Supplemental information for:

## FAN1 activity on asymmetric repair intermediates is mediated by an atypical monomeric VRR-Nuc domain

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#### **Experimental Procedures**

#### **Crystallisation conditions**

For crystallisation, all proteins were concentrated in a buffer containing 300mM NaCl, 50mM Tris-HCl pH 7.0, 0.5mM TCEP and mixed 1:1 with well buffer in sitting drops at 18° C as below.

stNUC - 0.2M sodium citrate, 20% w/v PEG3350 stNUC-Se - 0.2M sodium citrate, 20% w/v PEG3350 saNUC - 2.2M ammonium sulphate, 0.1M Tris-HCl pH 8.0 psNUC-Se - 12.5 % w/v PEG 1000, 12.5% w/v PEG 3350, 12.5% v/v MPD, 0.02 M sodium Lglutamate, 0.02 M DL-alanine, 0.02 M glycine, 0.02 M DL-lysine, 0.2 M DL-serine, 0.1 M MES/imidazole pH 6.5

#### Gel mobility shift assay and binding buffers

Optimised binding buffers were as follows:

FAN1 orthologues - 50 mM Tris-HCI (pH 8.0), 30 mM NaCl, 1 mM DTT, 0.1 mg/ml BSA; for psNUC - 20 mM Tris-HCI (pH 7.5), 50 mM NaCl, 1 mM DTT, 0.1 mg/ml BSA stNUC - 20 mM Tris-HCI (pH 8.0), 50 mM NaCl, 1 mM DTT, 0.1 mg/ml BSA saNUC - 20 mM Tris-HCI (pH 8.0), 100 mM NaCl, 1 mM DTT, 0.1 mg/ml BSA. Binding assays were performed with 1 nM substrate and the protein concentration was varied from 62.5 pM to 256 nM in 4-fold increments for psNUC and from 78.1 nM to 10  $\mu$ M in 2-fold increments for stNUC.

#### Activity assay buffers and conditions

FAN1 orthologues (100 nM) were assayed with 10 nM substrate and 1 mM MnCl<sub>2</sub> at 30°C, psNUC (50 nM) with 5 nM substrate and 10 mM MgCl<sub>2</sub> at 25°C and stNUC and saNUC (5  $\mu$ M) with 5 nM substrate and 10 mM MgCl<sub>2</sub> at 37°C.

#### psNUC/T7 endonuclease I competition assay

Increasing amounts of the T7 endonuclease I D55A mutant were incubated with an h strand end labelled fixed HJ in binding buffer at 25°C for 5 min before adding 50 nM psNUC and assaying its activity as described in the main text.

#### Ligation of psNUC reaction products

The ligation experiment was performed using a version of Jbm5 with one arm extended by 10 bp (Jbm-lig). Oligonucleotide sequences were as follows:

### a: GCGTTACAATGGAAACTATTCGTGGCAGTTGCATCCAACG b-lig: CGTTGGATGCAACTGCCACGAATAGTGTCAGTTCCAGACGCGGGGGATCCG c-lig: CGGATCCCCGCGTCTGGAACTGACACTATTCGTGGCGAATGGTCGTAAGC d: GCTTACGACCATTCGCCACGAATAGTTTCCATTGTAACGC

This substrate was incubated with psNUC alone, T4 DNA ligase (New England Biolabs) alone or both enzymes simultaneously in 1X T4 DNA ligase buffer (New England Biolabs) supplemented with 50 mM NaCl. The reaction was stopped at various times with an excess of EDTA and the enzymes digested with proteinase K before analysing the products by denaturing PAGE.

#### **Supplementary Figures**

#### Figure Legends

**Figure S1 – Refers to Figure 1 - A.** Activity assay of pFAN1 against the Jbm5 HJ substrate labelled on the a (left) and b (right) strands demonstrating no activity **B.** Time course following pFAN1 5'-3' exonuclease activity on a 5' flap substrate labelled at the 3' end of the a (left) and b (right) strands.

**Figure S2** – **Refers to Figure 2 - A.** Table of crystallographic statistics **B.** A portion of the 1.3Å resolution stNUC electron density (2Fo-Fc) map contoured at 2.0σ.

