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SUPPLEMENTAL INVENTORY

SUPPLEMENTAL FIGURE LEGENDS

Figure S1. FEN1 Complexes with Substrate and Product DNA, related to Figure 1.

(A) The primary binding interface between FEN1 and DNA is to the template strand. Stereoview of FEN1 (surface representation) in complex with product DNA (cartoon). The template strand is colored brown, the 5′-flap strand is yellow, and the 3′-flap strand is magenta. The FEN1 surface is colored based on proximity (5 Å) to each strand. Surface not within 5 Å of a DNA strand is colored white.

(B) Cartoon representation of D181A mutant FEN1 in complex with substrate. The overall structure resembles the product complex, indicating that base unpairing is not required for hydrophobic wedge and helical cap ordering. Coloring is as in Figure 1.

(C) Cartoon representation of WT FEN1:Sm^{3+} in complex with substrate DNA. Like D181A:substrate, the overall structure resembles the product complex. Coloring is as in Figure 1.

(D) Cartoon representation of WT FEN1:Sm^{3+} in complex with product DNA. Coloring is as in Figure 1.

Figure S2. Close-up Views of the FEN1:Sm^{3+}:Product Complex Active Site and Tertiary Interactions in the Helical Cap and Gateway, related to Figure 3.

(A) Arg47 links 3′-flap binding with cap and gateway ordering. Surface of the protein shows the extensive interface between the hydrophobic wedge and the basepair next to the 3′-flap. Ribbon and stick representation of residues and DNA around Arg47 show how the main chain of Arg47 stacks against the base pair near the 3′-flap while the side chain acts to C-cap the α2 helix and links to α5 through van der Waals interaction with Lys128.

(B) Stick and cartoon representation of the FEN1 WT substrate complex showing how the side chain of Arg104 in α4 C-caps the α5 helix and how the helical cap ordering is reinforced. We suggest that Arg104 may electrostatically pull the 5′-phosphate of the +1 nt to promote double unpairing.

(C) In the FEN1 WT product complex, Arg104 C-caps the α5 helix, as in the substrate.

(D) Stick representation stereoview showing coordination between seven invariantly conserved active site carboxylates and the 5′-phosphate monoester of the product DNA to two Sm^{3+} ions (cyan spheres) through direct and water-mediated interactions.

Figure S3. Kinetic Analyses of FEN1 mutants, related to Figure 5.

(A) Purity of WT and mutant FEN1 proteins used for kinetic assays. Shown is an image of a TRIS-glycine 4-15% gradient SDS-PAGE stained with Coomassie R250. Each lane was loaded with 1 μg of the indicated protein.

(B) Sequence of the 6 nt flap substrate used in kinetic assays. For second order rate constants, the fluorescein was synthesized on the 5′-terminus of the 5′-flap strand, whereas for radiolabeled experiments, the DNA was labeled on the 3′-terminus. To highlight where the incision is made, bases on either side of the incision are colored orange and green.

(C) Schematic illustrating the incision reaction. Coloring and labels are as in (B).

(D) Image of a 20% 19:1 PAGE cleavage site assay to show that regardless of FEN1 protein used, the cleavage site on 6 nt flap is one nt into the downstream duplex. The amount and identity of each FEN1 protein and time at which the reaction was quenched is indicated above. The control (C; no enzyme added and incubated 6 minutes before quench) and size markers (M) are labeled. This result is different from previous results

from the Shen laboratory that showed changes in the site of incision, particularly for Arg47 (Qiu et al., 2002). We postulate that the incision site is more precisely positioned due to the optimized conditions with an unpaired 3′-flap and K^+ in our assay conditions.

(E) The plot of normalized initial rate (v_0/[E], min^{-1}) versus substrate concentration ([6 nt flap], nM) used for second order rate constant determination of WT. Circles and error bars represent the mean normalized initial rates and standards errors of at least three independent measurements, respectively.

