Sequential and Multistep Substrate Interrogation Provides the Scaffold for Specificity in Human Flap Endonuclease 1

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SUMMARY

Human flap endonuclease 1 (FEN1), one of the structure-specific 5’ nuclease, is integral in replication, repair, and recombination of cellular DNA. The 5’ nuclease share significant unifying features yet cleave diverse substrates at similar positions relative to 5’ end junctions. Using single-molecule Förster resonance energy transfer, we find a multistep mechanism that verifies all substrate features before inducing the intermediary-DNA bending step that is believed to unify 5’ nuclease mechanisms. This is achieved by coordinating threading of the 5’ flap of a nick junction into the conserved capped-helical gateway, overseeing the active site, and bending by binding at the base of the junction. We propose that this sequential and multistep substrate recognition process allows different 5’ nuclease to recognize different substrates and restrict the induction of DNA bending to the last common step. Such mechanisms would also ensure the protection of DNA junctions from nonspecific bending and cleavage.

INTRODUCTION

Structure-specific 5’ nuclease are highly conserved phosphodiesterases—endo- and exonucleases—that recognize a diverse range of DNA and RNA structures and therefore play a central role in all aspects of DNA metabolism (Finger et al., 2012; Grasby et al., 2012; Tsutakawa and Tainer, 2012). Eukaryotic members of this superfamily include: flap endonuclease 1 (FEN1), DNA replication and long patch base excision repair protein; Exo1, a mismatch repair protein; Xeroderma pigmentosum complementation group G protein (XPG), a nucleotide excision repair protein; and gab endonuclease 1 (GEN1), a homologous recombination protein. Mutations in the genes of these proteins lead to cellular stress and genome instability (Yang, 2011; Zheng et al., 2011). FEN1, Exo1, XPG, and GEN1 all break phosphodiester bonds primarily at the 5’ end positions of DNA junctions. Nevertheless, the substrates they recognize vary significantly in the 3’ and 5’ regions flanking the scissile phosphate ester. It is therefore of significant interest that we understand how these highly conserved proteins recognize and attack a diverse range of DNA structures.

FEN1 recognizes, as an ideal substrate, a double-stranded (ds) DNA bearing a double-flap (DF) nick junction that consists of 5’ single-stranded (ss) DNA or RNA flaps of various lengths and a one-nucleotide 3’ DNA flap (Figure 1A). These DF substrates are equilibrated structures that result from strand displacement synthesis by DNA polymerases (Finger et al., 2012; Liu et al., 2004) during maturation of Okazaki fragments on the lagging strand (Pol δ) as well as single nucleotide gap filling in long-patch base excision repair (Pol δ). The incision site is one nucleotide into the duplex junction of the 5’ flap (Figure 1A). The 3’ flap complements the newly unpaired template base at the junction to result in a nick that can be sealed by DNA ligase I (Figure 1A). Removal of the 3’ flap dramatically reduces FEN1 endonuclease activity and alters the incision site whereas removal of the 5’ or both the 5’ and 3’ flaps triggers the FEN1 5’–3’ exonuclease activity that hydrolyzes nucleotides from the nick junction (Finger et al., 2009; Kao et al., 2002; Liu et al., 2006).

The structure of FEN1 in complex with a nicked DNA substrate bearing a one-nucleotide 3’ flap but missing the most critical substrate feature, the 5’ flap, (termed a single flap, SF-0,1 substrate) (Figure 1B) illustrates how FEN1 utilizes structural elements that are conserved in the superfamily to mediate highly sophisticated interactions with the substrate (Tsutakawa et al., 2011). Two dsDNA binding sites spaced by one DNA helical turn interact primarily with the intact complementary strand and impose a 100° bent conformation on it. The first of these is a superfamilly-conserved helix-two turn-helix (H2TH) motif that binds K+ and interacts with the downstream dsDNA. The second site contains in addition to the superfamilly-conserved β pin and the hydrophobic wedge, a helix-loop-helix (HLH) motif. Together these two motifs form the upstream dsDNA-binding site. The HLH motif is absent from superfamilly members like Exo1 that recognize substrates with an upstream ssDNA (Orans et al., 2011). The hydrophobic wedge binds at the base of the flap junction and may induce a complementary strand bending. A binding pocket formed by residues from the hydrophobic wedge and HLH motif selects for the 3’ flap. A cluster of acidic residues forms a block on the 3’ flap-binding pocket that selects against a 3’ flap with more than one unpaired nucleotide.

