Supplemental Figures:

S1

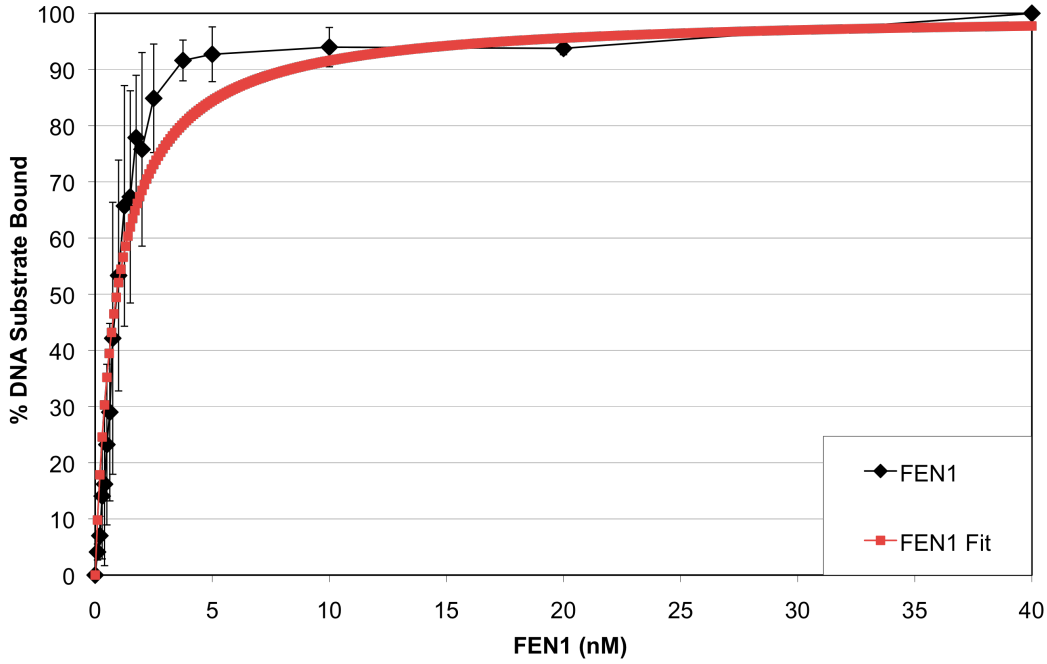

FIGURE S1: ***FEN1 binding to a 53 nt 5' flap with fit***: FEN1 binding was measured by EMSA as described in the Experimental Procedures section. Reactions were initiated by incubating increasing concentrations of FEN1 (0.10, 0.20, 0.30, 0.40, 0.50, 0.63, 0.75, 1.00, 1.25, 1.50, 1.75, 2.00, 2.50, 3.75, 5.00, 10.00, 20.00, 40.00 nM) with the 53 nt flap substrate (U1:T1:D1.53B) for 10 minutes at 37 ºC. The FEN1 binding line (represented by the diamond) shows the graphical quantitation of DNA substrate bound by FEN1 with error bars for at least three independent EMSA results. The FEN1 Fit line (represented by the red line) represents the fit to the hyperbolic equation 1 defined in the Experimental Procedures section.

