATM-dependent hyperphosphorylation following IR at serine 116 and that ATM phosphorylates APLF in vitro. Therefore, based on these findings we suggest that APLF is an ATM target that facilitates NHEJ-mediated repair of DSBs in human cells, likely via interactions with Ku and X4L4.

In addition to our own published work [70], three other groups have independently reported that APLF is a novel regulator of DNA strand break repair [64-66]. Collectively the results of these studies demonstrate that APLF possesses endonuclease and 3’-5’ exonuclease activities, that APLF is involved in FHA-dependent and CK2-mediated interactions with the SSB and DSB scaffold proteins XRCC1 and XRCC4, that APLF undergoes ATM-dependent phosphorylation following IR, that APLF accumulates at sites of SSBs or DSBs, and that downregulation of APLF results in impaired DSB and SSB repair kinetics following IR and reduced cellular resistance to various DNA damaging agents [64-66]. Some of their reported findings confirm the results presented here, while many serve to supplement and complement the work of this thesis, and taken together the combined findings from our group and others collectively demonstrate the importance of APLF in DNA repair.
CHAPTER 2: MATERIALS AND METHODS
2.1 Plasmid constructions

The sequence coding for the human APLF open reading frame was PCR-amplified from the human cDNA IMAGE clone ID 6042653 (Open Biosystems) and TOPO-cloned into the pcDNA3.1/V5-His mammalian expression vector (Invitrogen) to generate pcDNA3.1/V5-His-APLF (V5-APLF). The QuikChange site-directed mutagenesis kit (Stratagene) was then used according to the manufacturer’s instructions to create an arginine to alanine substitution at residue 27 of APLF, generating pcDNA3.1/V5-His-APLF\textsuperscript{R27A} (V5-APLF\textsuperscript{R27A}). Mutagenesis was also used to create serine to alanine substitutions at residues 116, 144 and 246 of APLF, generating the pcDNA3.1/V5-His-APLF\textsuperscript{S116A} (V5-APLF\textsuperscript{S116A}), pcDNA3.1/V5-His-APLF\textsuperscript{S144A} (V5-APLF\textsuperscript{S144A}), and pcDNA3.1/V5-His-APLF\textsuperscript{S246A} (V5-APLF\textsuperscript{S246A}) constructs, respectively.

The amplified APLF cDNA was further modified using PCR by the introduction of EcoRI restriction sites at the 5’ and 3’ ends, and then cloned in-frame into the EcoRI site of the bacterial expression vector pGEX4T3 (Amersham) to generate pGEXT4T3-APLF (GST-APLF). The sequence coding for the FHA domain of APLF (amino acid residues 1-108) was amplified with 5’ and 3’ PCR primers containing EcoRI sites and cloned in-frame into the EcoRI site of pGEX4T3 to generate pGEX4T3-APLF\textsuperscript{FHA} (GST-APLF\textsuperscript{FHA}). Mutagenesis was then used to create an arginine to alanine substitution at residue 27 of APLF, generating the pGEXT4T3-APLF\textsuperscript{R27A} (GST-APLF\textsuperscript{R27A}) and pGEX4T3-APLF\textsuperscript{FHA-R27A} constructs, respectively. Mutagenesis was also used to create cysteine to glycine substitutions at both of the zinc-coordinating cysteine residues in the first or second zinc finger motifs of APLF to generate the constructs pGEX4T3-APLF\textsuperscript{ZF1m} (GST-APLF\textsuperscript{ZF1m}) and pGEX4T3-APLF\textsuperscript{ZF2m} (GST-APLF\textsuperscript{ZF2m}) respectively. The pGEX4T3-APLF construct was digested with BamHI and BsmI (removing amino acids 1-99), blunt-ended and religatated in-frame to generate pGEX4T3-
APLF\textsuperscript{100-511} (GST-APLF\textsuperscript{100-511}). The pGEX4T3-APLF\textsuperscript{100-511} construct was then further digested with XhoI and XbaI, XhoI and SphI, XhoI and Accl, or XhoI and AvrII, followed by blunt-ending and religation in frame to generate the pGEX4T3-APLF\textsuperscript{100-469} (GST-APLF\textsuperscript{100-469}), pGEX4T3-APLF\textsuperscript{100-359} (GST-APLF\textsuperscript{100-359}), pGEX4T3-APLF\textsuperscript{100-263} (GST-APLF\textsuperscript{100-263}), and pGEX4T3-APLF\textsuperscript{100-166} (GST-APLF\textsuperscript{100-166}) constructs, respectively. The sequence coding for the human APLF open reading frame was excised from pcDNA3.1-V5/His-APLF using BamHI and XhoI and ligated in-frame into the pET28a(+) vector (Novagen) to generate pET28a-APLF (His-APLF). The human APTX cDNA was PCR-amplified from a human lymphocyte cDNA library and TOPO-cloned into the pcDNA3.1/V5-His vector to generate pcDNA3.1/V5-His-APTX (V5-APTX). The sequence coding for the FHA domain of APTX (amino acid residues 1-113) was amplified with 5’ and 3’ PCR primers containing EcoRI sites and cloned in-frame into the EcoRI site of pGEX4T3 to generate pGEX4T3-APTX\textsuperscript{FHA} (GST-APTX\textsuperscript{FHA}). The pSUPER.retro.neo+GFP vector was a generous gift of Dr. Homayoun Vaziri (University of Toronto). The following constructs were previously generated and used in another study [51]. The human PNK cDNA was PCR-amplified from a human lymphocyte cDNA library and TOPO-cloned into the pcDNA3.1/V5-His vector to generate pcDNA3.1/V5-His-PNK (V5-PNK). The sequence coding for the FHA domain of PNK (amino acid residues 1-136) was amplified with 5’ and 3’ PCR primers containing EcoRI sites and cloned in-frame into the EcoRI site of pGEX4T3 to generate pGEX4T3-PNK\textsuperscript{FHA} (GST-PNK\textsuperscript{FHA}). The human pET15b-XRCC4 (His-XRCC4) plasmid was a generous gift from Dr. Daniel Durocher (Department of Molecular and Medical Genetics, University of Toronto). From this plasmid, the sequence coding for the human XRCC4 open reading frame was PCR amplified and TOPO-cloned into the pcDNA3.1/V5-His vector to generate pcDNA3.1/V5-His-XRCC4 (V5-XRCC4). Mutagenesis
was used to create the threonine to alanine substitution at residue 233 of XRCC4 to generate the pET15b-XRCC4\textsuperscript{T233A} (His-XRCC4\textsuperscript{T233A}) and pcDNA3.1/V5-His-XRCC4\textsuperscript{T233A} (V5-XRCC4\textsuperscript{T233A}) constructs, respectively. All of the plasmid constructs used in this study were verified by sequence analysis. All restriction enzymes were from New England Biolabs.

2.2 Small interfering RNA duplexes (siRNA)

All siRNA used in this study were purchased from Dharmacon as a chemically synthesized pool of five RNA duplexes targeting unique regions of the gene of interest. The siRNAs used in this study include non-targeting (NT) siRNA (Dharmacon siCONTROL Non-Targeting siRNA Pool), APLF-specific siRNA (Dharmacon siGENOME SMARTpool for Accession NM_173545), and XRCC4-specific siRNA (Dharmacon siGENOME SMARTpool for Accession NM_003401).

2.3 Cell culture

All cell lines were grown at 37°C in a tissue culture incubator with a humidified atmosphere containing 5% CO\textsubscript{2}. The following human cell lines were cultured in Dulbecco’s Modified Eagle Medium (DMEM, Hyclone) supplemented with 10% fetal bovine serum (FBS, Hyclone) and antibiotics (50 U Penicillin and 50 µg Streptomycin, Gibco): HEK293T, HeLa, MDA-MB-231, U2OS, MO59J, MO59K, ATM\textsuperscript{-/-}, and ATM\textsuperscript{+/+}. The ATM\textsuperscript{-/-} and ATM\textsuperscript{+/+} cells were a generous gift of Dr. Stephen Meyn (Department of Molecular and Medical Genetics, University of Toronto) and were derived from SV40-transformed human fibroblasts from A-T patients or from normal controls, respectively [71]. The lymphoblast A-T cell line (Coriell Cell Repositories) was grown in RMPI-1640 media (Hyclone) supplemented with 15% FBS and
antibiotics. The Xrcc4-deficient Chinese hamster ovary (CHO) XR-1 cell lines (Coriell Cell Repositories) stably transfected with V5-XRCC4, V5-XRCC4_{T233A}, or empty vector were previously generated and used in another study [51]. The XR-1 cells were transfected with V5-XRCC4, V5-XRCC4_{T233A}, or empty vector, and cells were placed under selective pressure with 800 µg/ml of G418 (Gibco) at 48 hours post-transfection. Resistant colonies were isolated and maintained in F-12 media (Hyclone) with 5% FBS and 200 µg/ml of G418.

2.4 Transfections

Transient transfections of plasmid DNA were performed with the Effectene transfection kit (Qiagen), transient transfections of siRNA with the DharmaFECT 1 transfection reagent (Dharmacon), and transient co-transfections of plasmid DNA and siRNA with DharmaFECT Duo transfection reagent (Dharmacon), each according to the manufacturer’s instructions.

2.5 Antibodies

Rabbit anti-XRCC4, anti-DNA ligase IV, anti-Ku70 and anti-DNA-PKcs polyclonal antibodies were purchased from Serotec. Rabbit anti-Ku80 polyclonal antibody was from Cedarlane, rabbit anti-GST polyclonal antibody from Santa Cruz Biotechnology, mouse anti-Actín monoclonal antibody from Abcam, mouse anti-γ-H2AX (Ser-139) monoclonal antibody from Upstate, rabbit anti-ATM polyclonal antibody from Calbiochem, rabbit anti-APTX polyclonal antibody from Bethyl Laboratories, and mouse anti-V5 monoclonal antibody from Invitrogen. Rabbit anti-PNK polyclonal antibody (#004) was a generous gift of Dr. Michael Weinfeld (Cross Cancer Institute, Edmonton, Alberta, Canada). Secondary antibodies for immunoblotting (goat anti-mouse and goat anti-rabbit) were purchased from Jackson
ImmunoResearch, while secondary antibodies for immunofluorescence microscopy (goat anti-mouse or goat anti-rabbit Alexa 488) were purchased from Invitrogen. A rabbit anti-APLF polyclonal antibody was generated from antisera collected from two rabbits that were injected and serially boosted with purified recombinant GST-APLF from *E. coli* BL21 (DE3)/pLysS (Novagen) according to standard immunological protocols. The antisera were pre-cleared on a GST column, to remove GST-specific antibodies, and then the eluate was affinity purified using a His-APLF column to recover APLF-specific antibodies, both according to standard protocols.

### 2.6 Protein expression and purification

Recombinant proteins were purified from *E. coli* BL21(DE3)/pLysS. Transformed bacteria were grown to an OD600 of 0.6 and protein expression was induced by the addition of isopropyl-β-D-1-thiogalactopyranoside (IPTG, Sigma) to a final concentration of 1 mM for 2 hours at 37°C. The bacteria were then pelleted by centrifugation, washed with phosphate-buffered saline (PBS), and re-centrifuged. For the purification of GST-fusion proteins, the cell pellet was resuspended in extraction buffer (50 mM Tris-HCl pH 7.5, 150 mM NaCl, 1 mM Dithiothreitol (DTT), 1% Triton X-100, 1X Complete protease inhibitor cocktail (1X PIs, Roche)), and the bacteria disrupted by sonication. The lysates were clarified by centrifugation at 16,000 x g at 4°C for 20 minutes. The supernatant was collected and incubated with glutathione sepharose 4B beads (Amersham) for 2 hours at 4°C with gentle mixing. The beads were then washed and the protein eluted with extraction buffer containing 20 mM glutathione. The glutathione was then removed and the purified protein exchanged into a suitable buffer through three sequential rounds of dialysis. For the purification of Histidine-tagged proteins, cell pellets were resuspended in extraction buffer (20 mM Tris-HCl pH 8.0, 50 mM NaCl, 20 mM Imidazole...
pH 7.0, 5 mM β-Mercaptoethanol, 10% Glycerol, 0.1% Nonidet P40, and 1X PIs), and the bacteria disrupted by sonication. The lysates were clarified by centrifugation at 16,000 x g at 4°C for 20 minutes. The supernatant was collected and incubated with nickel agarose beads (Ni-NTA, Invitrogen) for 2 hours at 4°C with gentle mixing. The beads were then washed and the protein eluted with extraction buffer containing 250 mM imidazole. The imidazole was then removed and the purified protein exchanged into a suitable buffer through three sequential rounds of dialysis. All dialysis procedures were performed using Slide-A-Lyzer dialysis cassettes (Pierce). Unless otherwise specified, all chemicals were purchased from Sigma Aldrich.