http://dx.doi.org/10.1016/j.celrep.2013.05.001
Figure 1. Dynamics of FEN1 Bending of DF-6,1 by smFRET
(A) A schematic diagram of FEN1 reaction on DF-6,1.
(B) Structure of FEN1 in complex with SF-0,1; Protein Data Bank (PDB) code 3Q8L (Tsutakawa et al., 2011). The conserved and unique structural features that interact with the bent DNA conformation are depicted and labeled in the same color.
(C) smFRET efficiency histograms at different FEN1 concentrations. The fitted Gaussians are illustrated with brown and blue solid lines for bent and unbent substrate populations, respectively. An isotherm showing percentage of bent substrate versus FEN1 concentration; $K_{\text{D}}-\text{bending}$ is determined using nonlinear least-squares regression fit. Error bars represent SD from two or more experiments.
(D) Representative time traces showing the fluorescence intensities of a donor (green) and acceptor (red) and their FRET efficiency (black) at different FEN1 concentrations. The dwell times for FEN1 association ($t_{\text{bending}}$) and dissociation ($t_{\text{unbending}}$) are illustrated on the upper FRET time trace.
(E) Transition density plot (TDP) of the two FRET states at [FEN1] of 1,000 nM.
(F) Histogram of $t_{\text{bending}}$.
A superfamily-conserved helical gateway overseeing the active site guides the 5′ flap into the active site. The opening of this gateway restricts access of ssDNA or ssRNA, and a cap that extends from it forms a cavity through which a 5′ flap of only ssDNA or ssRNA can thread. The cap also limits access to the active site of a DNA with a free end. The capping of the helical gateway is conserved in the superfamily members that select for 5′ termini such as Exo1 and is absent from those that bind DNA bubbles or Holliday junctions, such as XPG and GEN1 (Orans et al., 2011; Grasby et al., 2012; Tsutakawa and Tainer, 2012).

The FEN1:SF-0,1 complex suggests that DNA bending provides a mechanism for substrate specificity by allowing FEN1 to interact with the 3′ and 5′ flaps and to place the 5′ flap into the capped-helical gateway (Tsutakawa et al., 2011). However, the absence of the 5′ flap and the structuring of the capped-helical gateway make it difficult to directly link DNA bending with 5′ flap recognition. Biochemical characterization suggests an alternate model for substrate recognition whereby FEN1 recognizes the 5′ flap by its unstructured capped-helical gateway, similar to that seen in the absence of DNA (Sakurai et al., 2005), and there is a transition into a structured form when the 5′ flap threads through the unstructured region (Gloor et al., 2010; Patel et al., 2012). This unstructured-thread-structured model would explain why FEN1 can incise substrates with 5′ flaps containing dsDNA, hairpins and small bulky groups when enough ssDNA is available at the base of the 5′ flap to support the capping of the helical gateway (Barnes et al., 1996; Bornarth et al., 1999; Liu et al., 2006). The structuring of the capped-helical gateway is critical to the correct assembly of catalytically important active-site basic residues (Tsutakawa et al., 2011).

In the substrate complex, the nicked 5′ end is paired and distant from the metal ions in the active site (Tsutakawa et al., 2011). The FEN1:product complex shows that unpairing of the two nucleotides flanking the scissile phosphate places the 5′ flap in the vicinity of the metal ions for hydrolysis (Tsutakawa et al., 2011). These metal ions and residues from the capped-helical gateway are proposed to unpair the two nucleotides flanking the scissile phosphate in a sequential manner by interacting with their backbone phosphate groups. Similar DNA bending and two-nucleotide unpairing is also reported in the substrate and product complexes of Exo1 (Orans et al., 2011), thus providing a mechanism that accommodates endo- and exonuclease activities in the 5′ nuclease superfamily and explains their uniform cleavage site.