Figure S3 – Refers to Figure 3 - A. Gel mobility shift experiments demonstrating psNUC (left) and stNUC (right) structure-selective complex formation with Holliday junction substrates. (S - specific, NS - non-specific). B. Ligation experiment, showing that HJ cleavage by psNUC generates ligatable nicked DNA duplexes. Junction Jbm-lig was 5'-<sup>32</sup>Plabelled on the 40 nt long d strand (thick line). Symmetrical cleavage by psNUC followed by nick ligation produces a radiolabelled 50-mer. The labelled substrate was incubated with no enzyme, psNUC, T4 DNA ligase or both enzymes for various times (indicated) and the reaction products analysed by denaturing PAGE. The lane on the far right is a 50-mer marker. A 50 nt product appears after incubation with both enzymes. C. psNUC displayed weak cleavage activity against a range of DNA substrates. Cleavage sites are indicated as arrows on the secondary structure diagrams below **D**. Competition experiment between psNUC and a slow-cutting mutant (D55A) of T7 endonuclease I (endo I), showing that psNUC targets the branchpoint of a four-way junction. A sample corresponding to a 2:1 endo I:psNUC ratio was also run alongside a chemical sequencing ladder in order to map the cleavage sites of both enzymes. The positions of cleavage sites are marked by blue (endo I) and black (psNUC) arrows on the diagram to the right. E. stNUC cleavage activity against a range of substrates. In order to distinguish structure specific cleavage events from nonspecific nuclease activity (see panel F), this assay was performed in the presence of unlabelled calf-thymus DNA (0.1mg/ml) to reduce the background band intensity. Incubations were at 30°C for 0, 15 or 60 minutes. F. stNUC cleavage of replication fork and Jbm5 HJ substrates demonstrating non-specific cleavage activity.

**Figure S4 – Refers to Figure 5 - A.** PRALINE sequence alignment of FAN1 and bacterial/bacteriophage VRR-Nuc sequences. **B.** N-J phylogenetic tree generated using Phylip based on PRALINE alignments of FAN1, viral/bacterial VRR-Nuc domains and HJC sequences. FAN1 proteins are in blue. HJCs are in green. VRR-Nuc domains are in orange with the subset of VRR-Nuc domains containing a short helical segment between  $\beta$ 1 and  $\beta$ 2 shown in red. **C.** In silico modelling of the VRR-Nuc domain from pFAN1 from the side (left) and top (right).  $\alpha$ -helices are in red and  $\beta$ -sheets in blue. The predicted helical insertion is shown in light pink **D.** Electrostatic surface representation of the pFAN1 VRR-Nuc domain model from the top showing the active site surface (red region in the centre).



Α				
	stNUC -Se	stNUC	psNUC -Se	saNUC
Protein Data Bank ID		4QBO	4QBL	4QBN
Data Collection				
Resolution (Å)	20.0 - 2.5	15.0 - 1.3	30.0 - 2.0	20.0 - 1.85
Wavelength (Å)	0.9791	0.9763	0.9804	0.9763
Space group	C222 <sub>1</sub>	C222 <sub>1</sub>	C2	F222
a, b, c (Å)	45.0, 60.0, 81.1	44.9, 60.5, 81.1	213.1, 51.2, 127.3	86.2, 99.3, 109.9
α, β, γ (°)	90.0, 90.0, 90.0	90.0, 90.0, 90.0	90.0, 125.5, 90.0	90.0, 90.0, 90.0
Molecules per AU	1	1	6	2
No. reflexions: total	74498	194996	733871	133292
No. reflexions: unique	7118	27396	147916	20335
Completeness (%)	97.0 (98.0)	99.5 (99.3)	100.0 (100.0)	99.8 (99.4)
Redundancy	10.5 (10.7)	7.1 (6.8)	5.0 (4.8)	6.6 (6.1)
Ι/σΙ	23.2 (26.1)	19.8 (5.3)	11.8 (2.0)	28.7 (3.5)
R <sub>merge</sub>	0.066 (0.085)	0.074 (0.492)	0.111 (0.686)	0.061 (0.534)
Refinement				
Resolution (Å)		15.0 - 1.3	30.0 - 2.00	20.0 - 1.85
Reflections		27354	76117	20234
R <sub>work</sub>		17.2 (33.1)	17.5 (25.5)	16.3 (19.9)
R <sub>free</sub>		18.0 (30.7)	20.3 (31.1)	19.7 (25.1)
rms∆ bonds (Å)		0.011	0.008	0.018
rms∆ bond angles (°)		1.27	0.97	1.68
Structure/Stereochemistry				
No. atoms: non-H protein		2111	5821	1564
No. atoms: Mg		1	6	0
No. atoms: water		155	493	98
Ramachandran: most favored		98.02	98.03	100
Ramachandran: additionally allowed		1.98	1.55	0

