SUPPLEMENTARY INFORMATION FILE to:

PAXX and its paralogues synergistically direct DNA polymerase λ activity in DNA repair.

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Running title: PAXX and its paralogs regulate DNA Polymerase λ function in NHEJ

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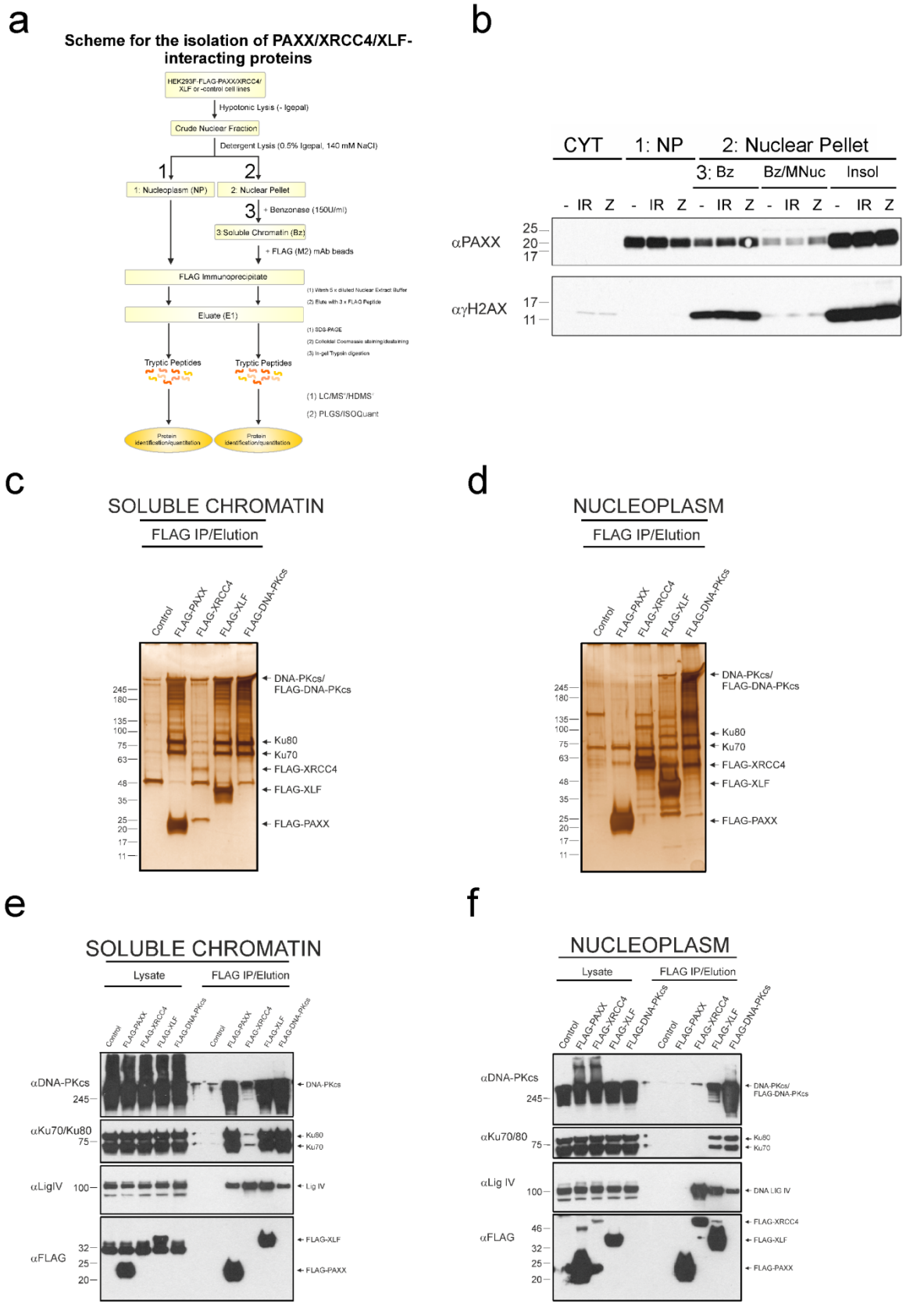
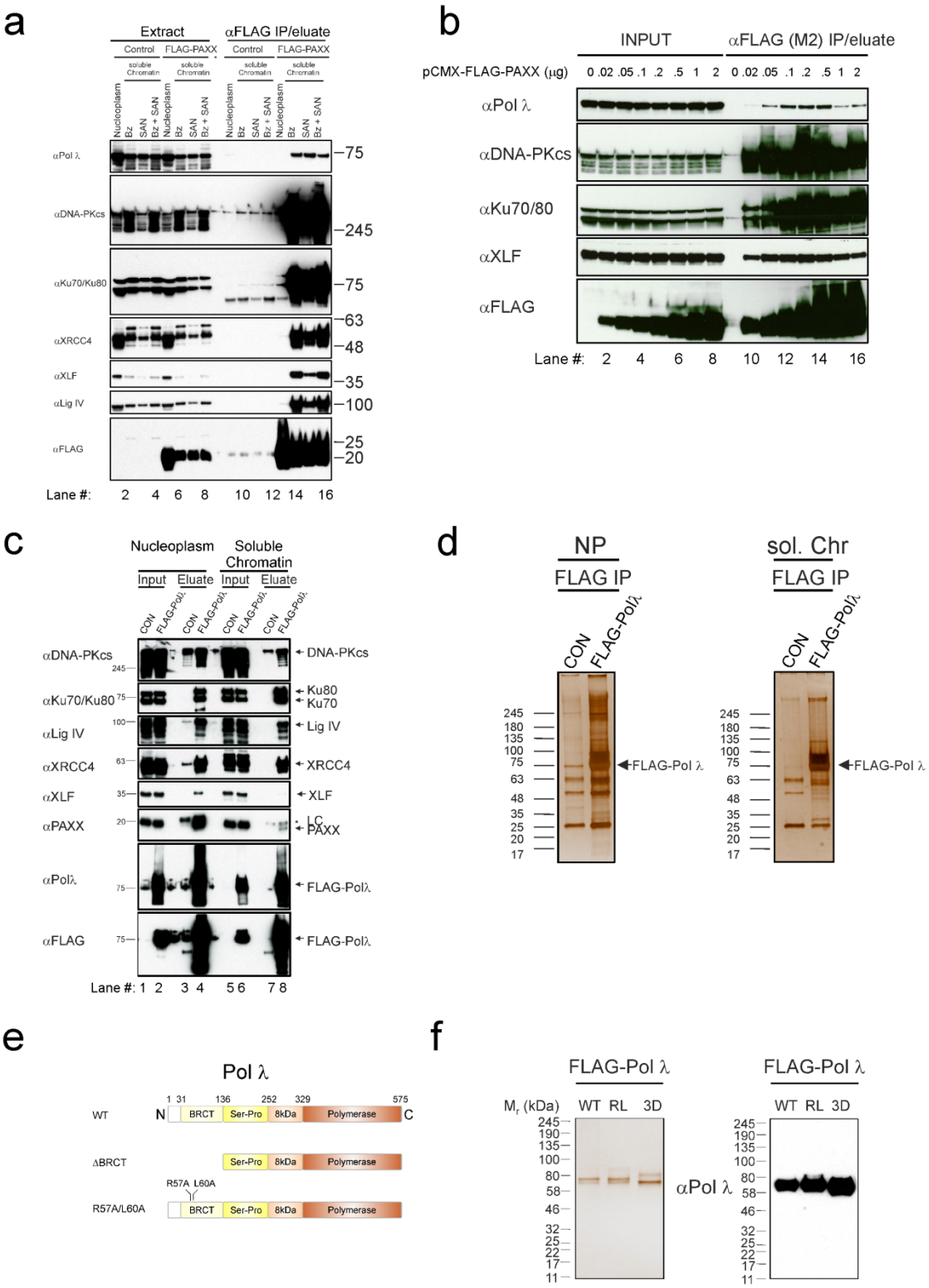
Supplementary Information File

Supplementary Figure 1



Supplementary Figure 1: Comparative Analysis of the Proteomes of XRCC4 Family Proteins. (a): Schematic figure showing a flow chart for the identification of FLAG-tagged PAXX, -XLF and –XRCC4 associated proteins by affinity chromatography and mass spectrometry from benzonase-treated "soluble chromatin" and nucleoplasmic fractions from HEK293 cells. (b): Untreated (-), irradiated (IR, 10Gy) or Zeocin (Z)-treated HEK293F cells were fractionated into non-nuclear soluble (CYT) or nuclear soluble (NP) or nuclear pellets. Nuclear pellets were either incubated with benzonase (Bz) or a combination of benzonase and micrococcal nuclease (Bz/MNuc). Fractions were resolved by SDS-PAGE and immunoblotted with the indicated antibodies. (c-d): Eluates of purified anti-FLAG IPs from (c) soluble chromatin or (d) nucleoplasmic fractions isolated from HEK293F-control, -FLAG-PAXX, -XLF, -XRCC4 or DNA-PKcs-expressing cells were resolved by SDS-PAGE and analysed by silver-staining. (e-f): As above, except the indicated NHEJ proteins were detected by immunoblotting using (e) soluble chromatin or (f) nucleoplasmic fractions.