In this study, we used single-molecule Förster resonance energy transfer (smFRET) to observe the intermediacy DNA bending step and deciphered the mechanism of substrate recognition by introducing variations in the substrate and directly linking their effects to DNA bending. We found that bending of the DF substrate requires threading of the 5′ flap into the capped-helical gateway of FEN1, structuring it to select for a 5′ flap with ssDNA, verifying the full base-pairing at the flap junction and binding to the one-nucleotide 3′ flap. The DNA bending is encoded by binding at the base of the flap junction but functions in coordination with 5′ flap threading into the capped-helical gateway. The engagement of the active site metal ions completes the structure of the intermediary bent DF species and facilitates two-nucleotide unpairing, which occurs in a subsequent step after DNA bending. Our results demonstrate that DNA bending on DF substrates is a consequence of correct recognition of the substrate rather than a prerequisite for substrate recognition. Collectively, we present a highly complex timeline of binding events prior to DNA bending and reconcile the previously published biochemical and structural findings. We propose that 5′ nucleases diversify their substrate specificity through unique collective structural features that delay the induction of the superfamily-unifying DNA bending intermediary step.

RESULTS

Observing DNA Bending by FEN1

We characterized the dynamics of DNA bending by FEN1 using smFRET with a variety of fluorescently labeled substrates. Standard substrate labeling consists of a donor (Cy3) that is placed on the 5′ flap end and an acceptor (Alexa Fluor 647) positioned on the upstream primer and linked internally to a base that is not in contact with FEN1 (Figure 1C). The complementary template has a biotin at the 5′ end to allow for the immobilization of the substrate onto a polyethylene glycol-coated coverslip via biotin-neutravidin (NA) linkage. The presence of the fluorophores does not interfere with FEN1 binding to DNA as determined by their direct interaction using surface plasmon resonance (SPR) (Figures S1A and S1C).

The specificity and stoichiometry of FEN1 interactions with different forms of DNA were largely influenced by the salt concentration as characterized by SPR. At 25 mM KCl, FEN1 bound ssDNA, dsDNA and the standard DF substrate, which consisted of a six-nucleotide 5′ flap and single nucleotide 3′ flap (termed DF-6,1) with stoichiometries of 1:1, 2:1, and 2.5:1, respectively. At 100 mM KCl, FEN1 bound DF-6,1 with 1:1 stoichiometry and discriminated against ss and dsDNA (Figures S1A and S1B). In a control experiment, we observed that additional FEN1 molecule(s) could interfere directly with its interaction with DNA when the smFRET measurements were carried out at 25 mM KCl (discussed below). Consequently, we worked at 100 mM KCl and with the shortest stable substrate to restrict the stoichiometry of FEN1 to DNA to 1:1 and to minimize the effect of nonspecific interactions with DNA. We also replaced the active site Mg2+ ions of FEN1 with the catalytically incompetent Ca2+ to stabilize its interaction with DNA (Harrington and Lieber, 1994).

(G) Histograms of $k_{\text{bending}}$, $k_{\text{unbending}}$ and $k_{\text{bending}}$ are calculated from fitting their histograms with a single-exponential decay function and their inverse values are the apparent first order rate constant for DNA bending ($k_{\text{bending}}$) and the rate constant for DNA unbending ($k_{\text{unbending}}$), respectively.

(H) The DNA bending association rate constant ($k_{\text{on-bending}}$).