2.7 Preparation of cell extracts

Whole cell extracts (WCEs) were prepared from indicated cell lines using lysis buffer containing 50 mM Tris-HCl pH 7.5, 100 mM NaCl, 1% Triton X-100, 0.5 mM DTT, 0.5 mM EDTA, 0.2 mM Na$_3$VO$_4$, 40 mM β-Glycerophosphate, 50 mM NaF, and 1X PIs. Lysates were clarified by centrifugation at 16,000 g for 30 minutes at 4°C. WCEs treated with lambda (λ) protein phosphatase (New England Biolabs) were prepared in lysis buffer lacking phosphatase inhibitors (50 mM Tris-HCl pH 7.5, 100 mM NaCl, 1% Triton X-100, 0.5 mM DTT, and 1X PIs). When indicated, lysates were pre-treated with 50 µg/ml of ethidium bromide (EtBr, Invitrogen) for 30 minutes on ice. The λ protein phosphatase incubations were performed at 30°C for 30 minutes, and were stopped by the addition of EDTA to a final concentration of 25 mM. For WCEs prepared from cells treated with VP16, cells were incubated with DMEM containing 25 µM of VP16 (Sigma) or dimethyl sulfoxide (DMSO, Sigma) as a control, for 2 hours at 37°C prior to lysis. For WCEs prepared from cells treated with ionizing radiation (IR), cells were irradiated with a MDS Nordion Gammacell 40 Irradiator ($^{137}$Cs source) at a dose-rate
of 0.88 Gy per minute and then incubated at 37°C for 1 hour prior to lysis. For WCEs prepared from cells treated with kinase inhibitors, cells were incubated with DMEM containing 10 µM of ATM inhibitor (KU55933, KuDOS), 1 µM of DNA-PK inhibitor (NU7441, KuDOS), or DMSO as a control, for 1 hour at 37°C prior to IR treatment. The inhibitors were a generous gift from Dr. Robert Bristow (Department of Medical Biophysics, University of Toronto).

2.8 Immunoprecipitations and pull-downs

For immunoprecipitations, clarified WCEs were incubated with 1 µg of the indicated antibody on ice for 60 minutes with occasional gentle agitation. Then 30 µL of 50% protein A-immobilized agarose bead slurry (Pierce) was added, and incubated for an additional 60 minutes at 4°C. Immunoprecipitates were washed three times with lysis buffer, and resuspended in 30 µL laemmli sample buffer. For pull-down assays, clarified WCEs were incubated with 1 µg of the indicated GST-fusion protein immobilized on glutathione sepharose 4B beads. The complexes were washed three times with lysis buffer, and resuspended in 30 µL laemmli sample buffer.

2.9 Immunoblotting

Samples were all resolved by sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) and proteins were transferred to polyvinylidene difluoride (PVDF) Immobilon-P membrane (Millipore) using a Trans-Blot SD semi-dry electrophoretic transfer cell (Bio-Rad). Membranes were immunoblotted with the indicated primary antibody and relevant secondary antibody, according to the manufacturer’s instructions. Detection was performed using the SuperSignal West Pico enhanced chemiluminescence kit (Pierce) and membranes were exposed to autoradiographic film (Marsh Bio) and developed using a Konica SRX-101A film processor.
2.10 Peptides and peptide binding studies

The peptides corresponding to the serine 116 epitope of APLF and the serine 15 epitope of tumour protein p53 were synthesized by Jason Ho (Dr. Vuk Stambolic, Department of Medical Biophysics, University of Toronto) on the automated MultiPep Multiple Peptide Synthesizer (Intavis AG) using Fmoc-chemistry according to the manufacturer’s instructions. They are (in single-letter amino acid code): CLRNSQVLDEDKK (S116 WT), CLRNAQVLDEDKK (S116A), and EPPLSQEAFADLWKK (p53). The peptides corresponding to the threonine 233 epitope of XRCC4 were synthesized and purchased from the Yale HHMI/Keck Biotechnology Resource Laboratory. They are (in single-letter amino acid code): Biotin-GGYDESTDEESEKK (T233), Biotin-GGYDESpTDEESEKK (T233P, where pT denotes phosphothreonine). For peptide pull-down assays, 25 µL of streptavidin-coupled magnetic beads (Dynal) were washed twice in binding buffer (PBS containing 0.2% Nonidet P40, 0.5 mM DTT, 1 mM Na$_3$VO$_4$, 30 mM β-Glycerophosphate, and 1X PI’s) and coupled to 500 ng of the indicated biotinylated peptide for 30 minutes at room temperature with gentle mixing in 500 µL of binding buffer. The coupled peptides were then washed twice with binding buffer, and incubated with 250 ng of the indicated GST fusion protein in 500 µL of binding buffer for 1 hour at 4°C. The complexes were then washed three times with binding buffer and resuspended in laemmli sample buffer. The bound proteins were detected by anti-GST immunoblotting.

2.11 In Vitro protein kinase assays

CK2 kinase assays were performed by incubating 3 µg His-XRCC4 or His-XRCC4$^{T233A}$ purified recombinant protein either with or without 100 ng of purified recombinant CK2 produced in Sf21 insect cells (Upstate) in 50 µl of kinase reaction buffer (20 mM MOPS pH 7.2,
25 mM β-Glycerophosphate, 5mM EGTA, 1 mM Na₃VO₄, 1 mM DTT, 100µM ATP, and 13.5 mM MgCl₂) for 15 minutes at 30°C. Then 1 µg of GST-APLF FHA protein immobilized on glutathione sepharose 4B beads was added to the reaction and incubated for 2 hours at 4°C in 1 mL of binding buffer (50 mM Tris-HCl pH 7.5, 100 mM NaCl, 1% Triton X-100, 0.5 mM DTT, 0.5 mM EDTA, 0.2 mM Na₃VO₄, 40 mM β-Glycerophosphate, 50 mM NaF, and 1X PIs). Complexes were then washed three times with binding buffer and resuspended in laemmli sample buffer. The bound proteins were detected by anti-XRCC4 immunoblotting.

ATM kinase assays were performed by immunoprecipitating ATM from cells treated with 10Gy IR and lysed in 50 mM Tris-HCl pH 7.5, 150 mM NaCl, 1% Tween 20, 0.2% Nonidet P40, 50 mM β-Glycerophosphate, 1 mM Na₃VO₄, 1 mM DTT, 10% Glycerol, 1 mM NaF, and 1X PIs. Complexes were then washed three times with lysis buffer and three times with kinase wash buffer (10 mM HEPES pH 7.4, 50 mM NaCl, 50 mM β-Glycerophosphate, and 1 mM DTT). The ATM kinase was then activated by incubating the immunoprecipitates with 1 mL of phosphorylation buffer (20 mM TrisHCl pH 7.4, 20 mM β-Glycerophosphate, 1 mM Na₃VO₄, 20 mM MgCl₂, 10 mM MnCl₂, and 1 mM ATP) for 30 minutes at 30°C. Complexes were washed three times with kinase wash buffer and the kinase reactions initiated by resuspending the beads in kinase reaction buffer (10 mM HEPES pH 7.4, 50 mM NaCl, 50 mM β-Glycerophosphate, 1 mM DTT, 10 mM MgCl₂, 10 mM MnCl₂, and 5 µM ATP) containing 10 µCi [γ-³²P]ATP and 1 µg of purified recombinant GST, GST-APLF, or GST-p53 (Santa Cruz) protein substrate or 1 µg of S116 WT, S116A, or p53 peptide substrate (each described in Section 2.10). Reactions were incubated for 30 minutes at 30°C. The reactions containing protein substrates were stopped by the addition of laemmli sample buffer, samples were resolved by SDS-PAGE, and the gel was exposed to a storage phosphor screen (Molecular Dynamics) and analyzed on the Typhoon
PhosphorImager (GE Healthcare). The reactions containing peptide substrates were stopped by spotting the reaction mixture onto squares of P81 cellulose phosphate paper (Whatman). The squares were washed three times with 0.75% phosphoric acid and once with 100% acetone and were then transferred into 6 mL plastic scintillation vials (Perkin Elmer) containing 5 mL of Ultima Gold liquid scintillation cocktail (Perkin Elmer). The vials were then analyzed using the LS 6000SC scintillation counter (Beckman Instruments) to determine the average $\gamma^{32}$P radioactivity incorporation as measured by average counts per minute (cpm).

DNA-PK kinase assays were performed by incubating purified recombinant DNA-PKcs (a generous gift of Dr. Daniel Durocher, Department of Molecular and Medical Genetics, University of Toronto) with 0.5 µg His-APLF or His-XRCC4 protein in kinase reaction buffer (25 mM HEPES pH 7.5, 50 mM KCl, 12.5 mM MgCl$_2$, 20% Glycerol, 0.1% Nonidet P40, and 0.5 mM ATP) containing 500 ng sheared calf thymus DNA (Invitrogen) and 5 µCi [$\gamma^{32}$P]ATP. Reactions were incubated for 10 minutes at 30°C and stopped by the addition of laemmli sample buffer. Samples were resolved by SDS-PAGE and the gel was exposed to a storage phosphor screen and analyzed on the Typhoon PhosphorImager.

### 2.12 Electrophoretic mobility shift assays

Linear DNA substrate was produced by digesting the pcDNA3.1/V5-His-PNK plasmid with DraIII and PstI to produce a 500 bp fragment, which was recovered using the QIAquick Gel Extraction Kit (Qiagen). Then, 45 ng of the DNA fragment was incubated with 50, 100, or 200 ng of purified recombinant His-APLF alone or in combination with 280 ng of the Ku heterodimer purified from insect cells (Trevigen) in a reaction buffer containing 150 mM KCl, 25 mM Tris-HCl pH 7.5, 2 mM DTT, 0.1% Triton X-100, 5% Glycerol, and 100 µCi/mL bovine
serum albumin for 30 minutes at 37°C. DNA-protein complexes were resolved by non-denaturing PAGE, the DNA stained with SYBR Green I (Sigma), and the gel imaged using the Molecular Imager Gel Doc XR System (Bio-Rad).

2.13 Immunofluorescence microscopy

Cells were seeded into 35 mm dishes containing glass coverslips (Fisher) and incubated for 24 hours at 37°C in a tissue culture incubator. Coverslips were then immunostained, with all procedures performed at room temperature, all solutions prepared in PBS, and with extensive PBS washes performed between each step. Cells were fixed by incubation with 4% paraformaldehyde for 20 minutes, permeabilized by incubation with 0.2% Triton X-100 for 20 minutes, blocked by incubation with 2% normal goat serum (Sigma) for 1 hour, and immunostained by incubation with anti-V5 or anti-APLF antibodies for 2 hours at 37°C and with goat anti-mouse or goat anti-rabbit Alexa 488 antibodies for 30 minutes at 37°C. Coverslips were then mounted onto glass microscope slides (VWR) using Vectashield mounting medium (Vector Laboratories) containing DAPI nucleic acid dye to visualize the nucleus. Slides were then examined using a Zeiss Axiovert 200M microscope with a Plan-NEOFLUAR 40X objective. Image capture was achieved using a Roper Scientific Coolsnap ProCF colour CCD camera.

2.14 Continuous field gel electrophoresis (CFGE)

U2OS cells were plated into 6 well dishes at 750,000 cells/well and incubated for 16 hours at 37°C. Cells were then transfected with either NT, APLF or XRCC4 siRNA and incubated for 48 hours at 37°C. Cells were replated into 60 mm dishes and incubated for 24 hours at 37°C. Two plugs were then prepared as described below for each siRNA treatment,
corresponding to one sample lysed immediately prior to IR treatment (undamaged plug) and one sample lysed immediately following IR treatment (0h plug). The undamaged plug was lysed as described below, while the 0h plug was transferred to a 1.5 mL eppendorf tube containing chilled DMEM and subjected to an 80Gy IR dose alongside the remaining dishes, which were sealed with parafilm and chilled on ice during irradiation. Immediately following IR, the 0h plug was lysed. The dishes were incubated at 37°C in a tissue culture incubator for the indicated time periods prior to preparation of plugs and lysis. For all samples, the plugs were prepared by washing the cell monolayer with PBS and incubating with 500µL of 0.25% Trypsin-EDTA solution (Gibco) for 5 minutes at 37°C. Cells were then resuspended in 1 mL of DMEM and counted using a hemacytometer. Cells were then pelleted by centrifugation and resuspended in DMEM containing 0.8% UltraPure low melting point agarose (Invitrogen) to achieve a final concentration of 625 cells/µL. Re-usable plug molds (Bio-Rad) were chilled on ice and 80µL of the cell suspension (50,000 cells) was added to the mold and solidified on ice for 5 minutes. Plugs were lysed by incubating on ice for 1 hour in 500 µL of chilled lysis buffer (0.5% SDS, 0.1 mM Tris-HCl pH 7.5, 0.5 mg/mL Proteinase K, 500 mM EDTA). Lysis was then continued overnight in a 37°C water bath. The plugs were then washed three times for 15 minutes each with 3 mL of PBS containing 10 mM EDTA. Plugs were further digested with 1 mL of a 0.2 mg/mL Rnase A solution for 2 hours at room temperature. RNase A digestion was halted by the addition of EDTA to a final concentration 50 mM. Plugs were then inserted into the wells of a 0.8% normal melting point agarose gel (Sigma) prepared in 0.5X Tris-borate-EDTA (TBE) buffer. Gel was overlayed with a thin layer of 0.8% agarose-0.5XTBE solution to achieve a smooth gel surface after solidification. Gel was electrophoresed at 0.6 V/cm in 0.5X TBE buffer.
for 40 hours and then stained with 1 µg/mL EtBr solution for 8 hours. Gel was destained overnight in distilled water and imaged using the Gel Doc XR System.

