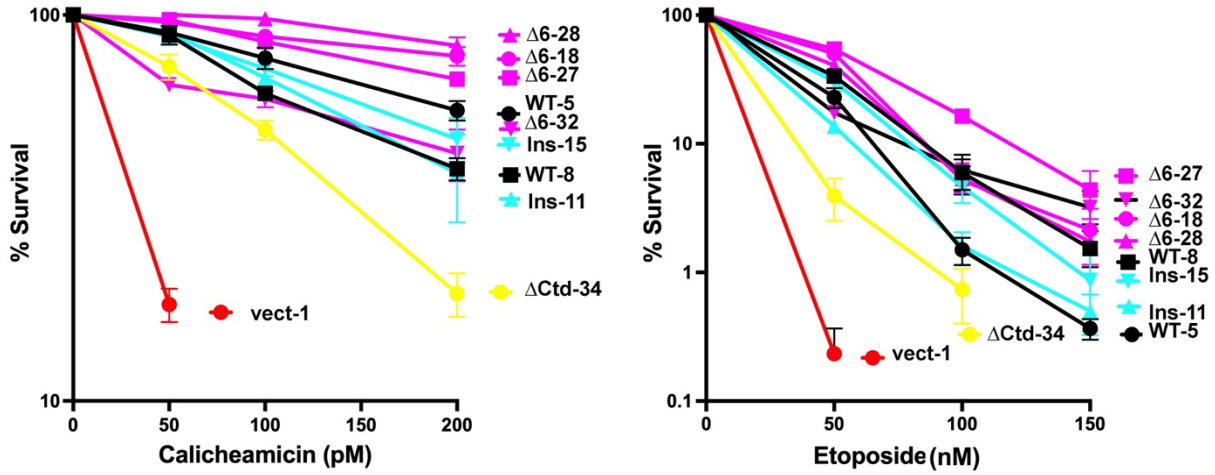
Table S1

DNA-PKcs mutations		
4XK/R>Ala; R1854A +K1857A+ K1913A+ K1917A	**4xala**	Disrupts interaction between DNA-PKcs and Ku80 C-terminus.
2569-2571; S2569A + D2570A	**2569**	Disrupts interaction with DNA-PKcs 898-900
898-900: F898A + R899A + E900A	**898**	Disrupts interaction with DNA-PKcs 2569-2571
2569-2571+898-900	**898/2569**	
4XK/R>Ala+898-900	**898/4xala**	
4XK/R>Ala+2569-2571	**2569/4xala**	
898-900: F898A + R899A + E900A + K902D +K944D	**898+902asp**	More severely disrupts the 898-900 interface
4XK/R>Asp; R1854D +K1857D+ K1913D+ K1917D	**4xasp**	More severely disrupts interaction between DNA-PKcs and Ku80 C-terminus
898-900+902D+4XK/R>Asp	**898+902asp+ 4xasp**	
Ku80 mutations		
Linker insert 1	**Ins1**	Inserts 5 residues in linker
Linker insert 2	**Ins2**	Inserts 15 glycine at the C terminus of linker
3 residue linker deletion	**Δ3**	Deletes 3 residues in linker
6 residue linker deletion	**Δ6**	Deletes 6 residues in linker
10 residue linker deletion	**Δ10**	Deletes 10 residues in linker
13 residue linker deletion	**Δ13**	Deletes 13 residues in linker
15 residue linker deletion	**Δ15**	Deletes 15 residues in linker
Linker/Ctd deletion	**ΔCTD**	Deletes entire linker and C-terminal helix
XLF mutations		
Disruption of XRCC4/XLF interaction	**L**	L115A
Disruption of XLF KBM/Ku80 interaction	**B**	L297W
Disruption of XLF/Ku70 interaction	**K**	RDR176-178AAA
L115A+L297W	**L+B**	
L115A+RDR176-178AAA	**L+K**	
RDR176-178AAA+L297W	**K+B**	
L115A+RDR176-178AAA+L297W	**L+K+B**	