Supplementary Figure 2

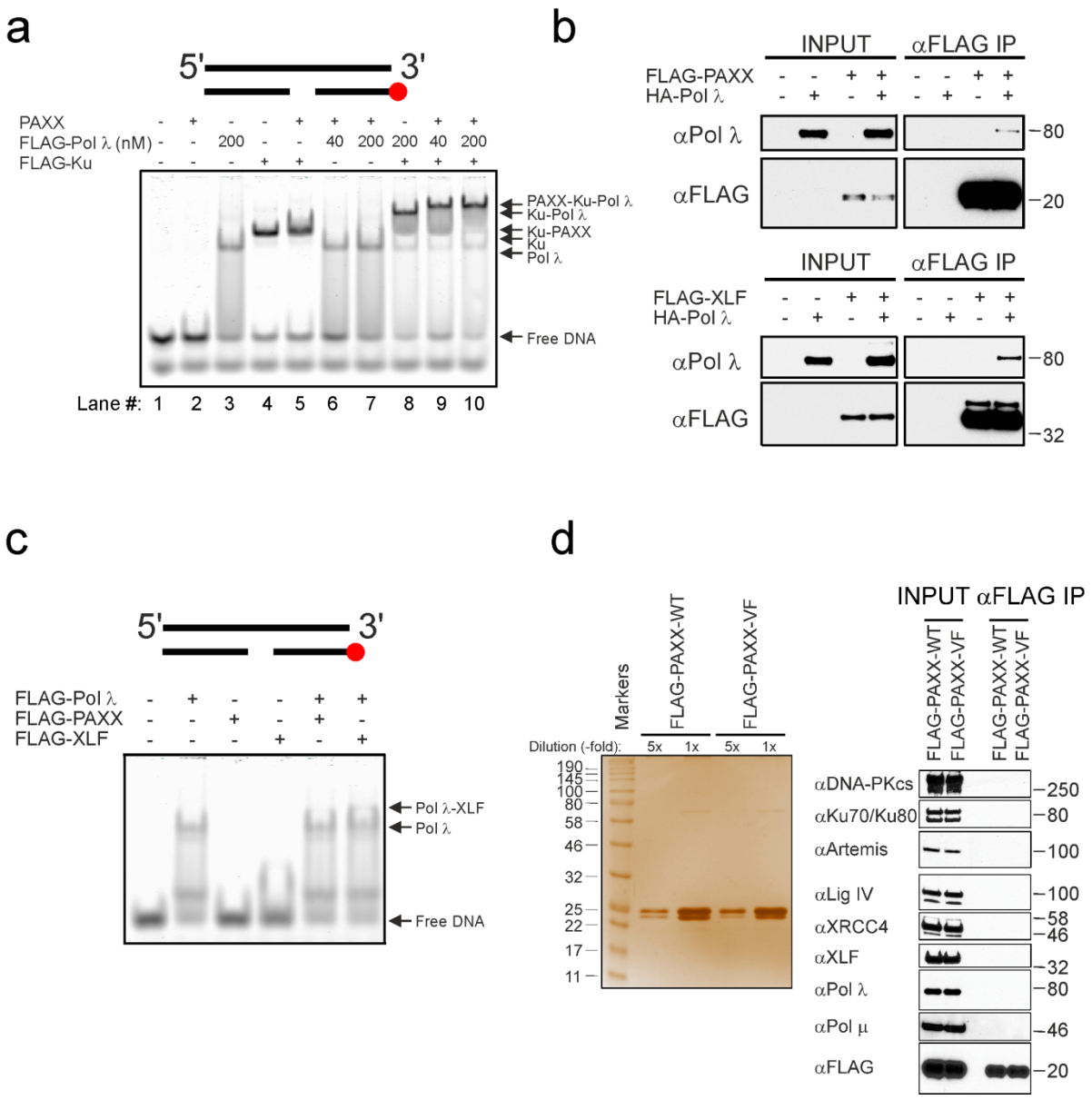


Supplementary Figure 2: Pol λ interacts with endogenous PAXX and its paralogs in cells.

(a): HEK293F or (b): U2OS cells were transiently transfected with either pCMX-LacZ (Control) or pCMX-FLAG-PAXX and nucleoplasmic or soluble extracts from benzonase- and/or salt active

nuclease (SAN)-treated nuclear pellets were isolated. Following anti-FLAG IP and FLAG peptide elution, FLAG-PAXX and associated proteins were eluted, resolved by SDS-PAGE and immunoblotted for the indicated proteins. (c): As described in Panel A, except that HEK293F cells were transiently transfected with either pCMX-LacZ (Control) or pCMX-FLAG-Pol λ. (d): Extracts from either nucleoplasmic (NP) or soluble chromatin (sol. Chr.) fractions or eluates of purified anti-FLAG IPs from HEK293F cells transiently transfected with either empty vector or FLAG-Pol λ were resolved by SDS-PAGE and visualized by silver staining. The position of FLAG-Pol λ was confirmed by immunoblotting and is indicated by a black arrow. (e): Schematic representation of Pol λ BRCT domain deletion mutant and α–helix 1 R57A/L60A mutant. (f): Silver-stained gel and anti-FLAG immunoblot for purified human Pol λ-WT, -R57A/L60A and -D427A/D429A/D490A.