(I) The DNA bending dissociation rate constant ($k_{\text{off-bending}}$). $k_{\text{on-bending}}$ and $k_{\text{off-bending}}$ are calculated from the slope of the linear fit of mean $k_{\text{bending}}$ and $k_{\text{unbending}}$ Versus concentration, respectively. Error bars correspond to the SE in fitting the dwell time distributions with single-exponential decay function. See also Figure S1 for SPR binding study, Figure S2 for histograms of $k_{\text{bending}}$ and $k_{\text{unbending}}$, and Figure S3 for DF-6,1 bending at low salt concentration.
The standard DF-6,1 substrate alone exhibited a single peak with high FRET efficiency ($E \sim 0.8$) (Figure 1C). The association with FEN1-induced DNA bending, which in turn caused the donor and acceptor to be farther apart, lowering the FRET efficiency and resulting in emergence of a side peak of the bent conformer at $E \sim 0.4$ (Figure 1C). The FRET efficiency time traces of individual substrate molecules in the absence of FEN1 displayed a single FRET state that was rarely interrupted by fast photoblinking events. In the presence of FEN1, the fluorescence intensities of the donor and acceptor exhibited anticorrelated fluctuations and their FRET efficiency alternated between high and low states (Figure 1D). The frequency of fluctuations and the proportion of time the substrate spent in the bent state increased with increasing FEN1 concentration (Figure 1D). FEN1 was able to cleave the 5′-flap of all DNA molecules that displayed FRET signals upon replacing Ca$^{2+}$ with the catalytically competent Mg$^{2+}$ (data not shown).

The transitions between the two states were used to construct a transition density plot (TDP) (Figure 1E). The different transition populations in the TDP were selected, and the dwell times in the low (DNA bending [association] dwell time, $\tau_{\text{bending}}$) and high FRET states (DNA unbending [dissociation] dwell time, $\tau_{\text{unbending}}$), as shown in Figure 1D, upper trace) were analyzed to generate plots of accumulated events against dwell times for each transition (Figures 1F and 1G). These distributions showed single-exponential decays, implying the dependence of each transition on a single rate-limiting step, and an apparent first-order rate constant for DNA bending ($k_{\text{bending}} = 1/\tau_{\text{bending}}$) and the rate constant for DNA unbending ($k_{\text{unbending}} = 1/\tau_{\text{unbending}}$) were obtained at various concentrations of FEN1 (Figures 1H and 1I). The value of $k_{\text{bending}}$ increased with increasing FEN1 whereas $k_{\text{unbending}}$ remained unchanged, as expected for a 1:1 binding equilibrium where $k_{\text{bending}}$ and $k_{\text{unbending}}$ correspond to association and dissociation of FEN1, respectively. The second-order association rate constant calculated from the slope of the linear fit of the concentration dependence of $k_{\text{bending}}$ was $(2.24 \pm 0.32) \times 10^{9}$ M$^{-1}$ s$^{-1}$ and the average value of $k_{\text{unbending}}$ was $0.28 \pm 0.01$ s$^{-1}$ (Figures 1H and 1I), giving a dissociation constant, $(K_{D,\text{bending}}) = (K_{D,\text{unbending}}/K_{\text{on,\text{bending}}}) = 1,250 \pm 180$ nM. Moreover, this value of $K_{D,\text{bending}}$ agrees with that calculated from the binding isotherm based on percentage of the population in the bent form ($1,280 \pm 280$ nM; Figure 1C) and also the equilibrium dissociation constants ($K_d$) measured directly by SPR (1,020 nM; Figure S1A). However, $K_{D,\text{bending}}$ is ~15-fold higher than the Michaelis-Menten constant ($K_m$) determined from the bulk-phase endonuclease cleavage assays of the DF substrate (80 nM) (Patel et al., 2012). We attribute this difference to the sharp increase in the stability of the FEN1:bent DNA complex as the length of the 5′ flap is increased and possible reduction in the overall stability of the FEN1:bent-DNA conformation in the smFRET experiment (discussed below).