(F) As in (E), the plot of normalized initial rate versus substrate concentration for R47A FEN1. The inset shows that the scale of the R47A data was changed to allow data visualization.

(G) Table of second order rate constants used to generate Figure 5B.

(H) Crystallographic evidence that incision occurs if the 1 nt flap DNA substrate has a 5′-phosphate. WT FEN1 was crystallized with a 1 nt double flap DNA substrate that has a 5′-phosphate. A simulated annealing omit mF_o-DF_c map of the -1 and +1 nts is shown contoured at 1.7 sigma and overlaid with a stick model of WT FEN1 with product DNA and Sm^{3+} ions. Electron density around the terminal -1 nt model indicates that the DNA has been incised and that it is unpaired and positioned in the active site with the Sm^{3+} ions.

Figure S4. XFX2 substrate sequence, related to Figure 6.

The sequence of the 30 nt bubble and 5′-flap substrates are shown with base pairing schematic. The strand that is radiolabeled on the 5′-end in the assays in shown in black type.

Movie S1. Hydrophobic Wedge and Helical Cap Becomes Ordered upon DNA

Binding, related to Figure 7.

Models of DNA-free FEN1 were created from X,Y, and Z chains in the PCNA:FEN structure (1ul1.pdb). Random coil of regions missing in the structure was added to each chain. Full-length models with proper distances and bond lengths were then created in the program BILBOMD (Pelikan et al., 2009) where only the missing regions were allowed to move. Morphing between the Y, X, and Z chains and the WT:Sm^{3+}:product DNA structure (protein only) were done in CHIMERA. The movie shows how mobile certain regions of the DNA-free structures and how they become ordered in the DNA-bound structure. The helical cap (magenta) and hydrophobic wedges (green) are flexible in the DNA-free structure and become ordered in the DNA-bound form. The β pin moves back upon DNA binding. There are additionally some small movements in the H2TH region (purple).

Table S1. Crystallographic Statistics of FEN1 Product and Substrate DNA Complexes, related to Figures 1-4.

Table S1				
Diffraction Data Statistics				
Data Set		FEN1 w/ Product DNA	FEN1 w/ Substrate DNA	FEN1 (D181A) w/ Substrate DNA
Synchrotron Beamline		SSRL 11-1	ALS 12.3.1	ALS 12.3.1
Space Group		P 3_1 2 1	P 3_1 2 1	P 2_1
Cell Dimension	a (Å)	105.44	105.22	59.52
	b (Å)	105.44	105.22	94.94
	c (Å)	105.06	104.04	104.52
	α (°)	90	90	90
	β (°)	90	90	105.06
	γ (°)	90	90	90
Wavelength (Å)		0.97945	1.11586	1.11584
Resolution range (Å)		91-2.20	91-2.32	100-2.6
Observations		122220	124938	122047
Unique Reflections		34291	29262	66819
Completeness Total/Final (%)		99.1 / 100.0	99.8 / 100.0	99.7 / 99.0
<I/σI> Total/Outer Shell		7.5 / 2.0	8.3 / 1.9	12.0 / 2.3
R-merge Total/Outer Shell (%)		0.083 / 0.557	0.083 / 0.578	0.049 / 34.4
Refinement Statistics				
Resolution Range (Å)		34-2.2	20 -2.32	100-2.6
R-work/R-free (%)		0.165 / 0.213	0.161 / 0.202	0.217 / 0.272
Number of Refined Atoms	Protein	2878	2771	5251
	DNA	732	748	1496
	Solvent	560	402	110
	Sm	4	4	
	K	1	1	2
Ramachandran (core/allowed/generous)		286 / 6 / 2	305 / 9 / 4	621 / 31 / 13
R.M.S. Deviations	Bonds (Å)	0.014	0.010	0.014
	Angles (°)	1.026	1.030	1.167
Mean B-factors (Å^2)		44	47	62
PDB ID		3Q8K	3Q8L	3Q8M

Table S2. Catalytic parameters for hFEN1-catalysed reactions*, related to Figure 5

Enzyme	k_{cat} (min^{-1})	K_M (nM)	k_{cat}/K_M (nM^{-1} min^{-1})
WT	238.3 (14.7)	95.8 (14.1)	2.5
$\Delta 336^{\Pi}$	397.3 (12.1)	718.6 (61.5)	0.6

*Model parameter estimates from R are reported with the standard errors in parentheses. $^{\Pi}$This data set was best fit using a Hill slope of 0.9.