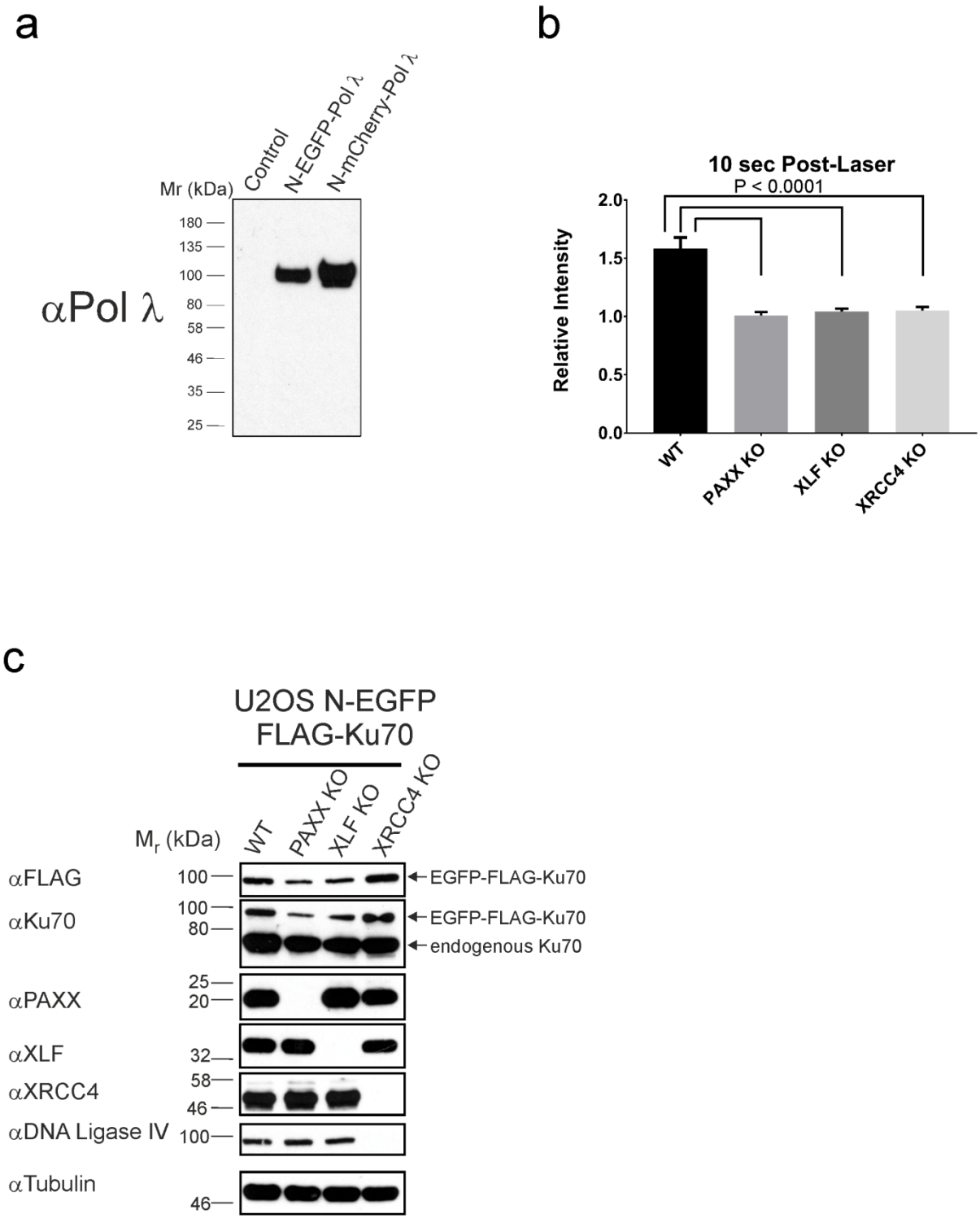
Supplementary Figure 3



Supplementary Figure 3: Interaction of PAXX with Pol λ requires the Ku binding motif of PAXX. (a): EMSA showing that interaction of PAXX with Pol λ requires DNA-bound Ku. Reactions were performed with 20 nM IRDye® 700-labelled 5nt-gapped dsDNA (30-mer) and the following concentrations of FLAG-Ku70/80 (20 nM), FLAG-Pol λ (40 or 200 nM) or cleaved PAXX (0.5 μM). (b): *In vitro* interaction of PAXX or XLF with Pol λ. Affinity purified FLAG-PAXX (*upper panel)* or FLAG-XLF (*lower panel*) were mixed with affinity purified HA- Pol λ and immunoprecipitated with FLAG (M2)-agarose. Following FLAG peptide elution, eluates were immunoblotted with the

indicated antibodies. (c): As shown above in Panel A, except that reactions contained the indicated proteins (d): Silver-stained gel (*left* panel) and immunoblots (*right* panel) of purified FLAG-tagged human PAXX-WT or a V199A/F201A (VF) mutant. Immunoblots were probed with the indicated antibodies.

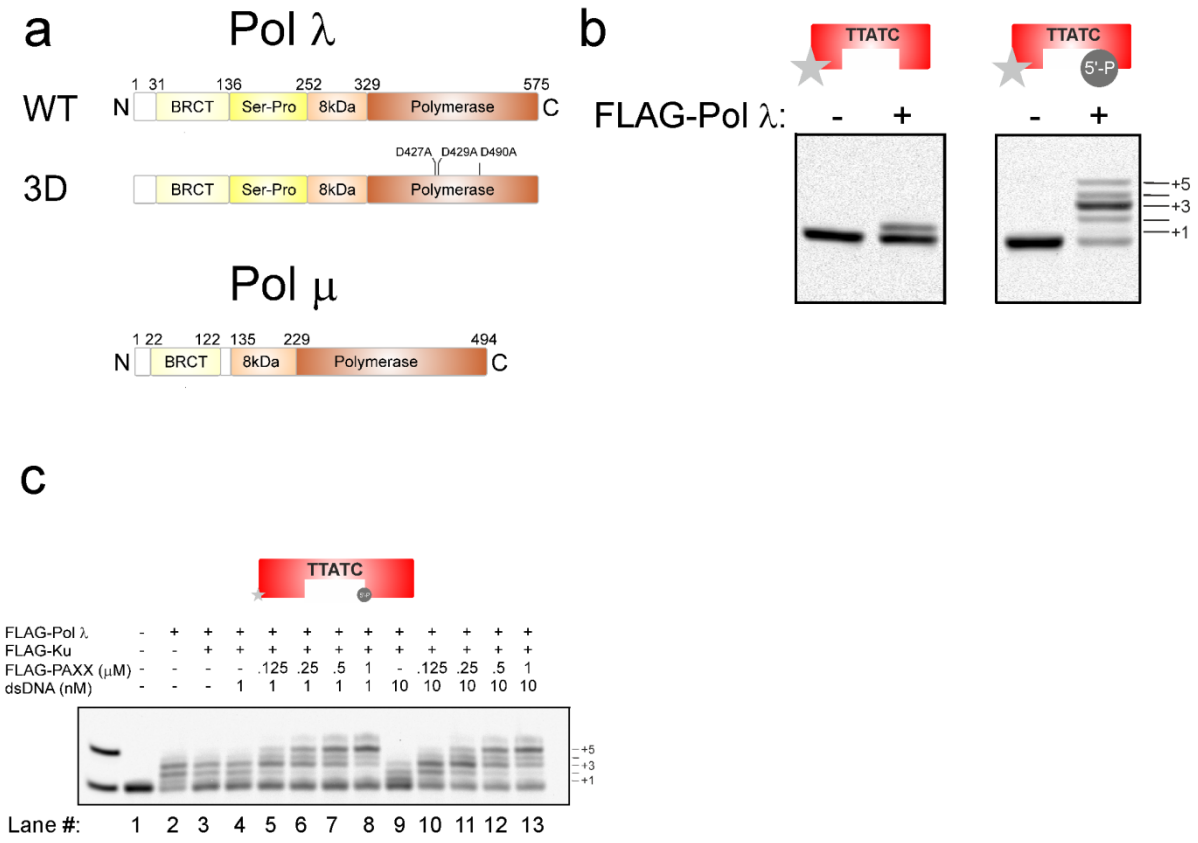
Supplementary Figure 4



Supplementary Figure 4: Role of PAXX and its paralogs in the recruitment of Pol λ to laser microirradiation-induced DNA damage sites. (a): Immunoblot showing N-EGFP- and -mCherry-Pol λ fusion proteins are expressed as 100kDa proteins in U2OS cells. (b): Bar graph indicates the

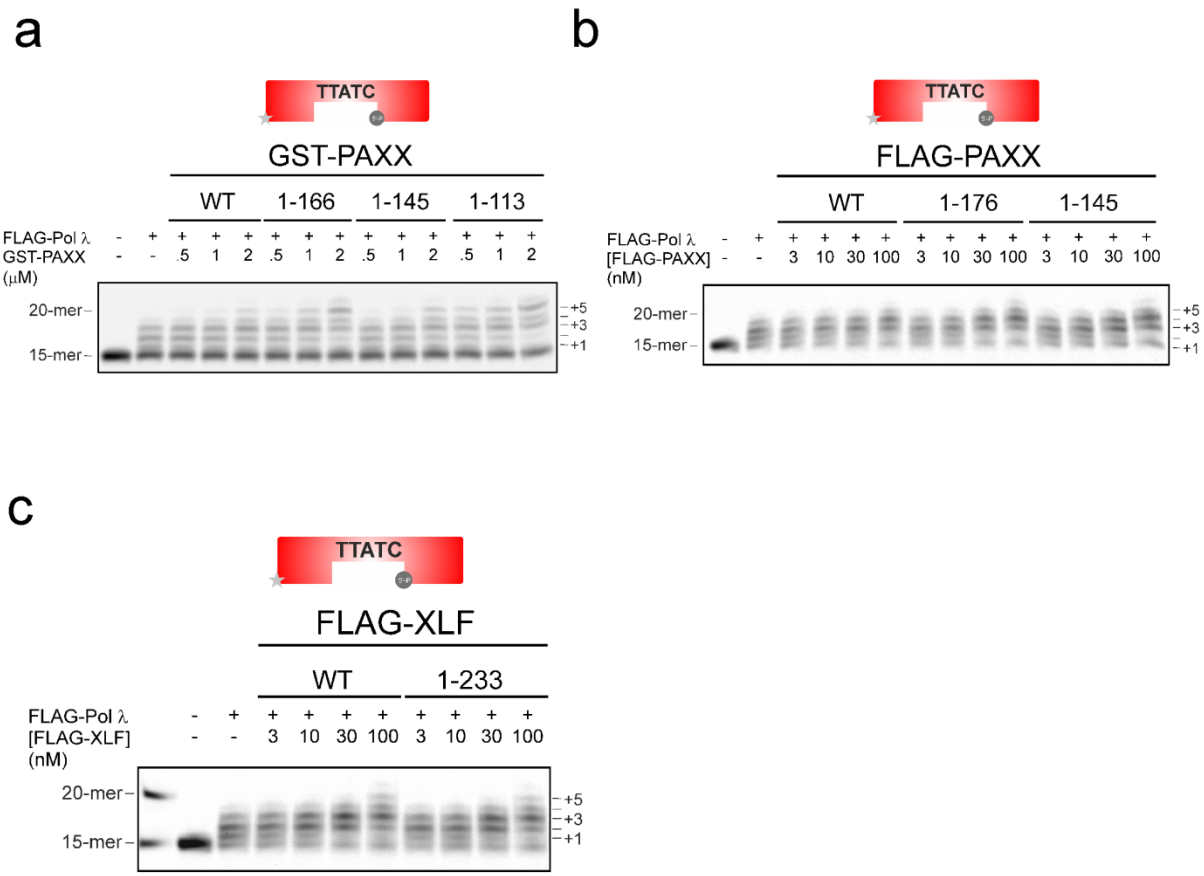
mean ± SEM for normalised N-EGFP- Pol λ fluorescence at the laser-induced microirradiation site 10 sec following laser pulse. P-values were calculated using a 2-tailed students t-test with Graphpad. (c): Immunoblot analysis of N-EGFP-FLAG-Ku70-expressing U2OS cells deficient in PAXX, XLF or XRCC4 generated by CRISPR-Cas9. WCL were resolved by SDS-PAGE and the indicated proteins detected by immunoblotting.