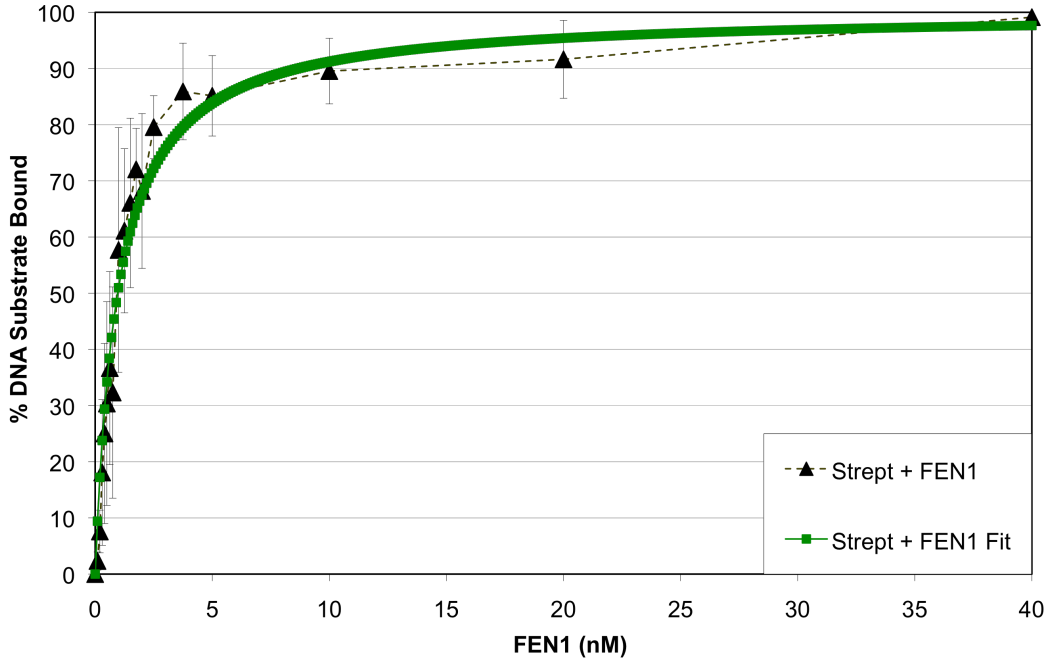

FIGURE S2: ***FEN1 binding to a streptavidin pre-blocked 53 nt 5' flap with fit***: FEN1 binding was measured by EMSA as described in the Experimental Procedures section. Reactions were initiated by incubating increasing concentrations of FEN1 (0.10, 0.20, 0.30, 0.40, 0.50, 0.63, 0.75, 1.00, 1.25, 1.50, 1.75, 2.00, 2.50, 3.75, 5.00, 10.00, 20.00, 40.00 nM) with the 53 nt flap substrate (U1:T1:D1.53B) for 10 minutes at 37 ºC. The Strept + FEN1 binding line (represented by the triangle) shows the graphical quantitation of streptavidin pre-blocked DNA substrate bound by FEN1 with error bars for at least three independent EMSA results. The Strept + FEN1 Fit line (represented by the green line) represents the fit to the hyperbolic equation 1 defined in the Experimental Procedures section.

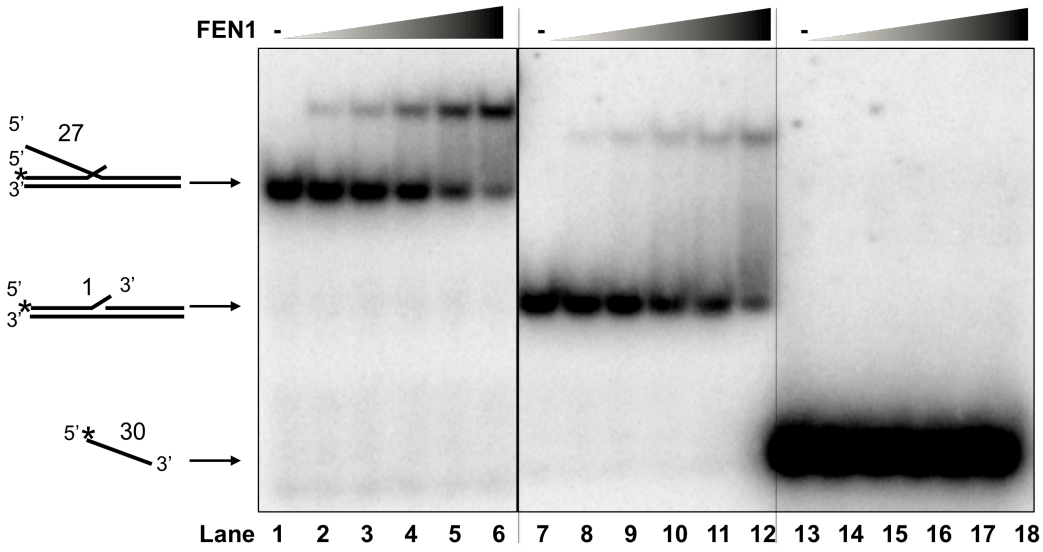

FIGURE S3: ***FEN1 preferentially binds the double flap substrate***. FEN1 binding was measured by EMSA as described in the Experimental Procedures section. Reactions were initiated by incubating increasing concentrations of FEN1 (as noted in Figure 3*A*) with the experimental substrates for 10 minutes. The gel shows FEN1 bound to a 27 nt double flap substrate (U2:T2:D2.27) in lanes 1 - 6, an upstream 1 nt 3' flap with no downstream 5' flap substrate (U2:T2:D0) in lanes 7 - 12, and a 30 nt ssDNA segment (D2.F) in lanes 13 - 18. The substrate configurations and label location are indicated to the left of the figure.