В





### Figure S3 (cont)

D



4 2

1044 -6

112220



Α

α-helix

β-strand

Helical insertion

	10	20	30	40	. 50		70	80	00	
	- 09¥2M0	DLCTDSE	REPALEARIO		DAPERSLEAM	VAATWHEORG	RVASINSWDR	F-TSLOOAOD	LVSCLGCPVL	SOVCENLAND
	D3ZVU2 L	DLLTDSFFSS	REQALEARLO	LIH	SAPAESLRAW	VTEAWOAOQG	RAASLVSWDR	F-TSLOQAOD	LVSCLGGPVL	SGVCRRLAAD
	H2Q925 L	DLCTDSFFTS	RRPALBARLQ	LIH	DAPEESLRAW	VAATWQEQEG	RVAS <mark>LVS</mark> WDR	F-TSLQQAQD	LVSCLGGPVL	SGVCRHLAAD
	Q69ZT1 L	DLLTDSF <mark>FAS</mark>	REQALEARLQ	<mark>LIH</mark>	SAPAESLRAW	VGEAWQAQQG	RVAS <mark>LVS</mark> WDR	F-TS <mark>LQQAQD</mark>	LVSCLG <mark>GPVL</mark>	SGVCRRLAAD
	F6UK14 L	DLYTDS <mark>FYEN</mark>	RREAIECRLQ	<mark>LLQ</mark>	ECSTETLSQL	I A D V W N A H E G	KVAA <mark>LVN</mark> WER	F-SS <mark>LQQAQS</mark>	LVSCLGGAFL	SGVCKKMSRD
	M3YI53 L	DLCTDSFCAS	RRPAIEARLQ	QIH	GAPMESLRAW	VAAAWQAQEG	RVASLVSWDR	F-SSLQQAQD	LVSCLGGPIL	SGVCRRLATD
	F1PPU4 L	DLCTDSFFAS	RGPAIEARLO		SAPAESLRAW	VAAAWQAQEG	RVASIVSWDR	F-ASLQQAQD	LVSCLGGPVL	SGVCRRLAVD
-	FIM288	DLUTDSFFES	PEDATEARLO		OAPPETLROL	TARVWUAUEG	KAAAVVSWDR	F-SSLQQAQD	LITCRCCCFL	SCUCRELAND
Ż	G3WVX9 L	DLYTDNFYEN	RRDAIESRLO	MIR	EASPETLKVW	IANVWNAOOG	KVAS GVNWDR	F-SSLOOAOD	LVSCFGGPFL	SGVCRLLAID
₹	G3WVY1 L	DLYTDNF <mark>YEN</mark>	RRDAIESRLQ	<mark>MIR</mark>	EASPETLKVW	IANVWNAQQG	KVAS <mark>GVN</mark> WDR	F-SSLQQAQD	LVSCFGGPFL	SGVCRLLAID
Ē	H3D860 L	<b>DLHTDCF<mark>YEN</mark></b>	RREAIDSRVQ	<mark>F L N</mark>	EAPVETLCSM	LEDVWTSQEG	KMCS <mark>LIS</mark> WER	F-SS <mark>LQQAQS</mark>	LVTCWGGAFL	GGVIARMSKD
_	D2HNY3	- LCTD <mark>SFFAS</mark>	RGPAIEARLQ	<mark>RIH</mark>	SAPAESLRAW	VAAAWQAQ	RVAS <mark>IVSW</mark> DR	F - AS <mark>LQQAQD</mark>	LVSCLGGPVL	SGVCRRLAAD
	H0Z586 L	DLYTDS <mark>FYEN</mark>	RRAVIEARLQ	QLH	TASSETLAEL	VADVWTAQEG	KAAA <mark>LVN</mark> WGR	F-IS <mark>LQQVQS</mark>	LVSCLGGRFL	SGIFQRLSRD
	M3W465 L	DLCTDSFFTS	RGPAIEARLQ	LIH	SAPAESLRAW	VAAAWQAQEG	RVASIVSWDR	F-ASLOQAOD	LVSCLGGPVL	SGVCRRLAVD
	F6QTP0 L	DLUTDSFFES	REQAIEARLO		GAPPESLEAW	TARAWGAGEG	RVASIVSWDR	F-ASLQQAQD	LVSCLGGRVL	SCURIDMAND
	21DWR4	DLS-NTLYSK	DEPEPEDPEV		FAROPLIERN	TRETWOLKHN	RTNR RCSWKO	FPMGAEDCVS	FROCTPRPAL	TLTLPRLAPN
	A8WZU5 CPS	DLS-NTLYLK	RKEKFEERFE	WLK	DADOETIEDN	IRRIWGMKON	ETNRECSWKH	FOSGIEDCVS	IFOCIPRPAM	LSIFRRLAEN