Supplementary Figure 5



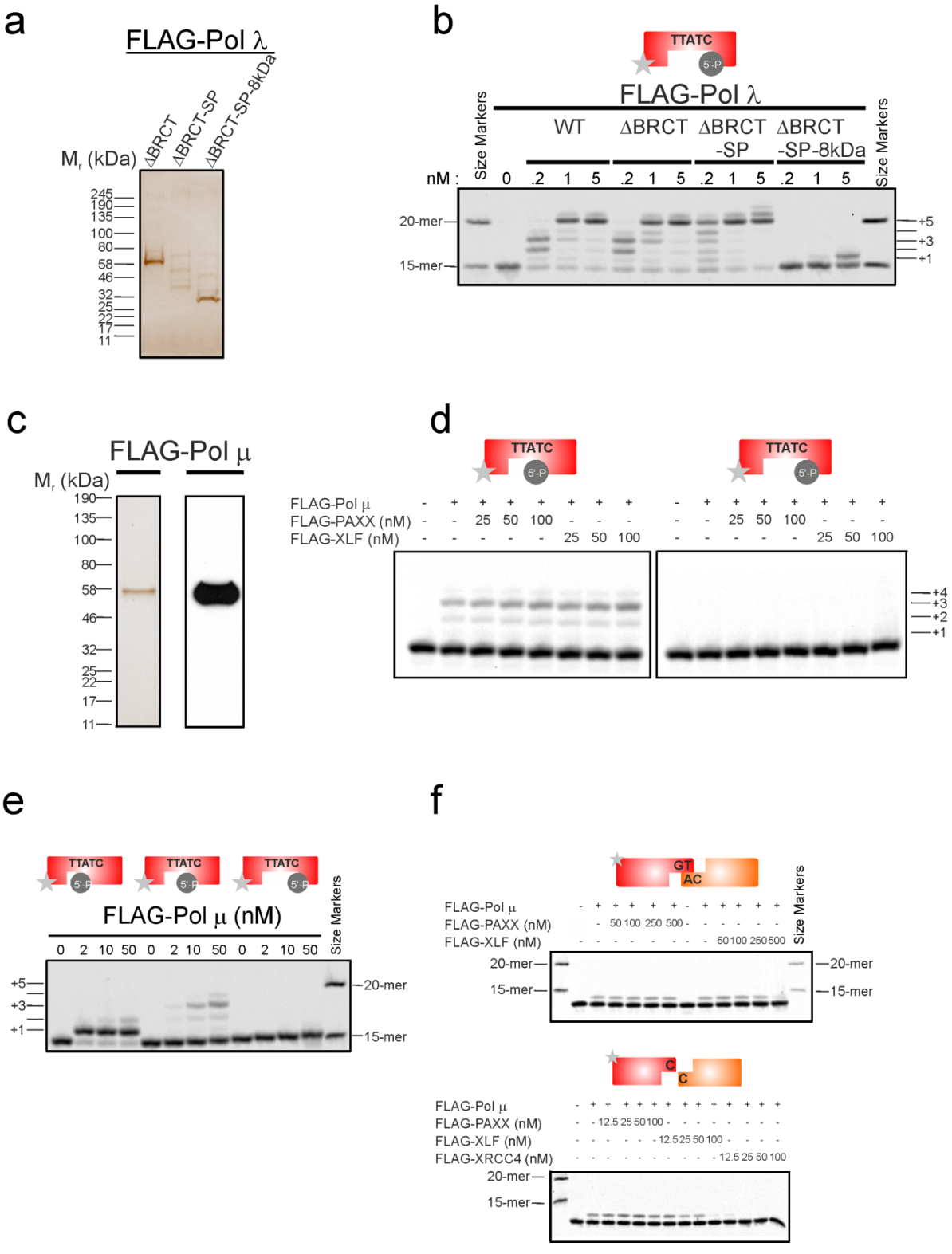
Supplementary Figure 5: PAXX stimulates Pol λ-dependent gap filling synthesis activity in the presence of Ku (a): Schematic representation of catalytically-inactive Pol λ D427A/D429A/D490A mutant and Pol μ. (b): Gap-filling synthesis activity of FLAG-Pol λ-WT using either an IRDye® 700-labelled non-phosphorylated (*left* panel) or 5'-phosphorylated (*right* panel) 5nt-gapped dsDNA (33-mer) substrate. (c): PAXX enhances Pol λ-dependent gap filling synthesis activity in a concentration-dependent manner in the presence of Ku.

Supplementary Figure 6



Supplementary Figure 6: C-terminal regions of PAXX and XLF are not required for stimulation of Pol λ-dependent gap filling synthesis activity. (a): Effect of GST-PAXX C-terminal deletion mutants on Pol λ-dependent gap filling synthesis activity. (b): Stimulation of Pol λ-dependent gap filling synthesis activity by PAXX is not affected by deletion of its C-terminal 59 amino acids. (c): Stimulation of Pol λ-dependent gap filling synthesis activity by XLF is not affected by deletion of its C-terminal 66 amino acids.

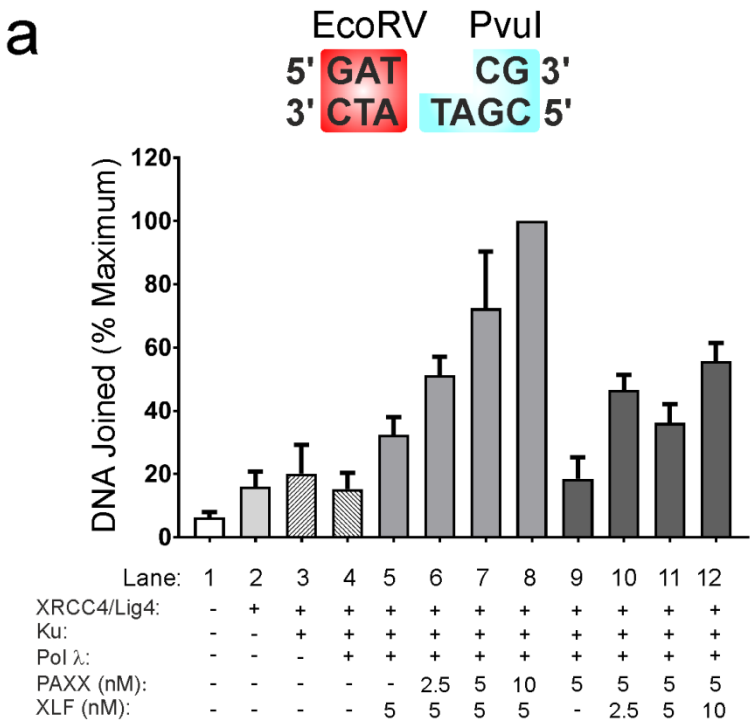
Supplementary Figure 7



Supplementary Figure 7: XRCC4 family proteins do not stimulate template-dependent or – independent activity of Pol μ. (a): Silver-stained gel showing purified FLAG-tagged human Pol λ-