### 2.15 γ-H2AX foci assay

For the analysis of DSB repair kinetics, U2OS cells were plated into 6 well dishes at 750,000 cells/well and incubated for 16 hours at 37°C. Cells were then transfected with either NT, APLF, or XRCC4 siRNA and incubated for 48 hours at 37°C. Cells were then collected and replated into either 96 well dishes at 20,000 cells per well for immunostaining or into 35 mm dishes for western blot analysis of siRNA knockdown efficiency, and incubated for 24 hours at 37°C. WCEs were prepared from the 35mm dishes and analyzed by immunoblotting with the indicated antibodies. Cells in the 96 well dishes were mock- or irradiated with 5 Gy of IR and incubated at 37°C for the indicated time points prior to fixation, permeabilization and immunostaining with mouse anti-γ-H2AX and goat anti-mouse Alexa 488 antibodies. Cell nuclei were stained with DAPI dye (Sigma). Cell imaging was then performed on the Opera™ automated spinning-disk confocal microplate imaging microscope (Evotec Technologies) using an air 20X/0.4NA lens objective. Ultraviolet (to visualize the DAPI stain) and 488 nm (to visualize the Alexa 488 dye) laser excitation lines were used and emission was detected simultaneously by two cameras using dichroic filters of 450 nm +/- 50 nm and 525 nm +/- 50 nm for the respective dyes. A single focal plane was imaged, derived from a 1 µm confocal slice. Data was collected from three independent experiments, and within each experiment 16 wells were analyzed at each time point for each siRNA treatment, corresponding to a total of 48 wells. Ten fields per well were imaged, corresponding to 300-600 cells per well. In total, 480 fields, or 15,000-30,000 cells, were imaged at each time point for each siRNA treatment. The acquired
images were stored and analyzed using Acapella™ image analysis software (Evotec technologies). First, the nuclei in every image was defined and quantified using the DAPI stain. Then, within each defined nucleus in every image, the number of γ-H2AX foci was defined and quantified. To obtain the average number of γ-H2AX foci per cell, the total number of γ-H2AX foci was then divided by the total number of nuclei. Error bars represent the standard error of the mean (S.E.M.). A two sample t-test at a 5% significance level was used to compare the difference in the mean values obtained for the NT- and APLF-siRNA data sets at each time point. To obtain the average percentage of nuclei both with and without γ-H2AX foci at the 24 hour time point, the number of γ-H2AX foci-positive nuclei or the number of γ-H2AX foci-negative nuclei was divided by the total number of nuclei. Error bars represent the standard error of the mean (S.E.M.). A two sample t-test at a 5% significance level was used to compare the difference in the mean values obtained for the NT- and APLF-siRNA data sets.

2.16 Random plasmid DNA integration assay

This assay was performed essentially as described previously [16, 72, 73] with some modifications. Linear DNA containing a neomycin resistance cassette was produced by enzymatic digestion of the pSUPER.retro.neo+GFP plasmid with BamHI and HindIII, followed by removal of the 18 bp fragment using the QIAquick PCR Purification Kit (Qiagen) and ethanol precipitation of the 8354 bp linearized plasmid DNA. U2OS cells were plated into 6-well tissue culture dishes at 750,000 cells per well and incubated for 16 hours at 37°C. Cells were transfected with NT, APLF or XRCC4 siRNA and incubated for 48 hours at 37°C. Subsequently, cells were transfected with linearized plasmid DNA along with NT, APLF or XRCC4 siRNA and incubated for 24 hours at 37°C. Cells were replated in triplicate at a low density of 10^6 cells
per 100 mm dish in DMEM containing 10% FBS and 800 µg/mL G418, and incubated for 10 days at 37°C in a tissue culture incubator. Colonies were stained with Coomassie Blue dye (Sigma), imaged using the Gel Doc XR System, and quantified using the ImageQuant TL Colony Counter image analysis software (Amersham). Data was collected from three independent experiments, and within each experiment three 100 mm plates were analyzed for each siRNA treatment, corresponding to a total of 9 plates for each siRNA treatment. The results were expressed as percentages of the control, with the number of colonies formed by cells treated with the NT siRNA fixed as 100% integration efficiency. The number of colonies obtained in each of the 9 samples treated with either APLF or XRCC4 siRNA were expressed as a percentage of the mean number of colonies obtained from cells treated with the NT siRNA. The mean of these percentages was then calculated. Error bars represent the standard error of the mean (S.E.M.).
CHAPTER 3: RESULTS
3.1 APLF is predominantly localized to the nucleus of human cells

To begin characterizing APLF we first sought to examine its subcellular localization, as we hypothesized that a protein involved in DNA damage signaling and repair would likely be localized to the nucleus. We examined human cells expressing V5-tagged wild-type APLF (V5-APLF) and determined that ectopically expressed APLF is largely localized to the nucleus, as judged by indirect immunofluorescence microscopy (Figure 3.1). We also generated a rabbit polyclonal antibody that specifically recognizes the human APLF protein. Using this antibody, we determined that endogenous APLF is also predominantly localized to the nucleus of human cells, as judged by indirect immunofluorescence microscopy (Figure 3.1).

![Figure 3.1 – APLF is predominantly localized to the nucleus of human cells. HeLa cells ectopically expressing V5-APLF were fixed and immunostained with DAPI nuclear stain and anti-V5 (APLF) antibody and the images were merged (left panel). MDA-MB-231 cells were fixed and immunostained with anti-APLF antibody or pre-immune sera (PI) and DAPI nuclear stain (right panel). This work was completed with the technical assistance of Nimerta Rajwans and Dr. Richard McCulloch.](image)

3.2 APLF interacts with core components of the NHEJ pathway

Given that the APLF FHA domain belongs to the same unique subclass of FHA domains as the PNK and APTX FHA domains, and that both of these DNA repair proteins are known to interact with the XRCC4 component of the NHEJ pathway, we initially examined the ability of APLF to co-immunoprecipitate with core NHEJ components in human cells. To do so,
HEK293T cells were transfected with either empty vector, V5-APLF, or V5-tagged APLF containing the FHA arginine-to-alanine substitution (R27A) that is predicted to abolish the phosphothreonine-binding ability of FHA domains [57] (V5-APLF\textsuperscript{R27A}). The whole cell extracts were immunoprecipitated with anti-V5 antibodies to recover APLF along with any associated proteins. The immunoprecipitated protein complexes were then immunoblotted with anti-XRCC4, anti-DNA ligase IV, anti-Ku70, anti-Ku80 and anti-DNA-PKcs antibodies to determine if any of these proteins associate with ectopically expressed APLF (Figure 3.2).

![Figure 3.2 – Ectopically expressed APLF complexes with XRCC4-DNA ligase IV and Ku in human cells.](image)

We detected interactions between V5-APLF and XRCC4 and DNA ligase IV. Because these interactions were abolished by the R27A substitution in the APLF FHA domain, the interaction of APLF with XRCC4 and DNA ligase IV appears to be FHA-dependent (Figure 3.2).
Therefore, these results indicate that APLF interacts with the X4L4 complex in human cells, and that this association is dependent on a functional APLF FHA domain. We also detected an interaction between APLF and both subunits of the Ku heterodimer, which is the DNA binding component of the DNA-PK holoenzyme. Interestingly, unlike the X4L4 interaction, APLF binding to Ku appeared to be FHA-independent, as the interaction was unaffected by the R27A substitution (Figure 3.2). No interaction was observed between APLF and the catalytic subunit of the DNA-PK holoenzyme (DNA-PKcs) (Figure 3.2).

We then wanted to confirm the interactions observed between APLF and X4L4 and Ku in an endogenous setting. To do so, HEK293T whole cell extracts were immunoprecipitated with anti-APLF antibodies to recover APLF along with any associated proteins. The resulting protein complexes were then immunoblotted with anti-XRCC4, anti-DNA ligase IV, anti-Ku70, and anti-Ku80 antibodies to determine if any of these proteins associate with APLF (Figure 3.3).

**Figure 3.3 – Endogenous APLF complexes with XRCC4-DNA ligase IV and Ku in human cells.** Whole cell extracts (WCE) from HEK293T cells were immunoprecipitated with either anti-APLF antibodies or pre-immune sera (PI) and the resulting protein complexes were immunoblotted with Ku70, Ku80, XRCC4, DNA ligase IV, or APLF antibodies as indicated.
We identified endogenous associations between APLF and XRCC4, DNA ligase IV, and the Ku heterodimer (Figures 3.3). Therefore, these results suggest that the interaction of APLF with the X4L4 and Ku complexes can occur in human cells in vivo. Therefore, the human APLF protein engages in interactions with several core components of the NHEJ pathway, supporting the idea that APLF may be involved in DSB repair.

3.3 APLF interacts with XRCC4 in an FHA- and phospho-dependent manner

Because the interaction of APLF with X4L4 appears to be FHA-dependent, we wanted to determine whether or not the FHA domain of APLF is sufficient to direct interactions with the X4L4 complex. To do so, we performed pull-down assays utilizing purified recombinant GST, as a negative control, and GST-fusion proteins of the isolated APLF FHA domain either without (GST-APLF<sup>FHA</sup>) or with (GST-APLF<sup>FHA-R27A</sup>) the R27A substitution that abolishes the phosphothreonine-binding ability of the FHA domain. The GST proteins were immobilized on glutathione sepharose beads, which were then incubated with HEK293T cell extracts to recover any interacting proteins. The resulting protein complexes were then immunoblotted with anti-XRCC4 or anti-DNA ligase IV antibodies to determine if either of these proteins were recovered. The results in Figure 3.4 demonstrate that the FHA domain of APLF is sufficient for and is required for the interaction of APLF with XRCC4 and DNA ligase IV, as GST-APLF<sup>FHA</sup> and not GST alone recovered XRCC4 and DNA ligase IV, while the mutant FHA domain (GST-APLF<sup>FHA-R27A</sup>) was incapable of sustaining these interactions. Furthermore, the interactions between GST-APLF<sup>FHA</sup> and X4L4 were retained in the presence of the DNA intercalating agent ethidium bromide, suggesting that these interactions are unlikely bridged by DNA (Figure 3.4). Given that the APLF-X4L4 interaction is FHA dependent, and that FHA domains recognize and
bind to phospho-threonine epitopes, we also sought to determine whether or not the interaction was phospho-dependent. To do so, we examined whether or not the APLF-X4L4 interaction could be disrupted by pre-treatment of HEK293T cell extracts with lambda (λ) protein phosphatase, an agent that will dephosphorylate all proteins present in the lysate, prior to performing pull-down assays. We utilized purified recombinant GST and GST-APLF<sup>FHA</sup> immobilized on glutathione sepharose beads, which were then incubated with λ protein phosphatase-treated HEK293T cell extracts to recover any interacting proteins. The resulting protein complexes were then immunoblotted with anti-XRCC4 or anti-DNA ligase IV antibodies to determine if the X4L4 complex was recovered. As demonstrated in Figure 3.4, λ protein phosphatase treatment completely abolished the binding of GST-APLF<sup>FHA</sup> to XRCC4 and DNA ligase IV, suggesting that the APLF-X4L4 interaction is indeed phosphorylation-dependent.

Figure 3.4 – The FHA domain of APLF associates with XRCC4-DNA ligase IV in a phospho-dependent manner. Pull-down assays were performed utilizing purified recombinant GST, GST-APLF<sup>FHA</sup> or GST-APLF<sup>FHA-R27A</sup> proteins immobilized on glutathione sepharose beads. Beads were incubated with HEK293T whole cell extracts (WCE) pre-treated with (+λ) or without (-λ) lambda protein phosphatase in the presence of 50 µg/ml of ethidium bromide and the resulting protein complexes were immunoblotted with anti-XRCC4 or anti-DNA ligase IV antibodies as indicated.
To further assess the phospho-dependency of the APLF-X4L4 interaction, we performed pull-down assays utilizing purified recombinant GST, as a negative control, and GST fusion proteins of either the isolated PNK FHA domain (GST-PNK$^{\text{FHA}}$), as a positive control, or APLF FHA domain (GST-APLF$^{\text{FHA}}$). The GST proteins were immobilized on glutathione sepharose beads and incubated with HEK293T cell extracts to recover any interacting proteins. The resulting protein complexes were then treated with $\lambda$ protein phosphatase and immunoblotted with anti-XRCC4 antibodies. We observed that phosphatase treatment of the pre-bound GST-APLF$^{\text{FHA}}$-XRCC4 complex did not result in the disruption of binding (Figure 3.5), suggesting that one or more phosphate groups required for the interaction are protected from phosphatase treatment by the binding of the APLF FHA domain. This phenomenon is seen with most FHA-phosphoprotein interactions [74], and was also observed in this experiment with binding of the PNK FHA domain to XRCC4 (Figure 3.5). These results further suggest that the interaction between the APLF FHA domain and XRCC4 is phospho-dependent.