SUPPLEMENTAL EXPERIMENTAL PROCEDURES

Purification of FEN1 for crystallization.

WT and D181A FEN1 were cloned with a prescission protease site and $(his)_6$-tag after residue 336 into pET29b using the NdeI and XhoI restricition sites. Protein was expressed in Rosetta2 DE3 pLysS cells at 20°C in Terrific Broth after induction with 0.5 mM IPTG. Cells were resuspended in 20 mM HEPES pH 7.5, 10 mM imidizole, 250 mM KCl, Roche EDTA-free protease tablets (1 per 50 mL) and lysed by sonication. The lysate was clarified by ultracentrifugation at 40,000 g for 45 min and again after 0.4% PEI addition. FEN1 was purified by Ni^{2+}-affinity, HiTrapQ, and Heparin chromatography. The C-terminal $(his)_6$-tag was removed by overnight incubation with GST-tagged prescission protease. The protease was removed with a HiTrap GST column. Final purification of FEN1 was achieved by gel filtration on a superdex 75 column, equilibrated with 10 mM HEPES pH 7.5, 100 mM KCl, 1 mM DTT, and 0.5 mM EDTA. FEN1 was concentrated and stored at -80 °C.

Purification of FEN1 for kinetic analysis.

Site-directed mutagenesis of the pET28b-FEN1-$(His)_6$ vector (Finger et al. 2009) was accomplished using the Stratagene quick change kit. Wild-type and mutant FEN1 were expressed in BL21(DE3) and purified by Ni^{2+}-immobilized affinity and cation exchange chromatography as previously described (Singh et al. 2007). Fractions containing protein were pooled, and solid $(NH_4)_2SO_4$ was slowly added at 4°C to a final concentration of 1.5 M. Solutions were filtered (0.2 μm) and then applied to a 2.6 X 10 cm phenyl sepharose (high sub, GE Life Sciences) column using buffer F (25 mM Tris pH 7.5, 1.5 M $(NH_4)_2SO_4$, 1 mM EDTA, 0.02% NaN_3, 20 mM β-mercaptoethanol (βME)).

Proteins were eluted using a 50 column linear gradient from buffer F to G (25 mM Tris pH 7.5, 1 mM EDTA, 0.02% NaN_3, 20 mM βME). Fractions were concentrated, and then applied to a 1.6 X 60 cm Sephacryl™ S200 column equilibrated with 50 mM HEPES pH 7.5, 100 mM KCl, 0.02% NaN_3, 20 mM βME. Protein concentrations were determined using A_{280} and calculated extinction coefficients and confirmed by Bradford assay. Proteins were stored as 100 μM stocks in 50 mM HEPES pH 7.5, 100 mM KCl, 5 mM tris-(3-hydroxypropyl)phosphine (THP), 50% glycerol, 0.02% NaN_3 at –20 °C. Protein sequences were confirmed by LC/MS/MS peptide mapping.

Human FEN1Δ336 was purified as described above except that before the S200 column, the protein was exchanged into TRIS buffered saline supplemented with 1 mM EDTA, 0.02% NaN_3, and 1 mM DTT using a 5 X 5 mL tandem Hitrap desalting column. Prescision protease was then added to the protein at a final concentration of 2U/mg of FEN1Δ336, and cleavage was allowed to occur overnight at 4°C. The protein was passed though a Hitrap GST column to remove prescision protease and then diluted in Buffer I (20 mM Tris pH 8, 1 mM EDTA, 20 mM βME, 0.02% NaN_3). The protein was applied to a HiTrap Q column and eluted using a 50 column linear gradient from buffer I to J (Buffer I plus 500 mM NaCl).