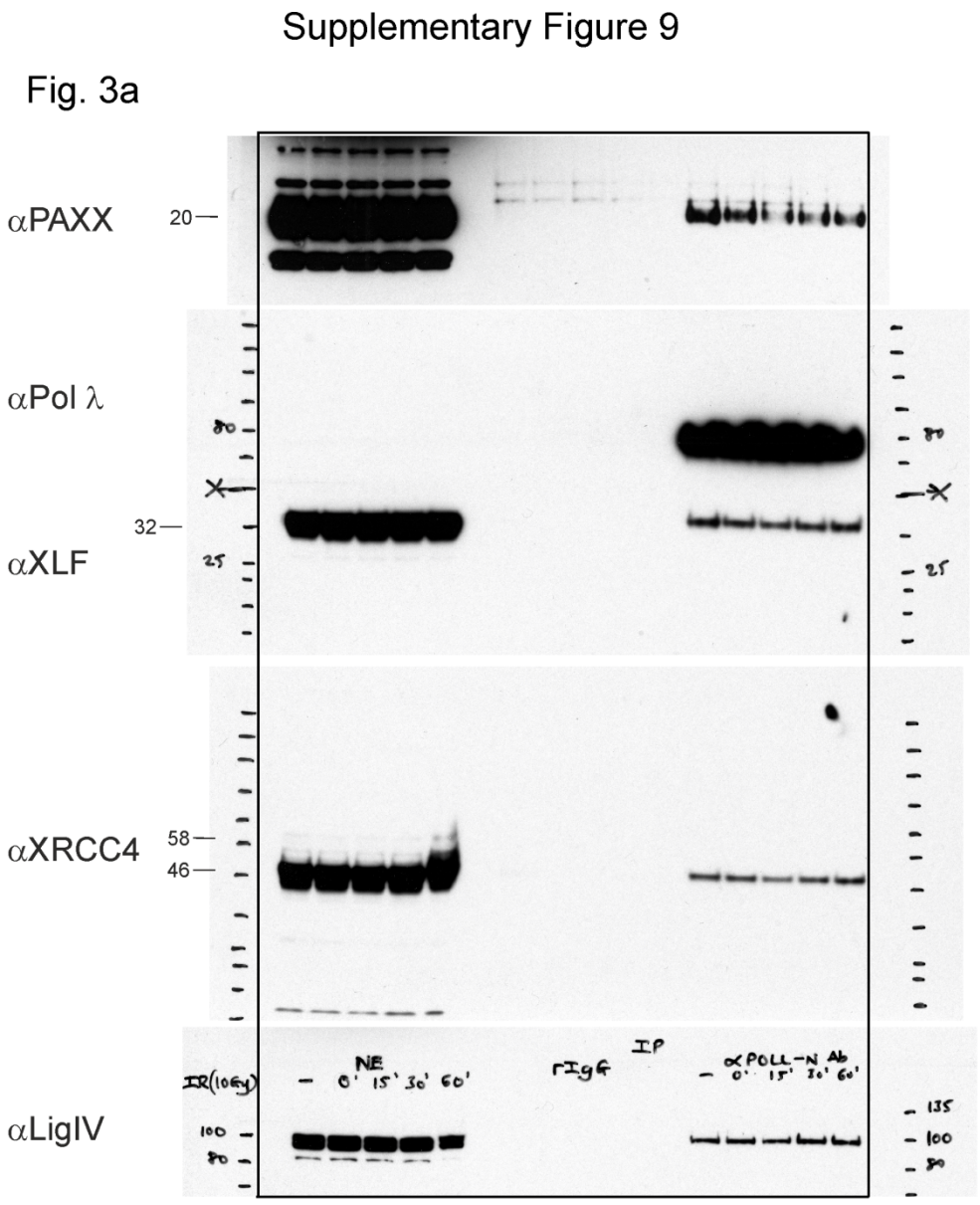
ΔBRCT, -ΔBRCT-Ser/Pro and ΔBRCT-Ser/Pro-8kDa domain mutants. (b): Gap-filling synthesis reactions were performed using an IRDye® 700-labelled 5'-phosphorylated 5nt-gapped dsDNA (33-mer) substrate with the indicated concentrations of FLAG-Pol λ-WT, -ΔBRCT, -ΔBRCT-Ser/Pro or ΔBRCT-Ser/Pro-8kDa domain mutants. (c): Silver-stained gel and FLAG immunoblot for purified human Pol μ. (d): Gap-filling synthesis reactions were performed with FLAG-tagged human Pol μ and IRDye® 700-labelled 3- or 5nt-gapped dsDNA (33-mer) substrates (*left* and *right* panels, respectively) with the indicated concentrations of FLAG-PAXX or –XLF (e): Gap-filling synthesis reactions were performed with IRDye® 700-labelled 1-, 3- or 5nt-gapped dsDNA (33-mer) substrates and FLAG-tagged human Pol μ (0-50 nM). IRDye®-800-labelled 15- and 20 nt oligonucleotides size markers are shown in the far-right lane. (f): Assays were performed using the indicated IRDye® 700-labelled NHEJ substrates containing either 1bp complementary template (*upper* panel) or no complementary template sequence (*lower* panel) with FLAG-Pol μ and the indicated concentrations of FLAG-PAXX, -XLF or –XRCC4.

Supplementary Figure 8



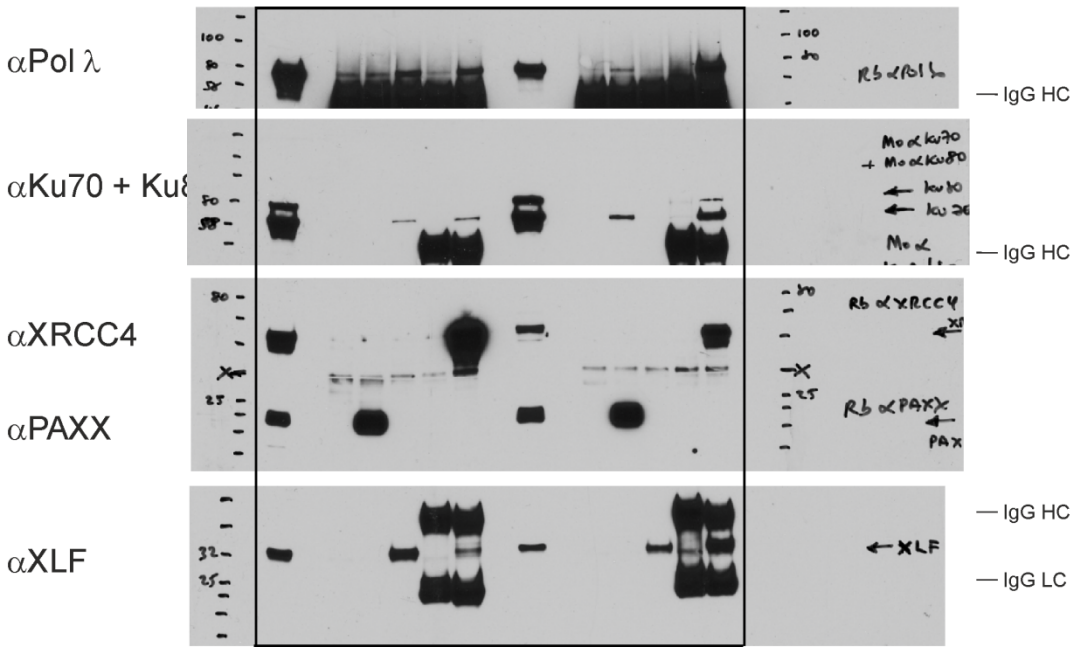
Supplementary Fig. 8: (a) PAXX and XLF synergise with Pol λ in a concentration-dependent manner to promote ligation of non-cohesive DNA ends which require Pol λ gap filling activity. Linear EcoRV-PvuI blunt-2nt 3' overhang DNA substrate was incubated with the indicated combinations of XRCC4/Lig IV, Ku70/80, PAXX, XLF and Pol λ and the joining efficiency quantified by qPCR with a TaqMan probe using a standard curve of $\log_{10}$ % joining efficiency versus C_t value generated using prejoined DNA fragments. Results shown are the mean ± SEM from 3 experiments performed in triplicate. Samples in lanes 7 and 11 contain the same reaction components, except for the order of addition of PAXX and XLF.