Figure 3.5 – The FHA domain of APLF associates with XRCC4-DNA ligase IV in a phospho-dependent manner. Pull-down assays were performed utilizing purified recombinant GST, GST-APLF$^{\text{FHA}}$ or GST-PNK$^{\text{FHA}}$ proteins immobilized on glutathione sepharose beads. Beads were incubated with HEK293T whole cell extracts (WCE) and the resulting protein complexes were then treated with ($+\lambda$) or without ($-\lambda$) lambda protein phosphatase and immunoblotted with anti-XRCC4 antibodies.
3.4 The APLF FHA domain binds CK2-phosphorylated threonine residue 233 of XRCC4

CK2 phosphorylation of threonine residue 233 (Thr-233) of XRCC4 has been shown to mediate binding of the PNK FHA domain to XRCC4 in mammalian cells [51]. Therefore, because the FHA domain of APTX belongs to the same unique subclass of FHA domains as does PNK, we next sought to determine whether or not the binding of the APTX FHA domain to XRCC4 was also dependent on the XRCC4 Thr-233 binding site. Whole cell extracts were prepared from the XRCC4-deficient CHO cell line XR-1 stably expressing either empty vector, wild-type V5-tagged XRCC4 (V5-XRCC4), or V5-tagged XRCC4 containing an alanine substitution of the PNK binding site at Thr-233 (V5-XRCC4\textsuperscript{T233A}), and pull-down assays were performed utilizing both immobilized GST-PNK\textsuperscript{FHA}, as a positive control, and GST-APTX\textsuperscript{FHA}. The resulting protein complexes were immunoblotted with anti-V5 (XRCC4) antibodies. As demonstrated in Figure 3.6, the Thr-233 residue of XRCC4 is required for the association of the FHA domains of APTX and PNK with XRCC4, as both of the interactions are abolished by the XRCC4 threonine-to-alanine substitution at residue 233.

![Figure 3.6](image-url)

**Figure 3.6** – Threonine 233 of XRCC4 is required for binding of the APTX FHA domain to XRCC4. Pull-down assays were performed utilizing purified recombinant GST-APTX\textsuperscript{FHA} or GST-PNK\textsuperscript{FHA} proteins immobilized on glutathione sepharose beads. Beads were incubated with whole cell extracts (WCE) from XRCC4-deficient cell lines stably expressing empty vector, V5-XRCC4, or V5-XRCC4\textsuperscript{T233A} and the resulting protein complexes were immunoblotted with anti-V5 (XRCC4) antibodies. This work was completed with the assistance of Dr. Anne Koch.
Therefore, it appears that the uniquely similar FHA domains of PNK and APTX both interact with XRCC4 at the Thr-233 binding site. Given that we have demonstrated that APLF also interacts with XRCC4, and that the FHA domain of APLF is the third remaining member of the unique subclass of FHA domains comprised of PNK and APTX, we then sought to determine if the APLF<sup>FHA</sup>-XRCC4 interaction was also dependent on Thr-233 of XRCC4. Whole cell extracts were prepared from the XRCC4-deficient XR-1 cell line stably expressing either empty vector, V5-XRCC4, or V5-XRCC4<sub>T233A</sub>, and pull-down assays were performed utilizing immobilized GST-APLF<sup>FHA</sup>. The resulting protein complexes were immunoblotted with anti-V5 (XRCC4) or anti-DNA ligase IV antibodies. As demonstrated in Figure 3.7, Thr-233 of XRCC4 is required for the association of the APLF FHA domain with XRCC4 and DNA ligase IV, as the interaction was abolished by the threonine-to-alanine substitution at residue 233 of XRCC4.

**Figure 3.7** – Threonine 233 of XRCC4 is required for binding of the APLF FHA domain to XRCC4. Pull-down assays were performed utilizing purified recombinant GST-APLF<sup>FHA</sup> protein immobilized on glutathione sepharose beads. Beads were incubated with whole cell extracts (WCE) from XRCC4-deficient cell lines stably expressing empty vector, V5-XRCC4, or V5-XRCC4<sub>T233A</sub>, and the resulting protein complexes were immunoblotted with anti-V5 (XRCC4) or DNA ligase IV antibodies as indicated.
These results suggest that the uniquely similar FHA domains of PNK, APTX, and APLF all interact with the X4L4 complex by binding to the Thr-233 residue of XRCC4.

The results of Figure 3.7 have demonstrated a requirement for the threonine 233 residue of XRCC4 to enable binding of the APLF FHA domain, and we have also shown that the APLF-XRCC4 interaction is phospho-dependent (Figures 3.4, 3.5). Given that FHA domains bind to phosphorylated threonine residues, we hypothesized that phosphorylation of Thr-233 may be required for the APLF<sup>FHA</sup>-XRCC4 interaction. Previously, it has been demonstrated that CK2 phosphorylation of XRCC4 Thr-233 directs binding of the PNK FHA domain [51]. Therefore, we sought to determine whether or not CK2 phosphorylation of XRCC4 at Thr-233 could also direct binding of the APLF FHA domain. To do so, purified recombinant His-XRCC4 or His-XRCC4<sup>T233A</sup> proteins were subjected to mock- or CK2-phosphorylation <i>in vitro</i> and then incubated with immobilized GST-APLF<sup>FHA</sup> protein in pull-down assays. The resulting protein complexes were immunoblotted with anti-XRCC4 antibodies. As shown in Figure 3.8, both the unphosphorylated and CK2-phosphorylated XRCC4<sup>T233A</sup> proteins were unable to bind GST-APLF<sup>FHA</sup>, confirming that the XRCC4 Thr-233 site is essential for APLF-XRCC4 interaction. Furthermore, only CK2 phosphorylated wild-type XRCC4 interacted with the APLF FHA domain, while no interaction was detected with unphosphorylated wild-type XRCC4, suggesting that CK2 phosphorylation at Thr-233 is required for binding of the APLF FHA domain to XRCC4, a situation similar to the binding selectivity noted for the PNK FHA domain.
Figure 3.8 – CK2 phosphorylation of Thr-233 of XRCC4 mediates binding to the APLF FHA domain in vitro. Purified recombinant His-XRCC4 or His-XRCC4^T233A proteins were phosphorylated with (+) or without (-) CK2 in vitro, and then subjected to pull-down assays utilizing purified recombinant GST-APLF^FHA protein immobilized on glutathione sepharose beads. The resulting protein complexes were immunoblotted with anti-XRCC4 antibodies. This work was completed with the technical assistance of Jackie Gilbert.

We then sought to demonstrate that the APLF FHA domain could directly bind the Thr-233 epitope of XRCC4 in a phosphospecific manner. To do so, we utilized peptides spanning the XRCC4 Thr-233 epitope. We coupled both phosphorylated (T233P) and unphosphorylated (T233) biotin-tagged XRCC4-dervied peptides to streptavidin magnetic beads, which were then incubated with purified recombinant GST-APLF^FHA or GST-APLF^FHA-R27A proteins. Peptide-protein interactions were then assessed by anti-GST immunoblotting. As predicted, only the peptide phosphorylated at Thr-233 (T233P) demonstrated efficient binding with GST-APLF^FHA (Figure 3.9), while we did not detect interactions between the unphosphorylated peptide (T233) and GST-APLF^FHA. Additionally, as expected, the APLF mutant FHA domain (GST-APLF^FHA-R27A) was unable to bind either epitope (Figure 3.9). Taken together, these results suggest that the APLF FHA domain is required for and sufficient to direct phospho-dependent interactions with X4L4 via the CK2-phosphorylated threonine residue 233 of XRCC4.
Figure 3.9 – The FHA domain of APLF can specifically bind the phosphorylated Thr-233 epitope of XRCC4. Pull-down assays were performed utilizing biotinylated unphosphorylated (T233) or phosphorylated (T233P) peptides spanning the threonine 233 epitope of XRCC4 immobilized on streptavidin beads. Beads were incubated with purified recombinant GST-APLF<sub>FHA</sub> or GST-APLF<sup>FHA-R27A</sup> proteins and the resulting protein complexes were immunoblotted with anti-GST antibodies. This work was completed with the technical assistance of Nimerta Rajwans.

Given the apparent overlapping binding specificities of PNK, APTX, and APLF for XRCC4, which all appear to interact at the Thr-233 binding site of XRCC4, we hypothesized that their interactions with XRCC4 may be mutually exclusive. To test this theory, we performed co-immunoprecipitation experiments to determine whether or not these proteins complex together. HEK293T cells were transfected with either empty vector or V5-APLF and whole cell extracts were immunoprecipitated with anti-V5 antibodies to recover APLF and any interacting proteins. The resulting protein complexes were then immunoblotted with anti-APTX, anti-PNK, anti-XRCC4, and anti-V5 (APLF) antibodies. Although we observed XRCC4 in the APLF immunocomplexes, APTX and PNK were notably absent, demonstrating that PNK and APTX do not complex with APLF in human cells (Figure 3.10).
Figure 3.10 – APLF co-immunoprecipitates with XRCC4 and not with PNK or APTX. Whole cell extracts (WCE) from HEK293T cells ectopically transfected with empty vector or V5-APLF were immunoprecipitated with anti-V5 (APLF) antibody and immunoblotted with APTX, PNK, XRCC4, or V5 (APLF) antibodies as indicated.

These preliminary results suggest that the uniquely similar FHA domains of PNK, APTX, and APLF all bind to Thr-233 of XRCC4 to complex with X4L4. APLF does not appear to interact with PNK and APTX \textit{in vivo}, suggesting that these three proteins may undergo mutually exclusive interactions with XRCC4.

3.5 The interaction of APLF with Ku is both FHA- and zinc finger-independent

In addition to the FHA-dependent interactions between APLF and the X4L4 complex, FHA-independent interactions were also observed between APLF and the Ku heterodimer. The APLF-Ku interaction was unaffected by the R27A substitution in the FHA domain of APLF, as both V5-APLF and V5-APLF$^{R27A}$ were found to interact equally well with Ku (Figure 3.2). To further confirm that APLF was interacting with Ku independently of the FHA domain, we performed pull-down assays utilizing purified recombinant GST, as a negative control, and GST-
fusion proteins of either full-length APLF (GST-APLF) or the isolated APLF FHA domain (GST-APLF\textsuperscript{FHA}). The GST proteins were immobilized on glutathione sepharose beads, which were incubated with HEK293T cell extracts to recover any interacting proteins. The resulting protein complexes were then immunoblotted with anti-Ku70 and anti-Ku80 antibodies to determine if the Ku heterodimer was recovered. We observed that while the full-length GST-APLF protein was able to bind to Ku, the isolated APLF FHA domain (GST-APLF\textsuperscript{FHA}) was unable to associate with Ku (Figure 3.11). Furthermore, the APLF-Ku interaction was unaffected by the presence of ethidium bromide, suggesting that the interaction is not bridged by DNA. The results of this experiment demonstrate that the APLF-Ku interaction is FHA-independent, and that Ku interacts with the carboxy-terminal portion of APLF.

**Figure 3.11 – APLF interacts with the Ku heterodimer in an FHA-independent manner.** Pull-down assays were performed utilizing purified recombinant GST, GST-APLF\textsuperscript{FHA}, or GST-APLF proteins immobilized on glutathione sepharose beads. Beads were incubated with HEK293T whole cell extracts (WCE) in the presence of 50 µg/ml of ethidium bromide and the resulting protein complexes were immunoblotted with anti-Ku70 and anti-Ku80 antibodies.

The only domains identified in APLF include the N-terminal FHA domain and the C-terminal tandem zinc finger motifs. Therefore, we next tested whether the two zinc finger motifs of APLF were important for the interaction with Ku. The functionality of these motifs was
disrupted via cysteine to glycine substitutions of the zinc-co-coordinating cysteine residues in either the first or second APLF zinc finger (denoted as ZF1m and ZF2m, respectively), a method which has been shown to disrupt the binding function of other zinc fingers [75]. We then performed pull-down assays utilizing purified recombinant GST, GST-APLF, GST-APLFZF1m and GST-APLFZF2m proteins immobilized on glutathione sepharose beads, which were incubated with HEK293T cell extracts to recover any interacting proteins. The resulting protein complexes were then immunoblotted with anti-Ku70 and anti-Ku80 antibodies to determine if the Ku heterodimer was recovered. We observed that Ku interacted similarly with the wild-type and zinc finger mutant APLF proteins (Figure 3.12), demonstrating that these substitutions do not affect the association of Ku with APLF and suggesting that, like the FHA domain, the zinc fingers are not essential for the APLF-Ku interaction. Once again, the APLF-Ku interaction was unaffected by ethidium bromide treatment, indicating that the interaction is not bridged by DNA.