FEN1 Activity Assays Using 3′-Radiolabeled Substrates. The desired DNA strand was ^{32}P-radiolabled at the 3′-terminus using [α-^{32}P] cordycepin 5′-triphosphate and recombinant Terminal deoxynucleotide transferase (rTdT) (Invitrogen Inc.) according to protocol. After addition of EDTA and heat denaturation of rTdT by incubation at 72ºC for 20 minutes, the T3F strand was added in a 1:1.1 ratio. The mixture was heated and annealed, and excess radiolabel and undesired salts were subsequently removed using

Micro-Biospin 6 columns (BioRad, SSC buffer). Reaction mixtures containing 5 nM radiolabeled substrate were incubated with the indicated concentration of protein in 50 mM HEPES pH 7.5, 0.05 mg/mL BSA, 2.5 mM THP, 10 mM $Mg(OAc)_2$ 100 mM KCl at 37ºC. Aliquots of the reaction mixture were removed at the appropriate time intervals and quenched by the addition of formamide loading buffer containing 50 mM EDTA. Product formation was visualized using a phosphoimager after 20% denaturing PAGE (19:1). Bands were quantified using TotalLab.

Purification of FEN1 for Comparison with XPG and XFX2.

WT FEN1 with a C-terminal $(his)_6$-tag (Finger et al., 2009) was induced in Rosetta2 cells with 1 mM IPTG for 3 hours at 37 °C. Cells were resuspended in 50 mM NaH_2PO_4, 5 mM Tris, 300 mM NaCl, 10 mM imidazole, 1 mM PMSF, 0.1% NP-40, 0.025 mg/mL DNaseI, EDTA-free Protease Inhibitors (1 tablet for 10 mls), pH 8.0, sonicated and clarified by centrifugation. FEN1 was purified by affinity chromatography on a HisPur Co^{2+}-Superflow resin and step concentrated on Ni-NTA Superflow resin. Protein was pure by Coomassie-stained SDS-PAGE and flash frozen in 20 mM Tris (pH 8.0), 150 mM NaCl, 5 mM β-mercaptoethanol.

Purification of XPG and XFX2

XPG and XFX2 was purified as described previously (Sarker et al., 2005). For XFX2, the purification was similar to that of the first generation XFX construct.

Crystallization of FEN1:DNA Complexes

To obtain crystals of the FEN1:DNA complex, FEN1 (25 mg/ml) was mixed with DNA at a 1:1.1 molar ratio. 3′-Phosphoramidate DNA was synthesized in the Grasby laboratory. The other oligonucleotides were purchased from IDT. The FEN1:DNA complex was