Supplementary Figure 9



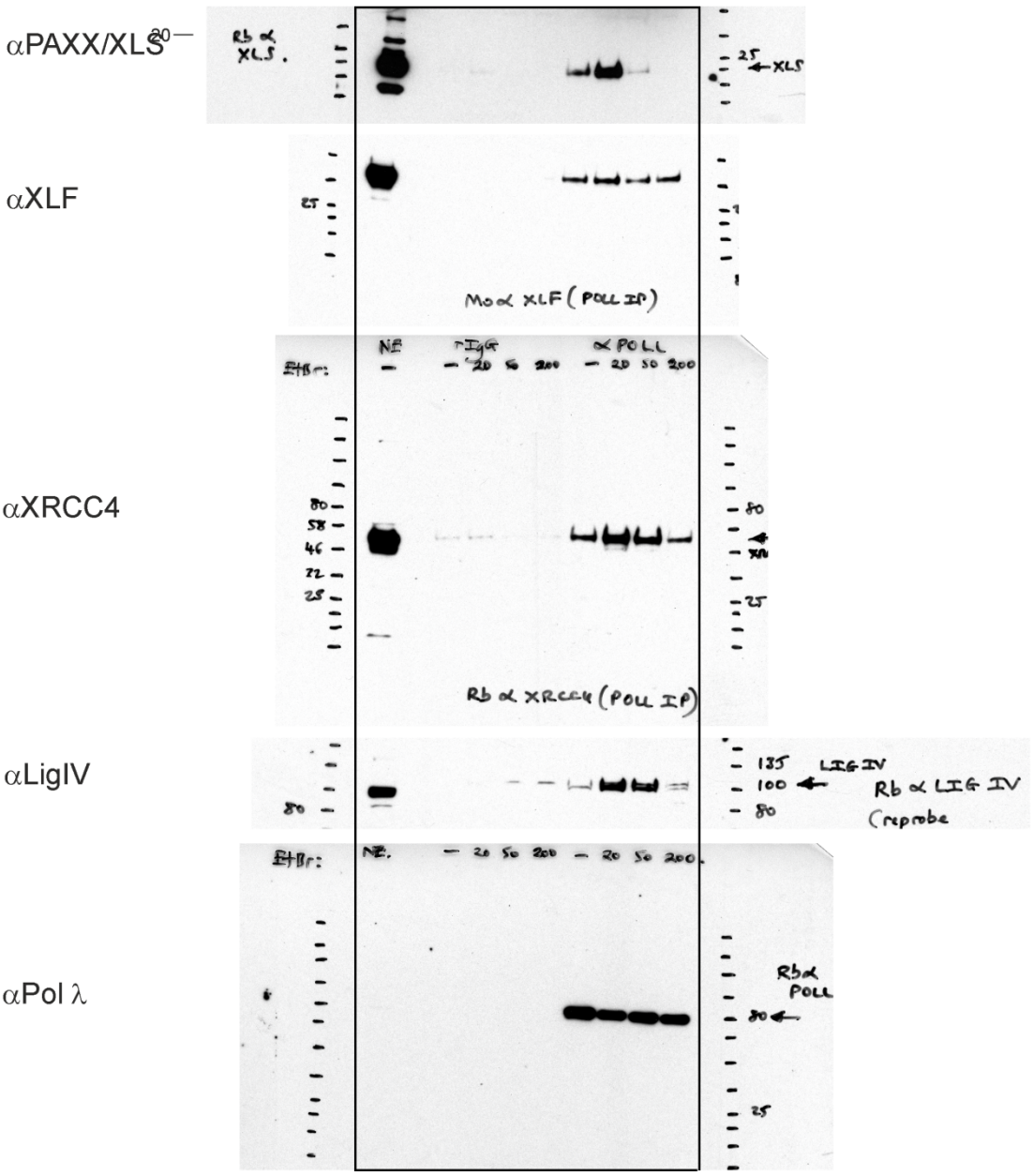
Supplementary Figure 9

Fig. 3b



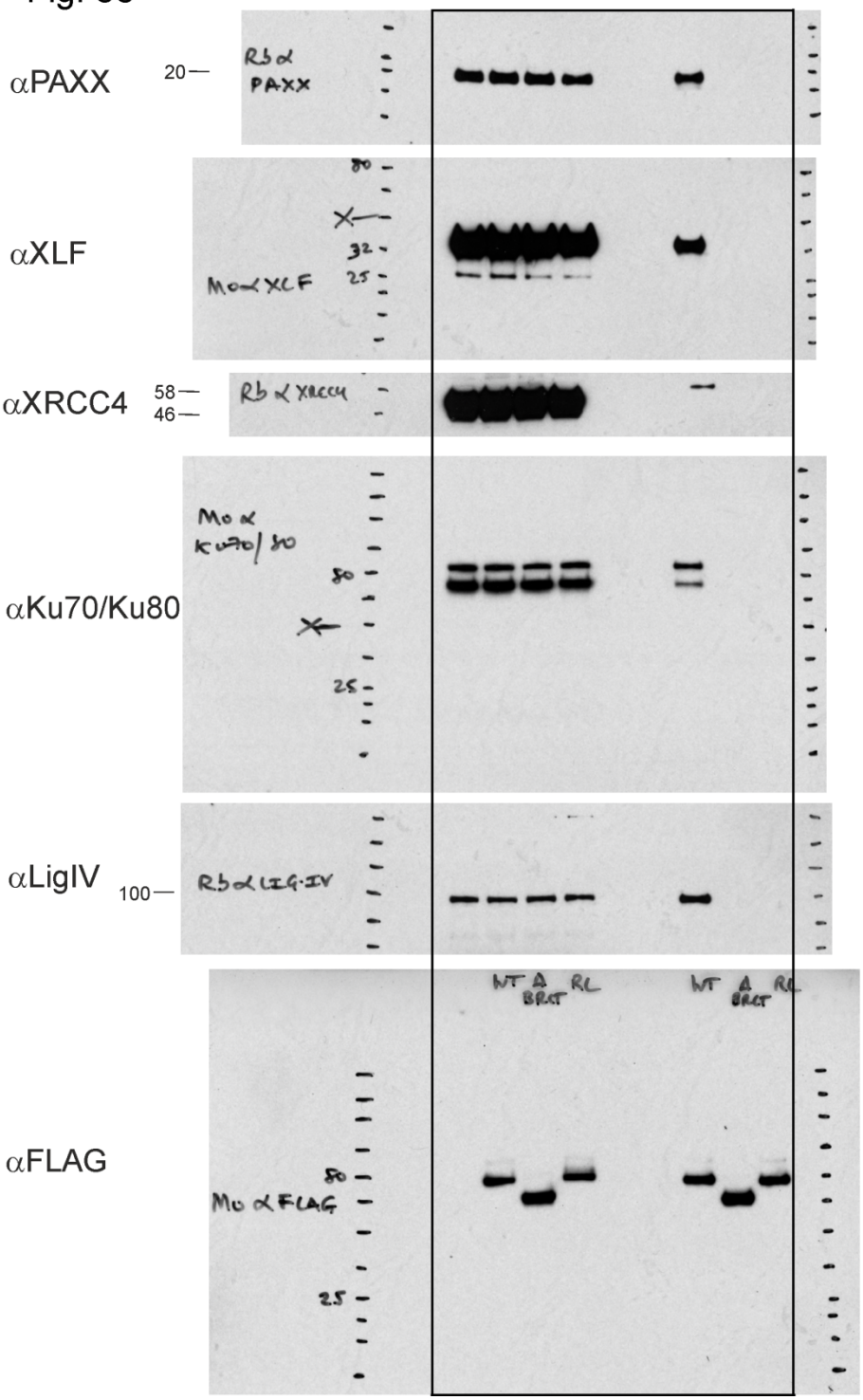
Supplementary Figure 9

Fig. 3c



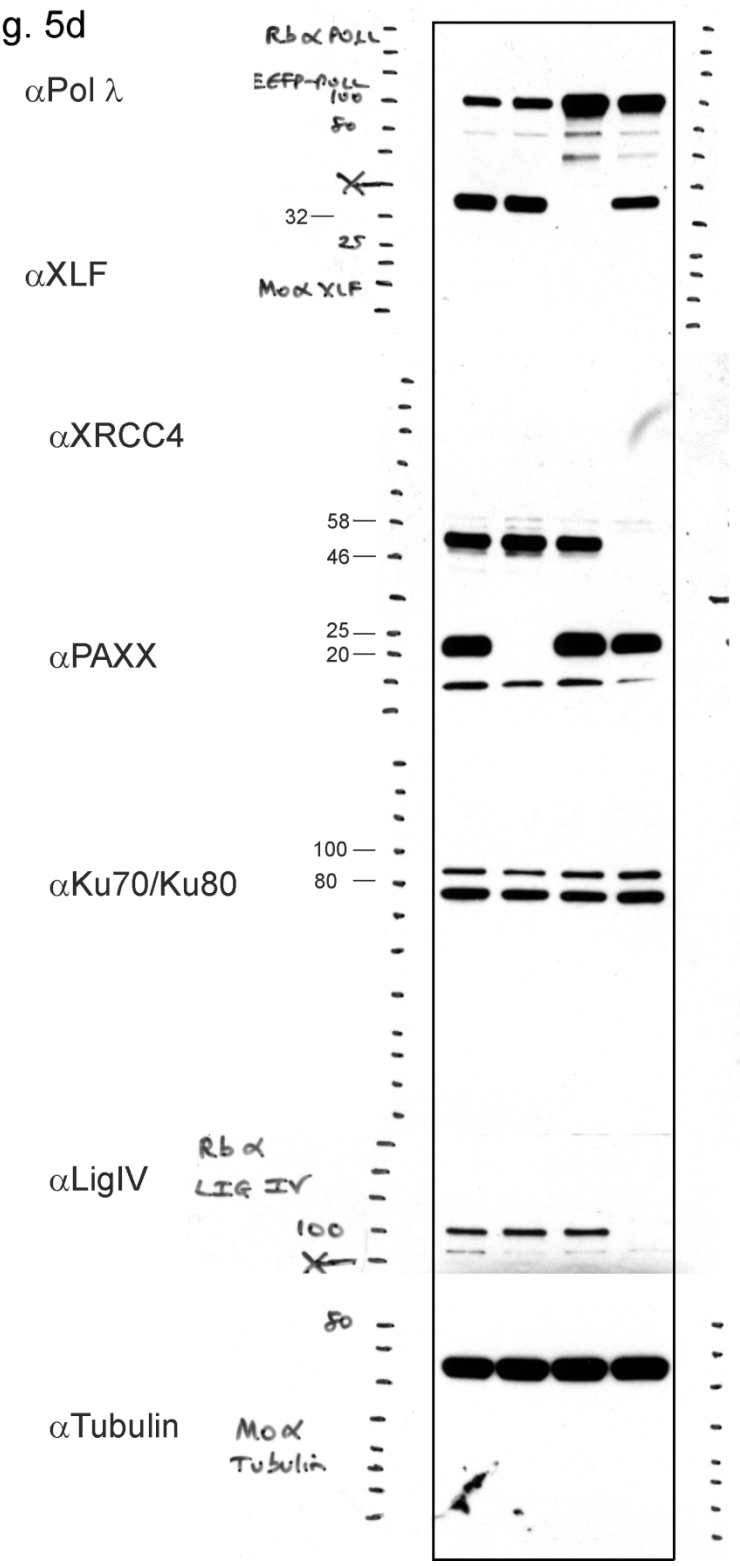
Supplementary Figure 9

Fig. 3e



Supplementary Figure 9

Fig. 5d



Supplementary Figure 9

Fig. 7c-left panel

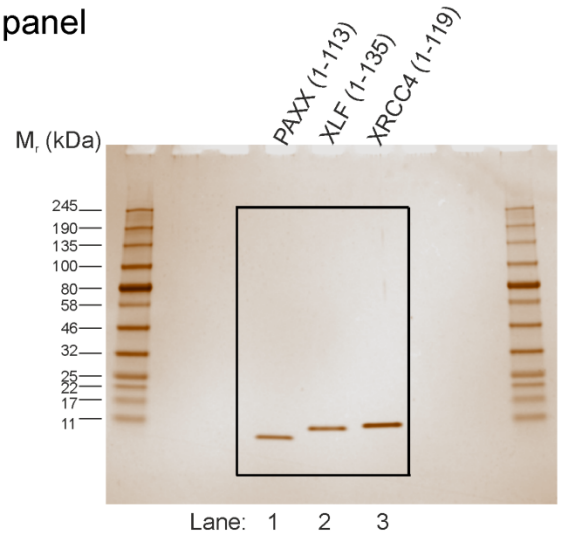
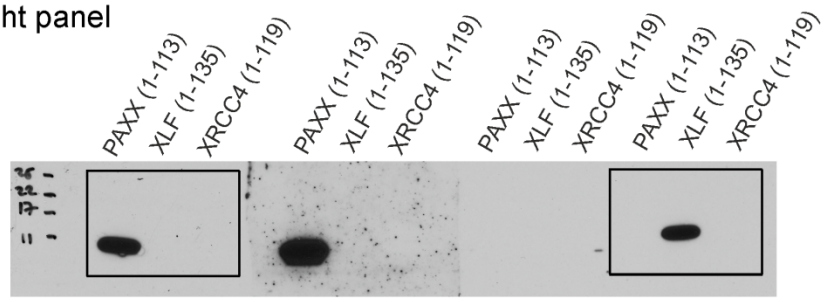
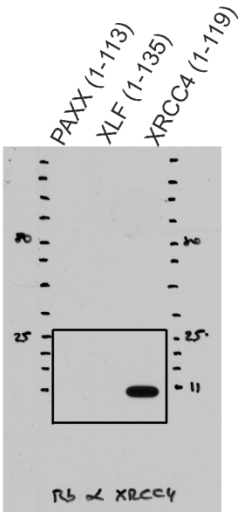


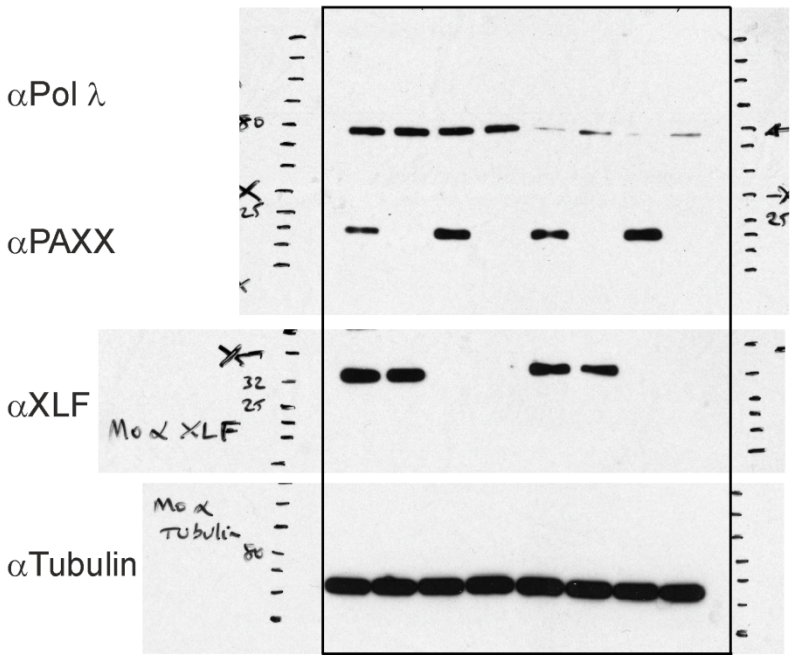
Fig. 7c-right panel





Supplementary Figure 9

Fig. 10a



Supplementary Table 1:

DNA Substrate	Primer	Sequence
5nt gap 33-mer (5'-phosphorylated)	33-mer	5'-ACTGGCCGTCGTTCTATTGTACTCACTGTGATC-3'
	15-mer	5'- IRDye® 700 -GATCACAGTGAGTAC-3'
	13-mer	5'-pAACGACGGCCAGT-3'
3nt gap 33-mer (5'-phosphorylated)	33-mer	As above
	15-mer	As above
	15-mer	5'-pAGAACGACGGCCAGT-3'
1nt gap 33-mer	33-mer	As above