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<tr>
<th>Pull-down</th>
<th>WCE Input</th>
<th>GST</th>
<th>GST-APLF</th>
<th>GST-APLFZF1m</th>
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**Figure 3.12** – The APLF zinc fingers are not required for the association with Ku. Pull-down assays were performed utilizing purified recombinant GST, GST-APLF, or GST-APLF proteins harboring mutations in either the first (GST-APLFZF1m) or second (GST-APLFZF2m) zinc finger motifs, each immobilized on glutathione sepharose beads. Beads were incubated with HEK293T whole cell extracts (WCE) in the presence (+) or absence (-) of 50 µg/ml of ethidium bromide (EtBr) and the resulting protein complexes were immunoblotted with anti-Ku70 and anti-Ku80 antibodies. This work was completed with the technical assistance of Dr. Richard McCulloch.
Figures 3.11 and 3.12 have demonstrated that Ku interacts with a region on APLF that is C-terminal to the FHA domain, and that the interaction is not dependent on the APLF ZFs. To map the site of Ku interaction on APLF, we generated a GST-APLF protein corresponding to the C-terminal region of APLF downstream of the FHA domain (GST-APLF<sup>100-511</sup>), which was used to generate a series of progressively truncated GST-APLF proteins (GST-APLF<sup>100-469</sup>, GST-APLF<sup>100-359</sup>, GST-APLF<sup>100-263</sup>, GST-APLF<sup>100-166</sup>) which were all immobilized on glutathione sepharose beads and used in pull-down assays alongside purified recombinant GST, and GST-APLF. The resulting protein complexes were then immunoblotted with anti-Ku70 and anti-Ku80 antibodies to determine if the Ku heterodimer was recovered. All of the GST-APLF fusion proteins, with the exception of GST-APLF<sup>100-166</sup> were able to bind the Ku heterodimer, suggesting that GST-APLF<sup>100-263</sup> is the minimum fragment capable of interacting with Ku (Figure 3.13). Therefore, amino acids 100-263 of APLF, which lie between the FHA and ZF motifs, are sufficient for interaction with Ku.

**Figure 3.13 – The Ku-binding region is located between the APLF FHA and zinc finger domains.** Pull-down assays were performed utilizing purified recombinant GST, GST-APLF, GST-APLF containing the full C-terminus (GST-APLF<sup>100-511</sup>), or a series of GST-APLF proteins truncated at the C-terminus (GST-APLF<sup>100-469</sup>, GST-APLF<sup>100-359</sup>, GST-APLF<sup>100-263</sup>, or GST-APLF<sup>100-166</sup>), each immobilized on glutathione sepharose beads. Beads were incubated with HEK293T whole cell extracts (WCE) and the resulting protein complexes were immunoblotted with anti-Ku70 and anti-Ku80 antibodies. This work was completed with the assistance of Dr. Richard McCulloch.
3.6 APLF complexes with Ku on DNA ends

The Ku heterodimer is the DNA end-binding subunit of the DNA-PK holoenzyme, and binds the DNA ends at DSBs in the first step of the NHEJ pathway. The identified interaction of APLF with Ku led us to investigate whether or not APLF could also interact with Ku when Ku is bound to DNA. To do so, we performed electrophoretic mobility shift assays that assess the ability of purified proteins to bind linearized plasmid DNA. These assays were performed by incubating a 500 bp linear double-strand DNA fragment with purified recombinant His-APLF alone or in combination with Ku heterodimer purified from insect cells. The DNA-protein complexes were resolved by non-denaturing PAGE and the DNA was detected with the SYBR Green I nucleic acid stain. As demonstrated in Figure 3.14, incubating increasing amounts of APLF with the DNA did not result in upward shifting, suggesting that APLF does not have intrinsic double-strand DNA-binding ability for this substrate. As expected, incubating the DNA end-binding Ku heterodimer with the DNA substrate resulted in the appearance of shifted DNA, demonstrating the ability of Ku to bind the DNA fragment. Interestingly, when increasing amounts of APLF were incubated with the DNA in the presence of the Ku heterodimer, a notable shift was observed that increased with increasing amounts of APLF (Figure 3.14). These results suggest that APLF is able to interact with DNA-bound Ku.
Figure 3.14 – APLF interacts with DNA-bound Ku. An electrophoretic mobility shift assay was performed to examine the ability of increasing amounts of purified His-APLF (50, 100, 200 ng) to interact with Ku heterodimer (280 ng) when bound to a 500bp linear DNA fragment (45 ng). DNA-protein complexes were resolved by non-denaturing PAGE, and DNA was detected using the SYBR Green I nucleic acid stain.
3.7 APLF is basally phosphorylated and is hyperphosphorylated following DNA damage

The interaction of APLF with the NHEJ components Ku and X4L4 suggests a role for this protein in DNA damage signaling and repair. Given that many proteins involved in cellular DNA damage responses are regulated via protein phosphorylation events, and undergo DNA damage-induced phosphorylation [76], we next sought to determine whether or not APLF was also phosphorylated. Because APLF interacts with Ku, a component of the DNA-PK holoenzyme, we hypothesized that APLF may be phosphorylated by the catalytic subunit of DNA-PK, an important kinase involved in DNA damage signaling [77-80]. ATM is another important kinase involved in the DNA damage signaling [78, 80-83], and we noted that APLF contains several putative DNA-PKcs and ATM sites of phosphorylation, broadly conforming to the [Ser/Thr]-Gln consensus [84]. Therefore, we sought to determine whether or not APLF is phosphorylated by either of these DNA damage signaling kinases. We initially examined the gel mobility of APLF from HEK293T cells treated with etoposide (VP16) or ionizing radiation (IR), two conditions that induce DSBs and promote the activation of ATM and DNA-PK. As shown in Figure 3.15, a proportion of APLF migrates more slowly by SDS-PAGE when cells are subjected either to etoposide or IR.

Figure 3.15 – APLF exhibits reduced electrophoretic mobility following DNA damage. HEK293T cells were untreated (-) or treated with either 25 µM VP16 or 10 Gy IR and the whole cell extracts were immunoblotted with anti-APLF antibodies.
This gel mobility shift is likely due to protein phosphorylation, as the APLF mobility shift was reversed when the lysates were treated with λ protein phosphatase (Figure 3.16).

![Figure 3.16 – APLF is phosphorylated following DNA damage.](image)

Interestingly, we further noted that APLF appears to be phosphorylated under basal conditions, as λ protein phosphatase treatment of whole cell extracts derived from untreated cells also increases the gel mobility of APLF (Figure 3.17).

![Figure 3.17 – APLF is phosphorylated in cells under basal conditions.](image)

Taken together, our results (Figures 3.15-3.17) are consistent with the notion that APLF exists as a phosphoprotein under basal conditions and undergoes hyperphosphorylation in response to DNA damage.
3.8 APLF is phosphorylated \textit{in vitro} by DNA-PKcs and ATM

Given that we observed DNA damage-induced phosphorylation of APLF, and that APLF contains several putative DNA-PKcs and ATM sites of phosphorylation [84], we first sought to determine whether or not either of these kinases can phosphorylate APLF \textit{in vitro}. To test whether ATM could phosphorylate APLF \textit{in vitro}, we performed ATM IP kinase assays using purified recombinant GST as a negative control, the known ATM substrate GST-p53 [85] as a positive control, and GST-APLF as a potential substrate. The protein substrates were phosphorylated with radiolabeled ATP \textit{in vitro} using ATM kinase immunoprecipitated from HEK293T cells, and the phosphoproteins were separated by SDS-PAGE and detected by autoradiography. In Figure 3.18, we show that, as expected, ATM phosphorylates p53. Interestingly, we observed that ATM can indeed phosphorylate APLF under these conditions. To test whether DNA-PKcs could phosphorylate APLF \textit{in vitro}, we performed DNA-PKcs kinase assays using no substrate as a negative control, the known substrate His-XRCC4 as a positive control [86], and His-APLF as a potential substrate. The protein substrates were phosphorylated with radiolabeled ATP \textit{in vitro} using purified recombinant DNA-PKcs, and the phosphoproteins were separated by SDS-PAGE and detected by autoradiography. In Figure 3.18, we show that, as expected, DNA-PKcs phosphorylates XRCC4. Interestingly, we observed that DNA-PKcs can indeed phosphorylate APLF under these conditions. Figure 3.18 demonstrates that both the DNA damage signaling kinases ATM and DNA-PKcs phosphorylate APLF \textit{in vitro}. 

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Figure 3.18 – ATM and DNA-PKcs phosphorylate APLF in vitro. Purified recombinant GST, GST-p53, or GST-APLF protein substrates were phosphorylated with radiolabeled ATP in vitro using ATM kinase immunoprecipitated from HEK293T cells. The phosphoproteins were then detected by autoradiography (left panel). No substrate (---) or purified recombinant His-XRCC4 or His-APLF protein substrates were phosphorylated with radiolabeled ATP in vitro using purified recombinant DNA-PKcs. The phosphoproteins were then detected by autoradiography (right panel).
3.9 APLF undergoes ATM-dependent hyperphosphorylation following DNA damage

We next sought to determine whether or not ATM and DNA-PKcs participate in the process of DNA damage-induced APLF hyperphosphorylation in vivo. Our initial investigation consisted of pre-treatment of either untransfected or V5-APLF transfected HEK293T cells with DMSO, as a negative control, or with either an ATM or DNA-PKcs inhibitor, prior irradiation with a 10 Gy dose. Whole cell extracts were then immunoblotted with anti-APLF or anti-V5 (APLF) antibodies to assess APLF phosphorylation, as indicated by changes in gel mobility. Both V5-APLF and endogenous APLF undergo IR-induced phosphorylation in DMSO and DNA-PKcs inhibitor pre-treated cells (Figure 3.19), indicating that inhibition of the DNA-PKcs kinase does not prevent APLF phosphorylation following DNA damage. However, we observed that IR-induced phosphorylation of APLF was prevented when the cells were pre-treated with the ATM inhibitor (Figure 3.19), indicating that the ATM kinase may play an essential role in the DNA damage-induced phosphorylation of APLF.

![Figure 3.19 – IR-induced hyperphosphorylation of APLF is prevented by ATM kinase inhibitors.](image)

HEK293T cells transfected with V5-APLF (top panel) or untransfected (bottom panel) were incubated with DMSO as a negative control, or with either ATM or DNA-PKcs inhibitors prior to mock (-) or irradiation (+) with a 10 Gy dose. WCEs were then immunoblotted with either anti-V5 (APLF) antibodies (top panel) or anti-APLF antibodies (bottom panel) as indicated.
To more clearly define the relative contributions of ATM and DNA-PKcs to APLF IR-induced hyperphosphorylation, we examined cell-lines deficient for each of these kinases. First, DNA-PKcs-deficient (MO59J) or -proficient (MO59K) cells were irradiated with a 5 Gy dose, and the whole cell extracts were immunoblotted with anti-APLF antibodies to assess APLF phosphorylation, as indicated by changes in gel mobility. As demonstrated in Figure 3.20, endogenous APLF undergoes IR-induced hyperphosphorylation to a similar extent in both cell types, indicating that DNA-PKcs is not essential for the observed mobility shift of APLF after DNA damage, and likely is not the primary kinase responsible for its phosphorylation. Intriguingly, we observed that the mobility of APLF was slightly reduced in the MO59J cells. Since MO59J cells also have low ATM protein levels and reduced ATM kinase activity [87, 88], we surmised that the reduction in ATM activity was responsible for the slight impairment of APLF phosphorylation in these cells. We then examined endogenous APLF phosphorylation in ATM deficient A-T lymphoblast cells following a 5 Gy dose of IR. As shown in Figure 3.20, a substantial defect in IR-induced APLF hyperphosphorylation was detected in the A-T lysates.

Figure 3.20 – IR-induced hyperphosphorylation of APLF is not DNA-PKcs-dependent. MO59K (DNA-PKcs+/+), MO59J (DNA-PKcs−/−), or A-T lymphoblast (ATM deficient) cells were mock (-) or irradiated (+) with a 5 Gy dose and the whole cell extracts immunoblotted with anti-APLF antibodies.
We also examined endogenous APLF phosphorylation in SV40-transformed human fibroblasts derived from either A-T patients (ATM\textsuperscript{−/−}) or from normal controls (ATM\textsuperscript{+/+}) following irradiation with a 10 Gy dose. As shown in Figure 3.21, a substantial defect in IR-induced APLF hyperphosphorylation was detected in the ATM\textsuperscript{−/−} cell extracts, as compared to APLF phosphorylation in ATM\textsuperscript{+/+} cells.

Although both ATM and DNA-PKcs can phosphorylate APLF \textit{in vitro} (Figure 3.18), taken together, these results (Figures 3.19–3.21) suggest that IR-induced hyperphosphorylation of APLF is largely ATM-dependent, although we cannot completely exclude the possibility of some DNA-PKcs-dependent APLF phosphorylation under certain conditions.