mixed 1:1 with precipitant and crystallized by vapor diffusion at 15°C. For the WT FEN1:product DNA complex, FEN1 was mixed with 4 nt double flap DNA and 16% mPEG 2k, 20% saturated KCl, 0.5 mM $Sm_2(SO_4)_3$, 10 mM $Mg(NO_3)_2$, 100 mM Hepes pH 7.0, 5% ethylene glycol. Crystals grew in 2 weeks. For the mutant D181:substrate complex, D181A was mixed with a 1 nt double flap DNA and 20% mPEG 2k, 20% KCl, 10 mM $CaCl_2$, 50 mM Bis-Tris pH 7.5, 5% ethylene glycol. Crystals grew in 3 weeks. For the WT FEN1:substrate complex, FEN1 was mixed with a 1nt double flap and 17% mPEG 2k, 20% KCl, 0.5 mM $Sm_2(SO_4)_3$, 50 mM Bis-Tris pH 7.0 and 5% Ethylene Glycol. Crystals grew in 1 week. To test the importance of the 5′-phosphate, WT FEN1 and 1 nt double flap DNA with a 5′-phosphate (no 3′-phosphoramidate) were mixed 1:1 with 14% mPEG 2K, 20% KCl, 5% ethylene glycol, 100 mM HEPES pH 7.0, 0.5 mM $Sm_2(SO_4)_3$. FEN1 was also crystallized with Mn^{2+} and 7 nt double flap DNA in 18% mPEG2K, 20% KCl, 5% ethylene glycol, 100 mM HEPES pH 7.0, and 80 mM $MnSO_4$. In the 7 nt flap DNA, the 5′-flap strand was 5′-TTTACTTTGAGGCAGAGT-3′ but is otherwise the same as the 1 and 4 nt flap DNA). In the crystallization screening, multiple metals were screened, but crystallization with Sm^{3+} resulted in the highest diffracting crystals.

X-ray Diffraction Data Collection, Phasing and Refinement

Data reduction and scaling for WT:Sm^{3+}:Product and D181:Substrate were done by ELVES, which is based on the CCP4 Program Suite (1994) (Holton and Alber, 2004). Data for WT:Sm^{3+}:Substrate was processed by HKL2000 (Otwinowski and Minor, 1997). Structures were solved by molecular replacement using PHASER and refined in Phenix with rounds of manual rebuilding in COOT (Adams et al., 2004; McCoy et al., 2007).

Late stages of the refinement utilized TLS group anisotropic B-factor refinement. Statistics are listed in Table S1. Although the D181A complex was crystallized in the presence of Ca^{2+}, no density consistent with a Ca^{2+} was observed in the active site. In the Sm^{3+} crystals, α4 formed a significant crystal contact. However, in the D181A crystals with a different crystallographic symmetry, α4 and α5 were shifted <1.2Å compared to the Sm^{3+} ones, indicating that the structures were not crystallographic artifacts.

For the WT FEN1 crystallized with 1 nt double flap DNA with a 5′-phosphate, data was collected at SSRL, beamline 11-1. Crystals diffracted to 2.8 Å. Preliminary refinement of a model without the DNA near the Sm^{3+} ions refined to R of 0.266 and Rfree of 0.323. Electron density near the Sm^{3+} ions was consistent with product DNA (Figure S3H). Since the data is essentially for WT:product DNA:Sm^{3+} which we already have a higher resolution data set, the data will not be deposited in the pdb.

The Mn^{2+} crystals diffracted to 3.5 Å with two molecules in the asymmetric unit. Data was collected at SSRL, beamline 11-1. A first rigid body refinement with two FEN1:product models with Mn^{2+} substituted at the Sm^{3+} positions (R of 0.0.3406, Rfree of 0.3265) showed clear $2F_o$-F_c density of a product complex with an unpaired -1 nt and no corresponding negative F_o-F_c density that would indicate that the model was wrong.

Structure figures and electrostatic calculations were done in PYMOL (Shrodinger). Morphing and movie generation were accomplished in CHIMERA (http://www.cgl.ucsf.edu/chimera). For alignment of DNA from Pol β (1TV9.pdb) to FEN product complex, Pol β chain P phosphates 7-11 were pairfitted to FEN chain F phosphates 2-6; Pol β chain T phosphates 7-11 to FEN chain D phosphates 14-18; Pol

β chain T phosphates 2-5 to FEN chain D phosphates 8-11; and Pol β chain D phosphates 4-5 to FEN chain E phosphates 5-6. The RMSD was 9.352 Å. For overlay of the DNA from Ligase I (1X9N) and FEN product complex, Ligase chain C phosphates 2-9 were pairfitted to FEN product chain E phosphates 3-10, and Ligase chain D phosphates 8-16 to FEN chain D phosphates 4-12. The RMS was 1.223 Å.

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