	- Q912N0 FHPFHSAPA	DLHSADFROR	RAALFEACLG	RLE	DGSYRDA	IRCRYRDE	LQSP <mark>FVY</mark> WEL	LGEELLEQ	ALDCLPAAHL	RAWFERLLED
	- E4RGB4 MNSP -	AIRPFRAK	PARAK	P	VDR <mark>EGQEQAA</mark>	LMQELQLRYP	Q A <mark>YKLIY</mark>	HVPNGGHRVK		<mark>a va ak lkg</mark> qg
	F8G187 MNAP -	ALR PFKAK	PARAK	P	VDR <mark>EGQEQAA</mark>	LMQELQLRYP	QAY <mark>KLIY</mark>	H A P N G G Y R V <mark>K</mark>		<mark>a va a k l k g</mark> Q G
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	D9R5D6		MSGGF	R	KRS <mark>EATEQEH</mark>	V V S W C S H R E G	MYP-DLKWIH	H C P N G G S R <mark>Q K</mark>		K E A A R L K A Q G
	psNUC		MKGRL	K	GLTEDEVQTV	VMNWSKROKF	KGRPLFDYIH	HSPNGGKRAA	KIGSSGKRYS	PEAAKFKRMG
_	BSIRJ7		MT	K	ARPEOVIQTE MESPHPIOTE	TLLALSPH-		PSNAGKTK		TDDGPPTMLE
a	ESTII45 MSAA -	PYRGAPTRTC	PAPAADTDLL	GDETOTPETN	SEPEAAALVE	VLKALRAH	PAVAW	AERMNTGAAK		V-GNRFIRFG
÷	D9RH97				MK-EST	LEKYLVKEIT	KL-N		G <mark>L</mark>	CLKWVAP
<	saNUC			MA-	TK - <mark>E</mark> GR	VQKYAKERFE	AL-G		G <mark>L</mark>	VRKLSYE
_	A7GFV9				ME- <mark>E</mark> SR	IEKRLKKEIE	K L - G		G <mark>K</mark>	ALKFVSP
g	G2IA12				ML- <mark>EKT</mark>	IENYFKIKIE	SN-G		G <mark>L</mark>	CIKLN
Ľ.	D0LED5			MST	RSSAPR-ESA	TERHLVORCA	EI-G		VL	CLKFTSP
te	B8DAH1				MK-ESY	VERVIPEOUN	R - G		GC	CWKET G
ğ	stNUC				MRTEKD	TENVLEKETE	<b>AK</b> - <b>G</b>		G	CLEETSP
ŝ	A8MGB3				MK-EKA	IQNKIVOYLK	QQPN		Tw	FFKTHGG
ш	I4D3D1				M-T <mark>EAE</mark>	FQKQVQEFLR	RQGV		<mark>w</mark>	YVKYWGG
	C0DAK7				MA <mark>EEKL</mark>	FEGQIKKYFH	SVGIYPAGFP	SDRM <mark>KVE</mark>	<mark>M</mark> VG <mark>W</mark>	YTKIWGG
	B2I822			MM-	TI <mark>PR</mark> - <mark>ERT</mark>	IERYLVAQVR	AK-G		G <mark>E</mark>	IRKVKWE
	B1JRR4			M-	SY <mark>IR</mark> - EDS	IEAHLVKEVK	<b>KA</b> -G		GI	AYKFISP
	F0L1A1			M-	AYNR-EDS	TECHLVKEVK	KV-G		GI	AYKFISP
	G0B258			M-	AVTR-ESS	VERHLGAKVE	A . G		G <b>T</b>	AVEFTS
	A9C2A1			M-	NMPR - ESA	IERADRKNHK	AA-G		RL	LLKFVSP
	E6UPE5				MS - ERS	IVTKVLRYLK	TV-P		G <mark>C</mark>	FCWKEHGGMY
	— G4Q4F7					MTETRKKG				- G <mark>LAVKFV</mark> SP
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FAN1		0	0	0	0	50	),	VAV GAKSQSL VAV GAKSQSL VAV GAKSQSL VAV GAKSQSL VSI GAKSRSL VAV GAKSRL VAV GAKSRL	)	
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FAN1		0	0	0	0	50	),	VAV GAKSQSL VAV GAKSQSL VAV GAKSQSL VSI GAKSRSL VSI GAKSRSL VAI GAKSRSL VI VAI GAKSRSL VI VI V	)	,