3.10 Serine 116 of APLF undergoes ATM-dependent IR-induced hyperphosphorylation

In order to delineate which site(s) on APLF are primarily phosphorylated in response to IR, we created V5-APLF constructs coding for serine to alanine substitutions at the sites most predictive of ATM phosphorylation (Ser-116, Ser-144 and Ser-246) [84] to yield the APLF mutant proteins V5-APLF\textsuperscript{S116A}, V5-APLF\textsuperscript{S144A}, or V5-APLF\textsuperscript{S246A} respectively. These proteins
were ectopically expressed alongside V5-APLF in HEK293T cells, which were mock-irradiated or irradiated with a 10 Gy dose, and the whole cell extracts were examined by anti-V5 (APLF) immunoblotting to assess APLF phosphorylation, as indicated by changes in gel mobility. As demonstrated in Figure 3.22, only the APLF mutant protein harboring the alanine substitution at serine residue 116 exhibited impaired IR-induced phosphorylation, as demonstrated by the lack of a hyperphosphorylated APLF species (indicated by the arrow in Figure 3.22), while the mutant proteins with alanine substitutions at serine residues 144 or 246 behaved similarly to wild-type APLF. This result suggests that the Ser-116 residue of APLF is an important site of DNA damage-induced phosphorylation.

Figure 3.22 – IR induces phosphorylation of APLF at serine 116. HEK293T cells ectopically transfected with V5-APLF or V5-APLF mutant proteins harboring alanine substitutions at serine residues 116, 144 or 246 (V5-APLF\(^{S116A}\), V5-APLF\(^{S144A}\), and V5-APLF\(^{S246A}\)) were mock (-) or irradiated (+) with a 10 Gy dose and the whole cell extracts were immunoblotted with anti-V5 (APLF) antibodies. IR-induced phosphorylation of APLF (indicated by the arrow) is not observed with the V5-APLF\(^{S116A}\) mutant protein.
3.11 ATM can phosphorylate the serine 116 epitope of APLF \textit{in vitro}.

Given that we have observed ATM-dependent IR-induced phosphorylation of APLF, and that we have shown that the Ser-116 residue of APLF is an important target for IR-induced phosphorylation, we next sought to determine whether or not ATM could phosphorylate the Ser-116 residue of APLF. We performed \textit{in vitro} ATM IP kinase assays using no peptide as a negative control, and a peptide corresponding to the serine 15 epitope of p53 as a positive control [85]. A peptide corresponding to the wild-type serine 116 epitope of APLF (S116 WT) and a peptide corresponding to the serine 116 epitope of APLF containing an alanine substitution at serine 116 (S116A) were also examined as potential substrates. The peptide substrates were phosphorylated with radiolabeled ATP \textit{in vitro} using ATM kinase immunoprecipitated from HEK293T cells and the phosphoproteins were transferred to P81 paper and analyzed using a scintillation counter to determine the average $\gamma^{32}$P radioactivity incorporation as measured by average number of counts per minute (cpm). In Figure 3.23, we demonstrate that, as expected, ATM phosphorylates the p53 serine 15 epitope. Interestingly, we observed that ATM can indeed phosphorylate the serine 116 epitope of APLF under these conditions, and this phosphorylation is impaired with the serine to alanine substitution of residue 116.
Figure 3.23 – The serine 116 epitope of APLF is phosphorylated by ATM in vitro. Peptide substrates were phosphorylated with radiolabeled ATP in vitro using ATM kinase immunoprecipitated from HEK293T cells. No peptide was used as a negative control and a peptide corresponding to the serine 15 epitope of p53 was used as a positive control. A peptide corresponding to the wild-type serine 116 epitope of APLF (S116 WT) and a peptide corresponding to the serine 116 epitope of APLF containing an alanine substitution at serine 116 (S116A) were examined as potential substrates. The phosphoproteins were transferred to P81 paper and analyzed using a scintillation counter to determine the average $\gamma^{32}$P radioactivity incorporation as measured by average number of counts per minute (cpm).

Therefore, ATM can phosphorylate the Ser-116 epitope of APLF in vitro. Although other APLF residues may undergo IR-induced phosphorylation, taken together, the data presented here suggests that APLF is phosphorylated in response to DNA damage by ATM at Ser-116, a residue that is highly conserved across mammalian APLF homologues.

3.12 Downregulation of APLF is associated with impaired DSB repair kinetics following IR

Given that we have observed interactions between APLF and the X4L4 complex and Ku heterodimer, which are both core components of the NHEJ DSB repair pathway, and that we have observed phosphorylation of APLF in response to DSB-inducing agents such as IR, we
hypothesized that APLF was likely involved in some aspect of DSB repair or signaling. We therefore sought to investigate the role of APLF in cellular responses to IR by examining the effect of APLF downregulation on the kinetics of DNA repair using the continuous field gel electrophoresis (CFGE) technique. Pulsed-field gel electrophoresis (PFGE) is a widely used technique that resolves large DNA fragments to directly quantify DSBs, however, the migration pattern is difficult to accurately analyze, and if CFGE is optimized, this technique can detect DSBs with similar efficiency [89, 90]. In addition, the running time for CFGE is shorter than for PFGE, and the equipment simpler and less expensive. We examined the XRCC4-deficient XR-1 cell line, as a positive control, alongside U2OS cells transfected with either non-targeting (NT) siRNA, as a negative control, or with APLF specific siRNAs to assess the effect of downregulated APLF protein expression on DNA repair. The cells were mock or irradiated with an 80 Gy dose and harvested in a time course following IR. Harvested cells were embedded into agarose plugs which were subjected to various treatments designed to lyse the cells, remove the proteins, and leave only the genomic DNA, which is then subjected to CFGE. Given that agarose gels separate DNA based on size, and that following induction of DNA damage the number of smaller DNA fragments increases, the fraction of DNA fragments released (FDR) from the bulk undamaged DNA (which remains in the agarose plug) can serve as a direct measure of the number of DSBs induced, while the decline in FDR over time can also serve as a measure of DNA repair [89, 90]. Therefore, genomic DNA subjected to CFGE will form a two band pattern that can be analyzed to determine the efficiency of DNA repair. The bottom band is fragmented DNA that has migrated from the agarose plug, while the upper band is undamaged DNA remaining in the plug. In Figure 3.24, we demonstrate that, as expected, the XRCC4-deficient XR-1 cell line exhibits defective DSB repair, with a significant amount of fragmented DNA
remaining in the bottom band even 24 hours following IR treatment. However, we were unable to observe any difference in the kinetics of FDR between U2OS cells treated with NT and APLF siRNAs. In both cases the majority of DNA was repaired and present in the upper band by 4 hours following IR.

![Figure 3.24](image)

**Figure 3.24 – The effect of APLF downregulation on DNA repair kinetics cannot be assessed using CFGE.** U2OS cells transfected with non-targeting (NT) siRNA (top panel), U2OS cells transfected with APLF specific siRNA (middle panel), or XRCC4-deficient XR-1 cells (bottom panel) were mock (Undamaged) or irradiated with an 80 Gy dose and harvested at 0, 1, 4 or 24 hours post-IR. Cells were lysed and the DNA subjected to CFGE to segregate undamaged DNA (slow migrating top band) and damaged DNA fragments (fast migrating bottom band). The efficiency of APLF knockdown was examined by immunoblotting whole cell extracts from NT or APLF-transfected U2OS cells with anti-Actin and anti-APLF antibodies as indicated (inset).

Although we were unable to observe an effect of APLF downregulation on DSB repair following IR using CFGE, this technique suffers from both a low sensitivity and detection threshold and the requirement for very high, non-physiological, doses of radiation. To more clearly investigate a role for APLF in cellular responses to IR, we employed an assay that would avoid these pitfalls. The earliest known marker for DSBs is the phosphorylation of histone H2AX at Serine 139 to form γ-H2AX [91]. Immunofluorescent staining with anti-γ-H2AX...
antibodies will produce quantifiable foci visible by fluorescent microscopy, that provide an extremely accurate marker of DSBs, with a nearly a 1:1 ratio between foci and DSBs [91]. Therefore, the number of γ-H2AX foci serves as a measure of the number of DSBs induced, while the decline in the number of γ-H2AX foci over time can also serve as an indication of DNA repair [91]. We examined the kinetics of γ-H2AX nuclear foci formation after IR in U2OS cells transfected with either non-targeting (NT) siRNA as a negative control, XRCC4-specific siRNA as a positive control, or with APLF specific siRNAs to assess the effect of downregulated APLF protein expression on foci formation. Forty-eight hours post-transfection, a fraction of the cells were analyzed for knockdown efficiency by immunoblotting the whole cell extracts to assess APLF and XRCC4 protein expression (Figure 3.25), while the remainder were irradiated with a 5 Gy dose and allowed to recover at 37°C prior to fixation over a 24-hour time-course at the indicated time points. The cells were then immunostained with DAPI nucleic acid stain to visualize the nucleus, and with anti-γ-H2AX antibody to visualize the foci that serve as a correlate for DSB formation. The cells were then imaged and analyzed using the Opera automated spinning-disk confocal microplate imaging microscope to quantify the IR-induced γ-H2AX foci. This is a large-scale, automated, high-throughput microscopy technique that provides an extremely accurate quantitative analysis of foci formation. We performed three independent experiments, examining at each time point as many as 30,000 cells for each siRNA treatment. In Figure 3.25 we demonstrate that, as expected, downregulation of XRCC4 results in defective DSB repair kinetics, with higher numbers of γ-H2AX foci observed at each time point, as compared to control cells transfected with the NT siRNA. Interestingly, we also found that downregulation of APLF results in impaired DSB repair kinetics, as evidenced by a statistically significant increase in the average number of γ-H2AX foci per cell at 30 minutes, 4 hours, and
24 hours post-IR in APLF-depleted cells, as compared to control cells transfected with NT siRNA (Figure 3.25). There was also an increase in the average number of γ-H2AX foci per cell at one hour post-IR in APLF-depleted cells relative to the NT control, although the difference was not statistically significant (Figure 3.25).

**Figure 3.25 –Downregulation of APLF results in impaired DSB repair kinetics.** U2OS cells transfected with XRCC4 specific, APLF specific, or non-targeting (NT) siRNAs were mock (No IR) or irradiated with a 5 Gy dose and γ-H2AX foci formation at the indicated time points was quantified using automated immunofluorescence microscopy. Each data point represents the mean from three independent experiments, and the error bars represent the standard error of the mean. The asterisks at the 30 minute, 4-hour, and 24-hour time points indicate statistically significant differences (p=0.0008282, p=0.02832, and p<2.2x10^{-16}, respectively) between the mean number of γ-H2AX foci per cell obtained from the NT and APLF siRNA data sets. The knockdown efficiency was examined by immunoblotting whole cell extracts from XRCC4, APLF, or NT transfected cells with anti-XRCC4, anti-APLF, or anti-Actin antibodies as indicated (inset).

Depletion of APLF and XRCC4 leads to higher numbers of γ-H2AX foci, or higher numbers of DSBs, at the indicated time points following IR, which suggests that there is a defect in DSB repair kinetics, with repair occurring more slowly in these cells. As time passes, the proportion of cells with foci decreases while the proportion of cells without foci increases, as cells complete repair of all IR-induced DSBs. If DSB repair kinetics are impaired, an increase in
the proportion of cells with foci is expected, indicating an increase in the number of cells with unrepai red DSBs. The most significant difference in the average number of foci per cell between APLF- and NT- treated cells was observed at 24 hours post-IR, therefore the percentage of nuclei with or without foci at this time point was determined. Figure 3.26 demonstrates that, as expected, downregulation of XRCC4 results in a larger percentage of nuclei with \(\gamma\)-H2AX foci (47.2%) at 24 hours post-IR, as compared to control cells transfected with the NT siRNA (33.0%). Interestingly, downregulation of APLF also leads to a statistically significant increase in the percentage of nuclei containing \(\gamma\)-H2AX foci at 24 hours post-IR in APLF-depleted cells (43.1%), as compared to control cells transfected with the NT siRNA (33.0%) (Figure 3.26).

**Figure 3.26 – Downregulation of APLF results in impaired DSB repair kinetics.** U2OS cells transfected with non-targeting (NT), XRCC4 specific, or APLF specific siRNAs were irradiated with a 5 Gy dose and \(\gamma\)-H2AX foci formation at 24 hours was quantified using automated immunofluorescence microscopy. The percentage of nuclei both with and without \(\gamma\)-H2AX foci was determined. Each data point represents the mean from three independent experiments, and the error bars represent the standard error of the mean. The asterisk indicates a statistically significant difference (p<0.0001) between the percentage of nuclei with foci for the NT and APLF siRNA data sets.
Taken together, these results demonstrate that downregulation of APLF in human cells results in impaired DSB repair kinetics following IR-induced DNA damage, therefore suggesting that APLF facilitates DSB repair in human cells.

