Supplementary Tables

Supplementary Table 1: k_{on} and k_{off} Values for Human/*Xenopus* XLF L115/117A

		***Xenopus* XLF$^{1-226}$**		**Human XLF$^{1-224}$**	
Concentration (nM)		**wild type**	**L117A**	**wild type**	**L115A**
2000	k_{on} ($s^{-1}M^{-1}$)	$218.9x10^3 \pm 1.9x10^3$	$229.2x10^3 \pm 0.5x10^3$	$272.1x10^3 \pm 7.1x10^3$	$226.1x10^3 \pm 12.1x10^3$
	k_{off} (s^{-1})	$69.0x10^{-3} \pm 5.8x10^{-3}$	$59.7x10^{-3} \pm 9.2x10^{-3}$	$56.7x10^{-3} \pm 0.8x10^{-3}$	$46.2x10^{-3} \pm 1.9x10^{-3}$
250	k_{on} ($s^{-1}M^{-1}$)	$312.6x10^3 \pm 7.9x10^3$	$342.8x10^3 \pm 8.7x10^3$	$294.5x10^3 \pm 11.6x10^3$	$233.8x10^3 \pm 1.7x10^3$
	k_{off} (s^{-1})	$49.5x10^{-3} \pm 0.4x10^{-3}$	$42.1x10^{-3} \pm 2.3x10^{-3}$	$36.2x10^{-3} \pm 1.0x10^{-3}$	$27.4x10^{-3} \pm 1.3x10^{-3}$
	Apparent K_d (M)	$2.4x10^{-7} \pm 0.5x10^{-7}$	$1.9x10^{-7} \pm 0.4x10^{-7}$	$1.7x10^{-7} \pm 0.3x10^{-7}$	$1.6x10^{-7} \pm 0.3x10^{-7}$

The k_{on} and k_{off} values reported in Table 1 are averaged from two experimental replicates for each condition, and the reported error represents the minimum and maximum values. The apparent K_d values for each sample are averaged from both replicates at both concentration points. The error represents the standard error of the mean.

Supplementary Table 2: Sample sizes for smFRET kinetics experiments, Fig. 2B-C.

Condition	**Number of trials**	**Total substrate molecules imaged**
ΔXLF	3	6436
ΔXLF + XLF^{WT}	3	8302
XLF^{L117D}	2	5136
XLF^{L68D}	2	6025
ΔXRCC4	3	8536
ΔXRCC4 + XRCC4^{WT}:LIG4	5	15078
ΔXRCC4 + XRCC4^{K104E}:LIG4	4	9478
ΔXRCC4 + XRCC4^{F111E}:LIG4	3	12009

Supplementary Table 3: Sample sizes for smFRET experiments, Fig. 2E and 4D

Sample	ΔXRCC4+ XRCC4^{WT}:LIG4^{K278R}	ΔXRCC4+ XRCC4^{K104E}:LIG4^{K278R}	ΔXLF + tdXLF$^{WT/WT}$	ΔXLF + tdXLF$^{WT/L68D,L117D}$
Molecules Tracked	1026	3539	365	414
High FRET Events	374	456	107	10
SR-complex Lifetimes Measured	81	50	N/A	N/A
Replicates	5	14	2	2

Supplementary Table 4: Plasmids

All NHEJ factors listed below are the *Xenopus laevis* orthologs unless otherwise noted.

Designation	Name	Source	Notes/associated figure(s) (Supplementary Figures are indicated by "S")
pTG024	pBluescript II KS(-) derivative ("pBS-RON")	5	Used as carrier DNA in all end joining experiments and as the source of the small DNA fragment in Supplementary Fig. 1D and 1E
pTG064	*parS*/pBluescript II KS(-)	1	Template for generating biotinylated DNA fragment in Supplementary Fig. 1D, 3E
pTG275	Flag-LIG4-H6/pFastBac1	1	1C; 2C
pTG276	XRCC4-StrepII/pFastBac1	1	1C; 2C
pTG296	H10-SUMO-XLF	1	1D; 2B; 3D; S1A, C-D, H-I; S2A; S3D-E; S4A
pTG322	XRCC4^{K104E}-StrepII/pFastBac1	2	1C; 2C
pTG324	pBirAcm	3	1A; S1B,E-F
pTG329	H10-SUMO-XRCC4-Avitag	2	1A; S1B,E-F
pTG330	H10-SUMO-XRCC4^{K104E}	2	S1C,D,G
pTG339	H10-SUMO-XLF^{L117D}	2	1D; 2B; S1A,C-D;
pTG343	H10-SUMO-Halo-XLF	2	3A-C
pTG349	H10-SUMO-XRCC4^{K104E}-Avitag	2	1A; S1E-F
pTG355	H10-XLF	2	S4C-D
pTG357	Flag-XLF	2	S4C-D
pTG369	H10-SUMO-XLF^{L68D}	2	1D; 2B
pTG370	H10-SUMO-XLF$^{1-226}$	2	1A; S1B,E-G
pTG371	H10-SUMO-XLF$^{1-226,L117D}$	2	1A; S1G
pTG373	XRCC4^{F111E}-StrepII/pFastBac1	2	1C; 2C
pTG387	H10-SUMO-Avitag-Halo	2	3A-C; S2C-F
pTG393	H10-SUMO-XRCC4^{F111E}	2	S1C-D,G
pTG436	H10-SUMO-XLF$^{1-226,L68D}$	2	1A; S1G
pTG437	H10-SUMO-XLF$^{1-226,L117A}$	2	5C; Supplementary Table 3
pTG441	H10-SUMO-XRCC4^{F111E}-Avitag	2	1A
pTG448	H10-XLF^{WT}:Flag-Avitag-XLF^{WT}	2	S4E-F
pTG449	H10-XLF^{WT}:Flag-Avitag-XLF^{L117D}	2	S4E-F
pTG454	H10-SUMO-tdXLF$^{WT/WT}$	2	4B-D; S4A-B
pTG458	H10-SUMO-tdXLF$^{WT/L68D,L117D}$	2	4B-D; S4A
pTG459	H6-SUMO-humanXLF$^{1-224,L115D}$	2	5C
pTG461	H6-SUMO-humanXRCC4-Avitag	2	5C; Supplementary Table 3
pSC7	H6-SUMO-humanXLF$^{1-224,L115A}$	2	5C; Supplementary Table 3
pSC8	H6-SUMO-humanXLF$^{1-224}$	2	5C; Supplementary Table 3

pBMS49	H10-SUMO-LIG4^{K278R}:XRCC4/pETDuet	4	2D-E

Sources:

1) Graham, T. G. W., Walter, J. C. & Loparo, J. J. Two-Stage Synapsis of DNA Ends during Non-homologous End Joining. *Mol. Cell* **61,** 850–8 (2016).
2) This work
3) Avidity, LLC
4) Benjamin Stinson
5) Courtney Havens