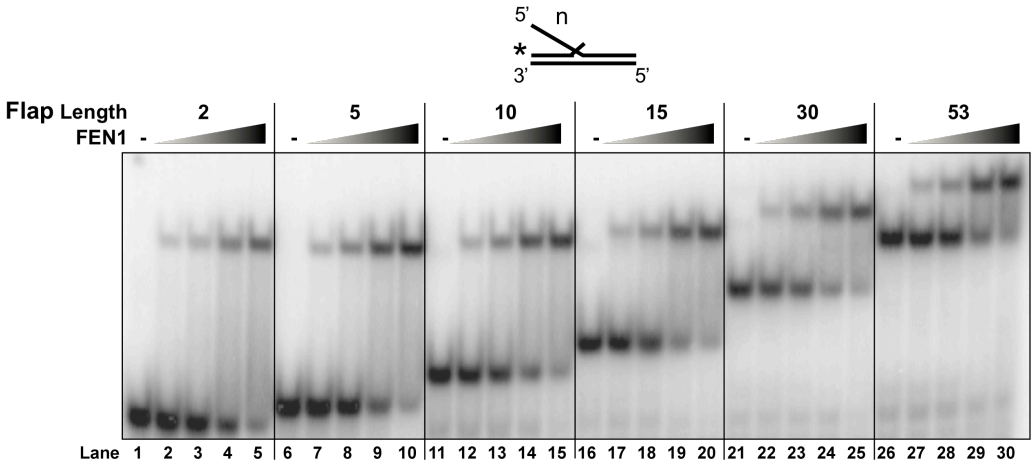

FIGURE S4: ***Long 5' flaps provide additional FEN1 binding affinity relative to a short flap***. FEN1 binding was measured by EMSA as described in the Experimental Procedures section. Reactions were initiated by incubating increasing concentrations of FEN1 (as noted in Figure 3*B*) with the experimental substrates for 10 minutes. The gel shows FEN1 bound to substrates with varying 5' downstream flap lengths of 2 nt (U1:T1:D1.2) in lanes 1 - 5, 5 nt (U1:T1:D1.5) in lanes 6 - 10, 10 nt (U1:T1:D1.10) in lanes 11 - 15, 15 nt (U1:T1:D1.15) in lanes 16 - 20, 30 nt (U1:T1:D1.30) in lanes 21 - 25, and 53 nt (U1:T1:D1.53) in lanes 26 - 30. The substrate configuration is indicated above the gel where "n" represents the length of the 5' flap listed.

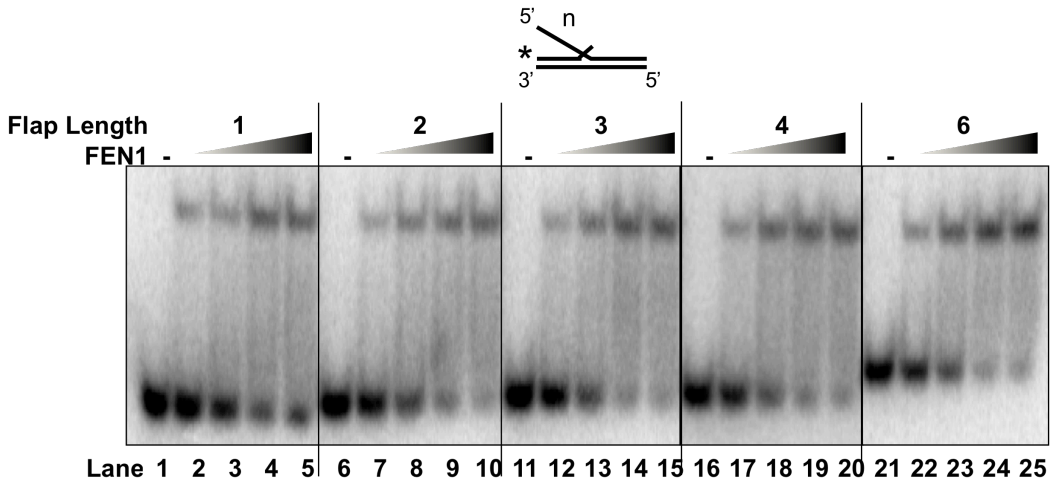

FIGURE S5: ***FEN1 binding affinity on short flaps increases with flap length.*** FEN1 binding was measured by EMSA as described in the Experimental Procedures section. Reactions were initiated by incubating increasing concentrations of FEN1 (as noted in Figure 3*C*) with the experimental substrates for 10 minutes. The gel shows FEN1 bound to substrates with varying 5' downstream flap lengths of 1 nt (U3:T3:D3.1) in lanes 1 - 5, 2 nt (U3:T3:D3.2) in lanes 6 - 10, 3 nt (U3:T3:D3.3) in lanes 11 – 15, 4 nt (U3:T3:D3.4) in lanes 16 - 20, and 6 nt (U3:T3:D3.6) in lanes 21 - 25. The substrate configuration is indicated above of the gel where "n" represents the length of the 5' flap listed.