3.13 Downregulation of APLF is associated with defective NHEJ

Given that we have observed that APLF is required for efficient DSB repair kinetics, and that we have observed interactions between APLF and core components of the NHEJ pathway, we next sought to determine whether or not we could detect a specific NHEJ defect in APLF-depleted cells. Foreign DNA can be inserted into the genome by two primary mechanisms: either homology-directed integration that seeks sequence similarity between the foreign and genomic sequences, or nonhomologous insertion of foreign DNA into random sites of the genome, an event occurring 1000–10,000 times more frequently than targeted integration [92]. Upon entering the nucleus, the majority of foreign DNA will be degraded, but a small percentage will integrate into the host genome, usually via nonhomologous insertion at sites of endogenously occurring DSBs. There are two possible scenarios for nonhomologous integration. The free DNA may simply arrive in the vicinity of a DSB, be recognized by NHEJ factors, and become modified and integrated into the genome. Alternatively, the free DNA ends may be recognized by DNA damage recognition proteins, such as the Ku heterodimer, and be extrachromasomally modified and purposely directed to DSBs through protein-protein interactions with NHEJ components for genomic integration [92].

An assay has been developed using these concepts, and has been implemented in other studies to demonstrate that the NHEJ machinery is essential for nonhomologous integration of foreign DNA into the genome of cells in culture, and that the efficiency of the random
integration of plasmid DNA can serve as a measure of the efficiency of the NHEJ process [16, 72, 73]. To perform this assay, linearized plasmid DNA containing an antibiotic resistance marker was transfected into U2OS cells treated with non-targeting (NT) siRNA as a negative control, XRCC4-specific siRNA as a positive control, or with APLF-specific siRNA to assess the effect of downregulated APLF protein expression on integration efficiency. The plasmid DNA is then integrated into the genome of host cells with varying efficiency to give rise to cellular antibiotic resistance that enables cell growth in selective medium, with each integration event resulting in formation of a single resistant colony. Colony counts indicate the efficiency of integration and NHEJ efficiency, and the number of colonies formed when cells are treated with the negative control siRNA is set as 100% integration.

As expected, downregulation of XRCC4, a core component of the NHEJ pathway, markedly reduced plasmid integration efficiency (Figure 3.27). Interestingly, we also found that depletion of APLF in U2OS cells decreased the frequency of plasmid integration to only 37% of the non-targeting siRNA control (Figure 3.27). These results demonstrate that downregulation of APLF in human cells results in defective NHEJ, and suggests that APLF is likely a DSB repair factor that facilitates NHEJ.
Figure 3.27 – Downregulation of APLF results in defective random genomic integration of plasmid DNA. U2OS cells were transfected with non-targeting (NT), APLF specific, or XRCC4 specific siRNAs, 48 hours later the cells were re-transfected with NT, APLF or XRCC4 siRNAs along with linearized plasmid DNA containing an antibiotic resistance cassette, and 24 hours later the cells were replated at low density in selective medium. The resulting antibiotic-resistant colonies were stained and quantified 10 days later. Representative plates are shown (inset). Data represent the mean from three independent experiments performed in triplicate. Error bars represent the standard error of the mean.
CHAPTER 4: DISCUSSION
4.1 APLF and the X4L4 complex

In this study we characterize APLF, an evolutionarily conserved nuclear protein containing an amino-terminal FHA domain and two unique carboxy-terminal zinc fingers. Because APLF was identified as the third FHA-containing protein belonging to the divergent FHA domain subgroup also comprised of the DNA end processors PNK and APTX [51], which both interact with XRCC4 and are implicated in NHEJ [50, 51, 54], we undertook a detailed characterization of the role of APLF in DSB repair and the NHEJ pathway. Although APLF does not contain an identifiable catalytic domain, APLF has recently been shown to possess endonuclease activity and 3’-5’ exonuclease activity against double-stranded DNA with overhangs that is dependent on its tandem zinc fingers [65]. Therefore, like PNK and APTX, APLF may also be a DNA end processor and may be required for the repair of specific types of DSBs prior to DNA ligation.

The results presented in this thesis show that, like PNK and APTX, APLF physically interacts with the X4L4 complex in an FHA-dependent and phospho-dependent manner, suggesting that a ternary APLF-XRCC4-DNA ligase IV complex may exist in human cells. We suggest that the interaction between APLF and DNA ligase IV is bridged by XRCC4, although we cannot exclude the possibility that APLF and XRCC4 may associate independently of DNA ligase IV under certain conditions. We demonstrate that the FHA domains of APTX and APLF interact with XRCC4 via an epitope comprising the phosphorylated Thr-233 residue, which overlaps with the PNK interacting site on XRCC4. Therefore, it appears that the FHA domains of all three of these DNA end processing proteins can bind to the same site on XRCC4 to form a tripartite complex with XRCC4 and DNA ligase IV. It is not clear what proportion of endogenous XRCC4 is engaged in APLF, PNK or APTX complexes under basal conditions, but
it is likely that these XRCC4 complexes are mutually exclusive. This notion is consistent with a report suggesting that the interactions of PNK and APTX with the analogous SSB repair scaffold protein XRCC1 are mutually exclusive [62], and with our preliminary evidence demonstrating that APLF does not complex with PNK and APTX in human cells. Further confirmation for this theory would include confirming the absence of APLF in anti-PNK and anti-APTX immunoprecipitates. It is also unclear how these various XRCC4-complexes may be regulated, but we have shown that CK2 phosphorylation of XRCC4 at threonine 233 can mediate binding of the APLF FHA domain in vitro. Accordingly, CK2 phosphorylation of XRCC4 is required for mediating interactions with PNK and APTX [51, 63], and we hypothesize that the PNK-, APTX-, and APLF-X4L4 complexes are similarly regulated.

Therefore, it appears that there are at least three ternary X4L4 complexes containing unique DNA end processing proteins that undergo FHA-directed interactions with the CK2-phosphorylated Thr-233 epitope of XRCC4. This supports the model that pre-formed X4L4-mediated DNA end processing complexes are recruited to DSBs for repair and ligation. It may prove fruitful to use the phosphorylated Thr-233 epitope in peptide pull-down assays to determine whether or not there are additional DNA end processing proteins that associate with X4L4 in a manner similar to PNK, APTX and APLF.

An interesting study to analyze the function of the PNK-, APTX- and APLF-X4L4 complexes involves downregulating these three end processors alone or in combination and measuring DSB repair using CFGE or γ-H2AX assays. This would be useful in determining whether or not PNK, APTX and APLF function in distinct non-overlapping DSB repair pathways, each acting to process a specific subset of DSBs. If this is the case, then the DSB repair defects observed following depletion of each protein individually could be determined,
and when all three proteins are depleted in combination, their defects should be additive. This would support the notion that the observed radiosensitivity of the XRCC4\textsuperscript{T233A} cell line [51], which demonstrates the requirement for Thr-233 of XRCC4 for full cellular resistance to IR, results from the combined disruption of the PNK-, APTX- and APLF-XRCC4 interactions. This is consistent with reports that stable downregulation of PNK in human cells is radiosensitizing [59], that downregulation of APLF results in increased radiosensitivity [66], and that APTX defective (AOA1) primary human fibroblasts also exhibit mild sensitivity to IR [63]. Future work could also include biophysical studies to assess the relative binding affinities of the FHA domains of PNK, APTX, APLF to a peptide spanning the phosphorylated Thr-233 epitope of XRCC4. Differences in XRCC4-binding affinity may affect the relative abundance of these complexes in the cell, which may in turn be related to the frequency of the specific types of DSBs each protein repairs, thereby explaining any differences in their relative contributions to DSB repair following different forms of DNA damage.

4.2 APLF and the Ku heterodimer

There are obvious functional similarities between the FHA domains of PNK, APTX, and APLF. However, despite these similarities, APLF also differs in some respects from these DNA repair proteins. In contrast to PNK and APTX [55, 93], we have demonstrated that APLF is devoid of intrinsic DNA binding ability, at least to double-strand plasmid DNA in electrophoretic mobility shift assays. Furthermore, we have demonstrated that, unlike PNK and APTX, APLF interacts with Ku. We have shown that endogenous APLF interacts with Ku under basal conditions \textit{in vivo} and with DNA-bound Ku \textit{in vitro}. We demonstrated that the interaction of APLF with Ku was not mediated by the FHA domain. Given that previously characterized
interact with proteins, in particular for proteins containing multiple zinc fingers [69], we sought
to determine if the APLF zinc fingers mediated its interaction with Ku. We demonstrated that
neither zinc finger was required for the interaction. We went on to localize the Ku-binding region
to amino acids 100-263 of APLF, which are located between the FHA domain and tandem zinc
finger motifs, a finding that has been confirmed independently in another study [65].

Therefore, the zinc fingers of APLF do not appear to bind Ku. In addition, they do not
appear to impart intrinsic DNA-binding ability. Whether or not the APLF zinc fingers can bind
to any DNA, RNA, or protein substrate(s) remains to be explored. We do have preliminary data
suggesting that the first APLF zinc finger motif (ZF1) is important for mediating protein-protein
interactions with the SSB-binding molecule PARP-1 (data not shown). Interestingly, this result is
consistent with recent reports demonstrating that the recruitment of APLF to SSBs is dependent
on both PARP-1 and the APLF zinc fingers [64-66], suggesting that APLF may be recruited to
SSBs through ZF1 binding to PARP-1. In contrast, it has been suggested that the APLF FHA
domain and the APLF Ku-binding region can accumulate at DSBs and are involved in the
recruitment of APLF to DSBs [65]. Our results support the idea that APLF is recruited to DSBs
via contacts with both the Ku heterodimer and the X4L4 complex.

The ability of APLF to interact with both the X4L4 complex and the Ku heterodimer is
unique and may constitute a larger multiprotein complex at DSBs. During NHEJ, the recruitment
of the X4L4 complex to DSBs is likely mediated by multiple protein-protein interactions, as
contacts have been identified between Ku and DNA ligase IV, and between DNA-PKcs and
XRCC4 [94]. Ku has been shown to bind to X4L4 and is required for the effective recruitment of
this complex to DNA ends, which is critical for efficient X4L4-mediated DNA end joining in
vitro [24, 25, 95]. Therefore, it is conceivable that Ku may facilitate the recruitment of a
preformed APLF-X4L4 complex to DSBs *in vivo*. Alternatively, a Ku-APLF complex may facilitate the FHA- and phospho-dependent recruitment of X4L4 to DSBs following DNA damage. These two scenarios are not necessarily mutually exclusive, and both may operate in tandem. In both scenarios, APLF may serve as an adaptor protein to bridge Ku and X4L4, thereby representing another mechanism for the recruitment of X4L4 to DSBs.

In addition to the scenario of APLF acting as an adaptor protein to bridge Ku and X4L4, APLF-Ku and APLF-X4L4 interactions may occur independently. There may exist distinct subcellular pools of APLF, with some APLF engaged in pre-formed X4L4 complexes that are recruited to DSBs in a manner similar to PNK- and APTX-containing complexes via contacts between Ku and X4L4, and with some APLF directly interacting with Ku. Because APLF possesses both endonuclease and exonuclease enzymatic activities [65] that may process different types of damaged termini, it is conceivable that there are distinct mechanisms of APLF recruitment depending on the type of DSB being repaired.

### 4.3 Phosphorylation of APLF

In this study, we observed that APLF was both basally phosphorylated and hyperphosphorylated at serine 116 in an ATM-dependent manner following DNA damage, a result that is consistent with a recent independent observation [66]. We also show that both APLF and the serine 116 epitope of APLF, can serve as ATM substrates *in vitro*. Taken together, these results suggest that ATM phosphorylates APLF at serine 116 following DNA damage.

It is unclear what function APLF basal phosphorylation performs or which protein kinase(s) are involved, but APLF does contain numerous predicted sites of phosphorylation, particularly in its highly acidic extreme carboxy-terminal region, for the constitutively active
CK1 and CK2 protein kinases [84]. Indeed, we have demonstrated that APLF is phosphorylated by CK2 in vitro (data not shown). Therefore, it is possible that one or both of these kinases may contribute to the basal phosphorylation of APLF, or may co-operate in some way with ATM-dependent APLF phosphorylation. A recent report has revealed the presence of co-regulated ATM, CK1, and CK2 phosphorylation sites in the transcription factor cyclic AMP-response element-binding protein (CREB), whereby basal phosphorylation and IR-induced phosphorylation events occur in cascade, with priming phosphorylation by CK1/2 being required for phosphorylation by ATM [96]. Interesting future work to study this possibility includes treatment of human cells with specific small chemical inhibitors of the CK1 and CK2 kinases, to determine if basal phosphorylation of APLF is abrogated. If basal phosphorylation can be prevented by pre-treatment with CK1/2 inhibitors, then IR treatment can be administered to determine if ATM-dependent phosphorylation of APLF at serine 116 is impaired, indicating that perhaps CK1/2 phosphorylation acts as a primer to allow ATM phosphorylation of APLF.

The function of APLF IR-induced hyperphosphorylation is also unclear. This event does not appear to alter either APLF subcellular localization, as assessed by immunofluorescence microscopy, or APLF interactions with Ku and X4L4, as assessed by co-immunoprecipitation from HEK293T cells (data not shown). It is interesting to note that serine 116 is within the Ku-binding region, and one might speculate that phosphorylation of a residue within this region may somehow affect the APLF-Ku interaction. There may exist specific pools of Ku molecules and phosphorylation of APLF at S116 may affect its interaction with either free or DNA-bound Ku, a distinction we have not yet addressed.