(5'-phosphorylated)	15-mer	As above
	17-mer	5'-pATAGAACGACGGCCAGT-3'
NHEJ (template-dependent)	13-mer	5'- IRDye® 700 –CCCTCCCTCCCGT-3'
	11-mer	5'-GGGAGGGAGGG-3'
	15-mer	5'-GCACTCACGTCCCCA-3'
	13-mer	5'-GGGACGTGAGTGC-3'
NHEJ (template-independent)	12-mer	5'- IRDye® 700 –CCCTCCCTCCCC-3'
	11-mer	5'-GGGAGGGAGGG-3'
	14-mer	5'-GCACTCACGTCCCC-3'
	13-mer	5'-GGGACGTGAGTGC-3'

Supplementary Table 2:

Primer	FWD/REV	Sequence
EcoRI-DNA1	**FWD**	CGGAATTCACAATTAATAGACTGGATGG
EcoRI-DNA1	**REV**	CGGAATTCATCTCAGTTCGGTGTAGGTC
KpnI-DNA1	**FWD**	CGGGTACCACAATTAATAGACTGGATGG
KpnI-DNA1	**REV**	CGGGTACCATCTCAGTTCGGTGTAGGTC
EcoRV-DNA1	**FWD**	CGGATATCACAATTAATAGACTGGATGG
EcoRV-DNA1	**REV**	CGGATATCATCTCAGTTCGGTGTAGGTC
BstEII-DNA1	**FWD**	CGGGTGACCACAATTAATAGACTGGATGG
BstEII-DNA1	**REV**	CGGGTGACCATCTCAGTTCGGTGTAGGTC
PvuII-DNA1	**FWD**	CGCGATCGACAATTAATAGACTGGATGG
PvuII-DNA1	**REV**	CGCGATCGATCTCAGTTCGGTGTAGGTC

Supplementary Table 3:

Primer	FWD/REV	Sequence
EcoRI-DNA2	**FWD**	CGGAATTCGGAGCTTCCAGGGGGAAACG
EcoRI-DNA2	**REV**	CGGAATTCGGCAGCTTCCACAGCAATGG
KpnI-DNA2	**FWD**	CGGGTACCGGAGCTTCCAGGGGGAAACG
KpnI-DNA2	**REV**	CGGGTACCGGCAGCTTCCACAGCAATGG
EcoRV-DNA2	**FWD**	CGGATATCGGAGCTTCCAGGGGGAAACG
EcoRV-DNA2	**REV**	CGGATATCGGCAGCTTCCACAGCAATGG
BstEII-DNA2	**FWD**	CGGGTGACCGGAGCTTCCAGGGGGAAACG
BstEII-DNA2	**REV**	CGGGTGACCGGCAGCTTCCACAGCAATGG
PvuII-DNA2	**FWD**	CGCGATCGGGAGCTTCCAGGGGGAAACG
PvuII-DNA2	**REV**	CGCGATCGGGCAGCTTCCACAGCAATGG

Supplementary Table 4:

Gene	Exon	Target Sequence
PAXX	1	GTGCAGAGCGGCGGCGACAG
XLF/NHEJ1	7	TTGGCTATCGATTCCTTGCA
XLF/NHEJ1	3	TGCATCTGCCACACAATCAC
XRCC4	4	GTTCTCTAATGACTTCAGCT

Supplementary Table 5:

Cell Line	Clone	Indel Size (nt)	Indel Sequence
PAXX KO	11	del 37 del 35	GGATCCGCTGTCGCCGCCGCTCTGCACGCTGCCGCCG CCGCTGTCGCCGCCGCTCTGCACGCTGCCGCCGGG
XLF KO	2-22	del 2 del 163	TT TCCTTGCAGGGAAGCACTGTTTGAGGTATGAGGATCT CCTGAAATCAGAAAGATCAAGAGAAGATAGTGATAA ATGCCTTCTTCTTACCAGGAAGATATTAGCATTCACC AAGCATTCTGATAAAGGCCAGGGTTTGGTACAAATAT ATAAGCAGACAGAAGT
XRCC4 KO	1-12	in 1 del 3 (+ 4 nt change)	T CCAGCTG to AAAT
PAXX/ XLF DKO	2-1	del 225 in 1	CACAAATATCCCTCAATATTTTTGAGGCAGTGCCAATGAAGCAGC AATTCCAGGGACCACTGGCTTGGGGGCTATAGGTGCCAACTCTCA GAACACCATCCAGGAAGCTGCTCATGACTCACCGTGGACTCTTTC TCAGGTGCTGAGAGGGTTGGGGCTGAGGAGACCAGTTGTTCTGG CTGGTTTACACATTGGCTATCGATTCCTTGCAGGGAAGCACTGTT T

Supplementary Table 6: NHEJ-related Proteins identified in purified FLAG IPs for FLAG-PAXX, -XRCC4, -XLF and –DNA-PKcs in the Benzonase-treated Nuclear Fraction identified by Mass Spectrometry

	Bait Protein				
Identified Protein (fmol)	**Control**	**PAXX**	**XRCC4**	**XLF**	**DNA-PKcs**
DNA-PKcs	46.8	1591.2	203.3	1454.2	1745
Ku70	31.7	1067.2	106	813.8	984
Ku80	39.7	825.7	150.8	792.5	1026.9
Lig IV	0	56.3	67	203.9	10
XRCC4	4.8	73.1	412	203.4	55
XLF	0	74.6	64.9	777.3	122.2
PAXX	112	1026.3	118.5	162.9	156.6
PARP1	34.4	81.5	8	51	63.9
PNKP	0	0	3.5	15.7	0
WRN	0	81.6	0	47.7	129
Pol λ	0	53.2	4.1	38.9	0
Artemis	0	0	0	0	0
APTX	0	0	7.5	0	0
APLF	0	0	0	6.8	0

Results shown are the averages (fmol) from duplicate technical repeats of two separate experiments. FLAG-tagged bait NHEJ factors and Pol λ are highlighted by shaded yellow and pale yellow boxes respectively. Abbreviations used are as follows: DNA-PKcs, DNA-dependent protein kinase catalytic subunit; Lig IV, DNA ligase IV; XLF, XRCC4-like factor; PAXX, paralog of XRCC4 and XLF; PARP1, poly(ADP-ribose) polymerase 1; PNKP, bifunctional polynucleotide phosphatase/kinase; WRN, Werner helicase; Pol λ, DNA polymerase lambda; APTX, Aprataxin; APLF, Aprataxin- and PNK-like factor.

Supplementary Table 7: NHEJ-related Proteins identified in purified FLAG IPs for FLAG-PAXX, -XRCC4, -XLF and –DNA-PKcs in the Nucleoplasmic Fraction identified by Mass Spectrometry

	Bait Protein				
Identified Protein (fmol)	**Control**	**PAXX**	**XRCC4**	**XLF**	**DNA-PKcs**
DNA-PKcs	0	0	20.1	254.3	1451
Ku70	2.8	10.1	22	180.6	396
Ku80	0	10.9	19	147.9	277.6
Lig IV	0	0	139.1	188.9	158.9
XRCC4	17.3	37.2	1396	554.2	89.9
XLF	0	9.1	133.6	1354.6	94.8
PAXX	143.7	1379	79.3	110.2	130.2
PARP1	0	0	12.2	101	145.9
PNKP	0	0	65.8	39.8	11.4
WRN	0	0	0	0	63.3
Pol λ	0	0	0	5.3	0
Artemis	0	0	0	0	20.3
APTX	0	0	41.7	0	0
APLF	0	0	0	0	0

Results shown are the averages (fmol) from duplicate technical repeats of two separate experiments. FLAG-tagged bait NHEJ factors and Pol λ are highlighted by shaded yellow and pale yellow boxes respectively.

Supplementary Table 8: Proteins identified by Mass Spectrometry which selectively interact with PAXX or XLF[1, 2]

PAXX		XLF	
Nucleoplasm	**Soluble Chromatin**	**Nucleoplasm**	**Soluble Chromatin**
	KV201_HUMAN	SP16H_HUMAN	KV206_HUMAN
	DYHC2_HUMAN	SSRP1_HUMAN	H2A2B_HUMAN
	MYOME_HUMAN	LAP2A_HUMAN	APOB_HUMAN
	PARK7_HUMAN	PNKP_HUMAN	PNKP_HUMAN
	PP6R3_HUMAN	KPYM_HUMAN	
	SMEK3_HUMAN	OGFD1_HUMAN	
	NEBU_HUMAN	RFA1_HUMAN	
	CREB1_HUMAN	SEP14_HUMAN	
	RYR3_HUMAN	IFFO1_HUMAN	
	CK5P2_HUMAN	PDIA1_HUMAN	
		EMD_HUMAN	
		IFFO2_HUMAN	
		BAF_HUMAN	
		TCPB_HUMAN	
		P4HA1_HUMAN	

[1]Proteins listed include only those detected above an average of > 10 fmol in 2 experiments (duplicate technical repeats per expt).

[2]All keratin, tubulin and ribosomal species of proteins were excluded from the list.

Supplementary Table 9: NHEJ-related Proteins identified in purified FLAG-Pol λ IPs of Nucleoplasmic and Benzonase-treated Fractions identified by Mass Spectrometry

	Nucleoplasm		Benzonase-treated Soluble Chromatin	
Identified Protein (fmol)	**Control**	**Pol λ**	**Control**	**Pol λ**
Pol λ	81.2	1400	96.7	557
DNA-PKcs	7.9	381.3	0	35.0
Ku70	19.2	371.0	0	206.1
Ku80	32.8	452.6	14	217.7
XRCC4	0	25.8	0	17.5
XLF	0	25.1	0	0
PAXX	0	47.9	0	0
Lig IV	0	21.5	0	36.3
PARP1	9.8	90.9	14.1	113.4
PNKP	0	0	0	119.0
WRN	0	13.6	0	0
Artemis	0	0	0	0
APTX	0	0	0	0
APLF	0	0	0	0

Results shown are the averages (fmol) from either duplicate or triplicate technical repeats of two separate experiments. FLAG-tagged Pol λ bait protein and XRCC4 paralogs are highlighted by shaded yellow and pale yellow boxes respectively. Abbreviations used are as follows: DNA-PKcs, DNA-dependent protein kinase catalytic subunit; Lig IV, DNA ligase IV; XLF, XRCC4-like factor; PAXX, paralog of XRCC4 and XLF; PARP1, poly(ADP-ribose) polymerase 1; PNKP, bifunctional polynucleotide phosphatase/kinase; WRN, Werner helicase; Pol λ, DNA polymerase lambda; APTX, Aprataxin; APLF, Aprataxin- and PNK-like factor.