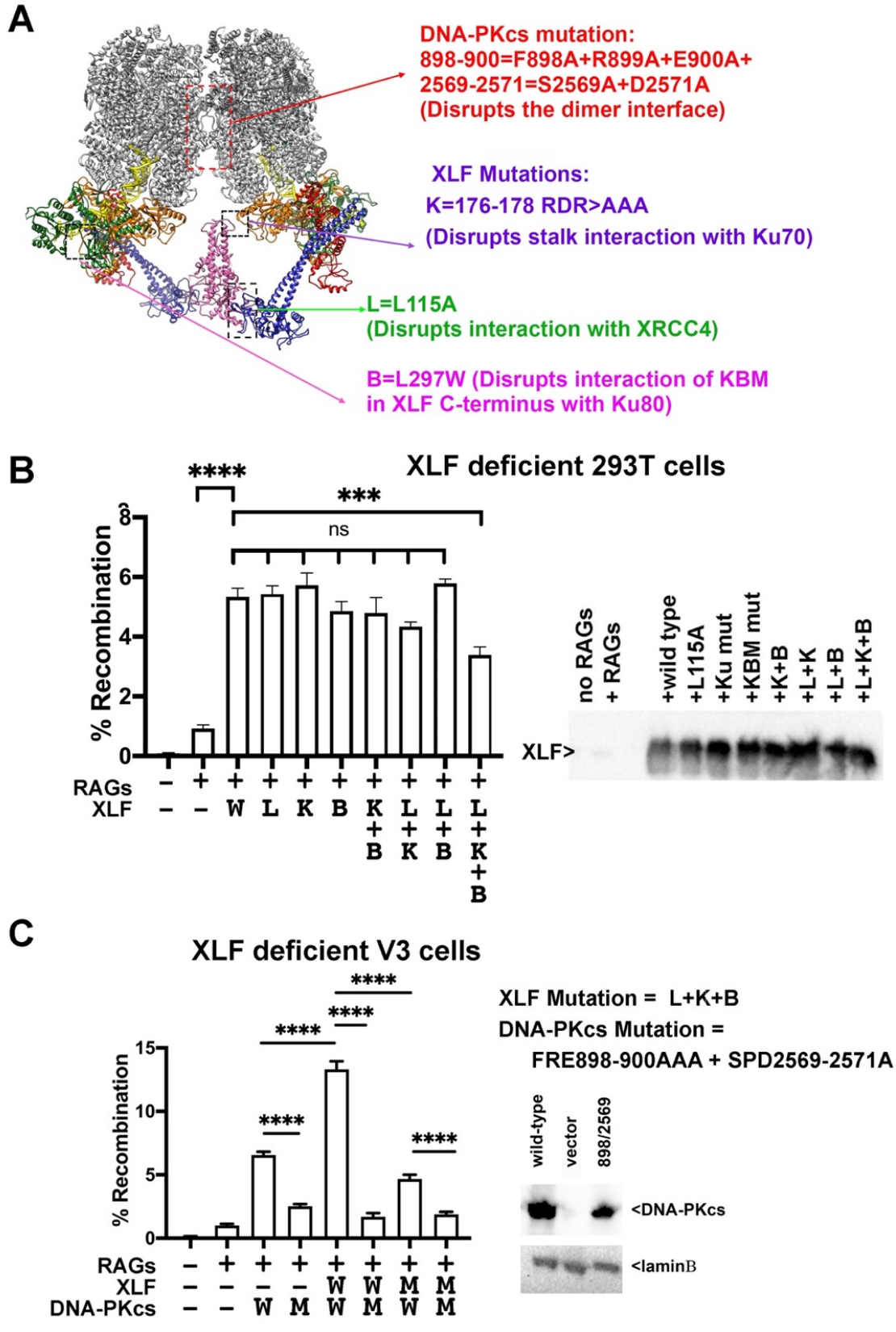
Supplemental Table 1. Related to Figures 1-7. DNA-PKcs, Ku80, and XLF mutants utilized in this study.

Figure S1.



Supplemental Figure 1. Related to Figure 1. Altering the length of the disordered linker in the C-terminus of Ku80 to favor cis or trans interactions alters end-joining. xrs6 clonal transfectants expressing wild-type human Ku80 (WT), the Δ6 linker shortening mutant (Δ6), the ins1 linker lengthening mutant (Ins), or ΔCtd linker/C-terminal+helix deletion mutant (ΔCtd), or no Ku80 (vect) were plated at cloning densities into complete medium with increasing doses of calicheamicin or etoposide. Colonies were stained after eight days, and percent survival was calculated. Error bars represent the standard error of the mean.

Figure S2.


A
DNA-PKcs mutation:
898-900=F898A+R899A+E900A+
2569-2571=S2569A+D2571A
(Disrupts the dimer interface)
XLF Mutations:
K=176-178 RDR>AAA
(Disrupts stalk interaction with Ku70)
L=L115A
(Disrupts interaction with XRCC4)
B=L297W (Disrupts interaction of KBM
in XLF C-terminus with Ku80)
B
XLF deficient 293T cells
****
***
ns
% Recombination
RAGs
XLF
W L K B K+B L+K L+B L+K+B
no RAGs
+ RAGs
+wild type
+L115A
+Ku mut
+KBM mut
+K+B
+L+K
+L+B
+L+K+B
XLF>
C
XLF deficient V3 cells
% Recombination
RAGs
XLF
DNA-PKcs
XLF Mutation = L+K+B
DNA-PKcs Mutation =
FRE898-900AAA + SPD2569-2571A
wild-type
vector
898/2569
<DNA-PKcs
<laminB

Supplemental Figure 2. Related to Figure 2. Via multiple protein-protein interactions, a single XLF dimer interacts with Ku70, Ku80, and XRCC4 to promote a long-range synaptic complex. (A) Ribbon diagram of the XLF-mediated long-range complex as reported by Chaplin *et al.*(Chaplin *et al.*, 2021b) (PDB:7NFC). DNA-PKcs is shown in grey, Ku80 in green, Ku70 in orange, XLF in pink, XRCC4 in purple, and DNA in yellow. (B) The fluorescent substrate 290-Crimson/ZS was utilized to detect coding end joining of RAG-induced DSBs in 293T cells lacking XLF as described previously(Roy *et al.*, 2015) (left). Immunoblot of transiently expressed XLF mutants in XLF-/- 293T cells (right). Percent recombination of episomal fluorescent coding-end joining substrate in cells transiently transfected with wild-type or mutant XLF expression constructs as described in 3A. Error bars indicate SEM from five independent experiments. ****P<0.0001, ***P<0.001; in two-way ANOVA with Holm-Sidak correction. (C) Fluorescent substrate 290-Crimson/ZS was utilized to detect coding end joining in XLF deficient V3 cells that lack both DNA-PKcs and XLF. Percent recombination assessed as in B. Error bars indicate SEM from three independent experiments. P values as in B.