ATM broadly functions in both DNA damage signaling and cell cycle checkpoint responses [45], and also functions with the DNA end processor Artemis in a NHEJ subpathway.
to repair a small subset (~10%) of IR-induced DSBs that are normally repaired with slow kinetics, which is also referred to as the repair of potentially lethal damage [29, 30, 97]. Like Artemis, APLF is likely a DNA end processor [65], and although our study does not define a specific function for ATM-dependent APLF hyperphosphorylation, this event may represent an additional novel link between ATM and DSB repair. It is tempting to speculate that APLF is involved in either the ATM-Artemis NHEJ subpathway or in a novel ATM-dependent NHEJ subpathway that is important for the processing of a specific subset of DSBs. Alternatively, it is also possible that ATM-dependent phosphorylation of APLF functions in some aspect of ATM-dependent DNA damage signaling or cell cycle checkpoint arrest.

Interesting future work could involve studies specifically examining the serine 116-phosphorylated species of APLF (pS116-APLF), which may represent a functionally distinct form of APLF. Our lab is currently in the process of developing phosphospecific antibodies that recognize pS116-APLF. We would like to use this antibody in immunofluorescence experiments to determine if pS116-APLF localizes to sites of DSBs or co-localizes with any proteins involved in DNA repair following DNA damage. We also hope to use this antibody for western blotting of various immunocomplexes, so that we can determine if pS116-APLF engages in any unique protein-protein interactions distinct from basally phosphorylated APLF. Although we have not observed loss of interaction between APLF and X4L4 and Ku following IR, we have been unable to monitor whether these proteins are preferentially associating with either the basally phosphorylated or hyperphosphorylated species of APLF. The work in this thesis has focused on the IR-induced phosphorylation of APLF, however, future studies could expand this work to assess the phosphorylation of APLF in response to a panel of various DNA damaging agents.
The pS116-APLF antibody will allow us to determine if any observed mobility shifts in response to specific DNA damaging agents are due to phosphorylation of the serine 116 residue of APLF.

4.4 Function of APLF

The depletion of APLF from human cells subjected to IR resulted in defective DSB repair kinetics, as measured by the γ-H2AX assay. Although the observed defect was small, it was statistically significant and reproducible, thereby implicating APLF in DSB repair. A small repair defect was not unexpected given that the XRCC4$^{T233A}$ cell line, which collectively disrupts PNK-, APTX-, and APLF-XRCC4 interactions, exhibits only a 20% reduction in survival compared to cells expressing wild-type XRCC4 after 2 Gy of IR [51]. Different types of DNA damage can arise from exposure to IR, and these results suggest that the PNK-, APTX-, and APLF-XRCC4 DNA end processing complexes are involved in repairing only a fraction (~20%) of this damage, although their relative contributions remain undefined. Although we have demonstrated that APLF interacts with core components of the NHEJ pathway, we cannot conclude from the γ-H2AX assay that the observed DSB repair defect is due to a disruption of NHEJ. However, we then went on to further analyze in a different system whether or not downregulation of APLF in human cells results in an NHEJ defect. Downregulation of APLF resulted in defective random genomic integration of foreign DNA, a process known to be dependent on the NHEJ pathway, suggesting that APLF functions in the NHEJ pathway to facilitate DNA end-joining.

The γ-H2AX assay was performed with proliferating U2OS cells that were not synchronized with regard to cell cycle position. While the majority of DSBs produced by IR are repaired via the NHEJ pathway, there may be some contribution of the HR pathway in

75
replicating cells, depending on cell cycle position [6]. Therefore, we cannot exclude the possibility that the impairment of DSB repair kinetics observed in the APLF-depleted cells might be attributable to a defect in HR. Given that our preliminary experiments have not revealed any association between GST-APLF and the HR proteins, Rad51, Rad51B, Rad51C, Rad54, XRCC3, or BRCA2 in pull-down assays (data not shown), and that others have observed similar DSB repair defects in differentiated non-cycling cells [64], we suggest that the defects likely arise from disruption of DSB repair via the NHEJ pathway, and not the HR pathway. An interesting companion study that could better determine the participation of APLF in the HR and NHEJ pathways of DSB repair involves performing the γ-H2AX assay in wild-type, NHEJ-defective, and HR-defective cell lines treated with either non-targeting (NT) or APLF-specific siRNA. If a specific DSB repair pathway is already completely non-functional, depletion of additional components within that same pathway would be redundant and would not result in any additional defect. However, if a specific DSB repair pathway is already completely non-functional and you then deplete a component of a different DSB repair pathway, you actually disrupt both repair mechanisms, and their individual DSB repair defects become additive. Therefore, in the context of the γ-H2AX assay, normal kinetics of DSB repair would occur in the NT-control wild-type cells, while we would expect to see a small DSB repair defect in the APLF-depleted wild-type cells. The NT-treated HR-defective and NHEJ-defective cells would both exhibit defects in DSB repair kinetics. If APLF does indeed function in the NHEJ pathway, then APLF downregulation in the NHEJ-defective cell lines should confer no additional defect in DSB repair. Accordingly, an additional additive DSB repair defect should be observed in the HR-defective cell lines. This would further suggest that APLF functions in DSB repair primarily via the NHEJ pathway.
Another result from the γ-H2AX assay that would be interesting to analyze further was the observation that downregulation of APLF led to an increased proportion of nuclei with unrepaired DSBs at 24 hours post-IR. Studies examining cells defective in either Artemis or ATM, which are both essential components of the NHEJ subpathway that repairs a small subset of IR-induced DSBs, have shown that disruption of this pathway results in substantial radiosensitivity because the DSBs linger in the cells and remain un-rejoined beyond 14 days following DNA damage [29], as these DSBs are essentially irreparable by other DNA repair enzymes. Because of the limitations of transient siRNA-mediated downregulation of protein expression, we were unable to assess the persistence of γ-H2AX foci beyond 24 hours. Interesting future studies will likely involve following the DSBs in cells stably-depleted of APLF to determine whether or not they persist indefinitely, or if they are repaired through other means.

4.5 Future directions

One of the most important avenues of APLF research is to determine the mechanism of APLF function in NHEJ. We are currently optimizing the purification of enzymatically active APLF for in vitro reconstitution NHEJ assays [98], in which purified recombinant NHEJ components including the Ku heterodimer, DNA-PKcs, and the X4L4 complex are used to re-join double-stranded DNA oligos with different termini modifications. We would like to assess the effect of APLF on the end-joining of oligos that are unligatable by DNA ligase IV if unprocessed and that have been shown to serve as APLF enzymatic substrates [65]. We would also like to begin using the previously described cell-free system [99], which uses nuclear extracts that contain both the core NHEJ proteins and the host of accessory proteins required to achieve full end-joining efficiency, to re-join oligos in vitro. Using this system, the requirement
for APLF can be assessed by using nuclear extracts obtained from wild-type cell lines and APLF-depleted cell lines. Additionally, nuclear extracts obtained from APLF-depleted cell lines can be reconstituted in vitro with purified recombinant APLF protein. We hypothesize that APLF participates in NHEJ as a DNA end processor, and these types of experiments can be used to demonstrate that APLF can specifically enable the re-joining of DSBs containing modified termini that are otherwise unligatable by the NHEJ pathway. In addition, mutations targeted to specific sites on APLF can give clues to their importance in APLF function. For example, if purified recombinant wild-type APLF but not APLF containing the R27A mutation, can mediate end processing and re-joining of oligos in vitro, then we can infer that the APLF-XRCC4 interaction is essential for the function of APLF in the NHEJ pathway.

Useful tools for the future study of APLF function include APLF knock-out cell lines, which can be generated using murine embryonic stem cells or chicken DT40 B lymphocyte cells. This will eliminate the need to transiently downregulate APLF using siRNA. In addition to enabling the study of persistent DSBs for extended periods of time following DNA damage, these cell lines will be useful for the in vitro end-joining assays described above. However, reconstitution experiments will be among the most important studies enabled by these APLF knock-out cell lines, whereby assays such as CFGE and γ-H2AX can be used to analyze the NHEJ/DSB repair defects of these cell lines, which can then be reconstituted by stable ectopic expression of wild-type APLF in order to rescue these defects. Concurrently, reconstitution using various APLF mutants can be performed to determine whether or not they can still mediate the full rescue of the defects, thereby indicating the impact of these mutations on APLF function.

For example, we hypothesize that APLF functions in NHEJ as a DNA end processor, and that the DSB repair defects we have observed in APLF-depleted cells are due to an impairment
in their ability to process a specific subset of DSBs normally repaired by APLF. Therefore, it would be interesting to reconstitute APLF knock-out cell lines with an ‘enzymatically dead’ form of APLF, which studies suggest may be at least partially achievable by mutation of the tandem zinc finger domain (ZF1/2m) [65]. If an ‘enzymatically dead’ form of APLF is unable to even partially rescue the DSB repair defects, then the DNA end processing activity of APLF is likely essential for its function in NHEJ. Given that we have observed APLF interactions with both Ku and XRCC4, we hypothesize that APLF may also increase the overall efficiency of NHEJ by acting as an adaptor protein to enhance the recruitment of the X4L4 complex to DSBs by bridging Ku to X4L4. Therefore, it would be interesting to reconstitute APLF knock-out cell lines with APLF containing either an FHA-inactivating arginine-to-alanine substitution at arginine 27 (R27A) to disrupt APLF binding X4L4, or an internal deletion of a portion of the Ku-binding domain at amino acids 100-263 to disrupt APLF binding to Ku. If a form of APLF that is unable to bridge Ku to X4L4 is unable to even partially rescue the DSB repair defects, then the Ku-to-X4L4 adaptor ability of APLF is likely essential for its function in NHEJ. The importance of IR-induced ATM-dependent phosphorylation of serine 116 for APLF function can also be assessed by reconstitution of APLF knock-out cell lines with APLF containing an unphosphorylatable serine-to-alanine substitution at serine residue 116 (S116A), to determine whether or not this event is essential for mediating full rescue of the DNA repair defects following IR. Similarly, the APLF knock-out cell lines can also be reconstituted with APLF containing a phospho-mimicking serine-to-aspartic acid substitution at serine residue 116 (S116D). If a form of APLF that is unphosphorylatable at serine 116 is unable to even partially rescue the DSB repair defects, and if a form of APLF that mimics phosphorylation at serine 116 is able to fully mediate rescue of these defects, then the IR-induced ATM-dependent
phosphorylation of APLF at this residue is likely essential for its function in NHEJ. As an aside, it may also be interesting to re-constitute ATM-null human fibroblast cells by stable ectopic expression of APLF S116D to determine if this could recover some of the DNA repair defect observed in these cells [29]. Collectively, these types of experiments will help us to better understand the function of APLF in NHEJ and DSB repair.

4.6 Summary

The work presented in this thesis is important because it demonstrates that APLF plays a role in DSB repair and NHEJ, a theory supported by the findings of other groups [64-66]. Our published research supports the idea that the unique DNA end processing proteins PNK, APTX, and APLF all engage in FHA-mediated interactions with CK2 phosphorylated Threonine 233 of XRCC4 to form mutually exclusive complexes with X4L4. We show that APLF undergoes an additional interaction with Ku that is mediated by a region between the APLF FHA and tandem zinc finger domains, and that APLF can bind to Ku while Ku is bound to DNA. Although we do not demonstrate a mechanism for the function of APLF in NHEJ, its participation is suggested by its interactions with core NHEJ components, by the ATM-dependent phosphorylation of APLF following IR, and by the deleterious effect of siRNA-mediated downregulation of APLF on DSB repair kinetics and NHEJ. We hypothesize that APLF acts to increase the overall efficiency of NHEJ by behaving as an adaptor to bridge Ku and X4L4, and that APLF functions as a DNA end processor that is required for the repair of a specific class of DSBs and is recruited via interactions with Ku and X4L4. In addition, it is tempting to speculate that APLF may function in an ATM-dependent NHEJ subpathway to repair a specific subset of DSBs. Figure 4.1 depicts a model for the potential role of APLF in NHEJ.
The research presented in this thesis supports a model for NHEJ wherein the DNA end processors PNK, APTX, and APLF engage in FHA-mediated interactions with CK2 phosphorylated Thr-233 of XRCC4 to form mutually exclusive complexes with X4L4. These proteins are likely recruited to DSBs by contacts between components of the X4L4 complex and the DNA-PK holoenzyme. Unlike PNK and APTX, APLF also appears to interact with the Ku heterodimer and is also able to complex with Ku on DNA ends. APLF may act to increase the efficiency of NHEJ by behaving as an adaptor to bridge Ku and X4L4. APLF may also function as a DNA end processor to repair a subset of DSBs, and may be recruited to sites of DNA damage via its interactions with Ku and X4L4. The ATM-dependent phosphorylation of APLF may affect its enzymatic activity or interactions with Ku and X4L4, and may also provide an additional link between ATM and NHEJ. Therefore, APLF may function in an ATM-dependent NHEJ subpathway to repair a specific subset of DSBs.
REFERENCES