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terial / viral FAN1		0	0	0	0	50	)	VAVGAKSOSL VAVGAKSOSL VAVGAKSOSL VSIGAKSOSL VSIGAKSOSL VSIGAKSOSL VAVGAKSKSL	S	,
acterial / viral FAN1		0	0	0	0	50	),	VAV GAKSOSL VAV GAKSOSL VAV GAKSOSL VAV GAKSOSL VAV GAKSOSL VAV GAKSOSL VAV GAKSKSL VAV GA		
3acterial / viral FAN1		0	0	0	0	50 INLALLOLLO INLALLOLLO INLALLOLLO INLELO INLELOLLO INLELOLLO INLELOLLO INLELOLLO INLELOLLO INLE INLELO INLELO INLE INLELO INLE INLELO INLE INLELO INLELO INLE	),	Y AV GAKSOSL VAV GAKSOSL VAV GAKSOSL VAV GAKSOSL VSI GAKSNEL VAV GAKSKSL VAV G		,
Bacterial / viral FAN1		0	0	0	0		),	VAVGAKSOSL VAVGAKSOSL VAVGAKSOSL VAVGAKSQSL VSIGAKSKSL VAVGAKSKSL VAVGAKSKSL VAVGAKSKSL VAVGAKSKSL VAVGAKSKSL VAVGAKSKSL VAVGAKSKSL VAVGAKSKSL VAVGAKSKSL VAVGAKSKSL VAVGAKSKSL VAVGAKSKSL VAVGAKSKSL VAVGAKSKSL VAVGAKSKSL VAVGAKSKL VAVGAKSKSL VAVGAKSKL		
Bacterial / viral FAN1		0	0	0	0	50	),	VAV GAKSOSL VAV GAKSOSL VAV GAKSOSL VAV GAKSOSL VAV GAKSOSL VAV GAKSOSL VAV GAKSKSL VAV GA		
Bacterial / viral FAN1		0	0	0	0	50	),	A AN SOLUTION A AN A AN AN A AN SOLUTION A AN A ANA SOLUTION A AN A ANA SOLUTION A AN A ANA SOLUTION A ANA ANA ANA ANA A ANA SOLUTION A ANA ANA ANA ANA A ANA ANA ANA ANA A ANA AN		
Bacterial / viral FAN1		0	0	0	0		),	VAV GAKSOSL VAV GAKSOSL VAV GAKSOSL VAV GAKSOSL VAT GAKSOSL VAT GAKSOSL VAT GAKSOSL VAT GAKSKSL VAV GA	)	
Bacterial / viral FAN1		0	0	0	0	50	),	VAV GAKSOSL VAV GAKSOSL VAV GAKSOSL VAV GAKSOSL VAV GAKSOSL VAV GAKSKSL VAV GA		
Bacterial / viral FAN1			0	0	0	50 INLARLOLU INLARLOLU INLELO INLELO INLELO INLELO INLE INLELO INLE I	),	VAV GAKSOSL VAV GAKSOSL VAV GAKSOSL VAV GAKSKGL VAV GAKSKGL VAV GAKSKGL VAV GAKSKSL VAV GA		
Bacterial / viral FAN1		0	0	0	0		) A EVEVCE - V A DVEVCE - V A EVEVCE - V I RA EVE - V E E E E E E E E E E E E E E E E E E E	VAVGAKSOSL VAVGAKSOSL VAVGAKSOSL VAGAKSQSL VSIGAKSKSL VAGAKSKSL VAGAKSKSL VAVGAKSKSL VAVGAKSKSL VAVGAKSKSL VAVGAKSKSL VAVGAKSKSL VAVGAKSKSL VAVGAKSKSL VAVGAKSKSL VAVGAKSKSL VAVGAKSKSL VAVGAKSKSL VAVGAKSKSL VAVGAKSKSL VAVGAKSKSL VAVGAKSKSL VAVGAKSKL VAVGAKS	9	
Bacterial / viral FAN1		0	0	0	0	50	),	VAV GAKSOSL VAV GAKSOSL VAV GAKSOSL VAV GAKSOSL VAV GAKSOSL VAV GAKSOSL VAV GAKSKSL VAV GA		

# Figure S4 (cont)

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