**The Length of the 5′ Flap Modulates the Kinetics of DNA Bending**

The mechanism by which FEN1 accommodates the 5′ flap is debatable, particularly because the 5′ flap is absent in the structure of the FEN1:substrate complex. Nevertheless, it has been proposed that DNA bending occurs prior to threading the 5′ flap into the capped-helical gateway (Tsutakawa et al., 2011). To investigate this proposition and to characterize the mechanism of substrate recognition in the presence of the 5′ flap, we started by studying the effect of 5′ flap length on inducing DNA bending; the FRET efficiency histograms of DF substrates with variable 5′ flap are shown in Figure S2. We observed a modest decrease in $K_{D,\text{bending}}$ (1.5-fold) upon increasing the 5′ flap length from two to six nucleotides (compare Figures 2A and 1C) and a dramatic decrease (39-fold) from 6 to 12 nucleotides (compare Figures 2C and 1C). FEN1 bound DF-12,1 with a $K_D$ that was 1.5-fold less than DF-6,1 as measured by SPR (compare Figures S1D and S1A). The transition in the $K_{D,\text{bending}}$ beyond six nucleotides was sharp—it decreased by 5-fold upon increasing the 5′ flap length from six to eight nucleotides (compare Figures 2B and 1C). Although the values of $K_{D,\text{bending}}$ for complexes with DF-2,1 and DF-6,1 were similar, analysis of their FRET efficiency time traces showed much faster bending and unbending transitions in DF-2,1; the $K_{\text{bending}}$ and $K_{\text{unbending}}$ were 15- and 8-fold higher than for DF-6,1, respectively (compare Figures 2D, 1H, and 1I). DF-12,1 and DF-8,1, on the other hand, displayed primarily fully bent conformations that appeared at all tested FEN1 concentrations throughout the standard experimental 60 s acquisition time (Figures 2E and 2F). When transitions were observed, their $\tau_{\text{bending}}$ and $\tau_{\text{unbending}}$ were significantly longer than those observed with the DF-6,1 substrate (Figures 2E and 2F).

The length of the 5′ flap in the DF substrate has been shown to have little effect on $K_m$ (Patel et al., 2012) whereas it has a strong effect on $K_{D,\text{bending}}$ (Figures 1C and 2A–2C). It is possible therefore that the overall stability of the bent conformation might be reduced in the smFRET experiment and because the bent

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**Figure 2.** The Effect of 5′ Flap on DF Bending by FEN1

(A–C) The effect of 5′ flap length. Isomers of bent substrate versus FEN1 concentration are shown for DF-2,1 (A), DF-8,1 (B), and DF-12,1 (C). Error bars represent SD from two or more experiments.

(D) $K_{\text{on,\text{bending}}}$ and $K_{D,\text{bending}}$ of DF-2,1. Rate constants were calculated as described in Figures 1H and 1I. Error bars correspond to the SE in fitting the dwell time distributions with single-exponential decay function.

(E and F) The donor/acceptor intensities and FRET efficiency time trace at 500 nM FEN1 are shown for DF-8,1 (E) and DF-12,1 (F).

(G) FRET efficiency histograms of DNA bending on 5′-flap-blocked DF. A schematic showing substrate and 5′ flap blockage (1) and FEN1 binding (2).

(H) DNA bending of trapped FEN1 on DF. A schematic showing binding of FEN1 at 2,000 nM (1) and then trapping it by blocking the 5′ flap (2). The FRET efficiency histograms of trapped FEN1 and after extensive washing with protein-free buffer are shown.

(I) A representative donor/acceptor intensities and FRET efficiency time trace of a trapped FEN1.

(J) FRET efficiency histograms of FEN1 bending of a DF containing a gapped 5′ flap hairpin.

(K) FRET efficiency histograms of FEN1 bending of a DF containing a fully duplex 5′ flap hairpin; the bent population is calculated after excluding the contribution from the substrate side peak.

See also Figure S1 for SPR binding study and Figure S2 for histograms of FRET efficiency, $k_{\text{bending}}$, and $k_{\text{unbending}}$. **
conformation in the DF substrate with a shorter 5’ flap was less stable, it was more prone to this reduction than was the DF with a longer 5’ flap. Results on DF-6,1 bending at low and high salt concentrations support this argument. At 25 mM KCl, bending significantly increased with increasing FEN1 concentration and was estimated to saturate at ~600 nM before inhibition was observed at higher concentrations (Figure S3A). SPR binding studies demonstrated that ~2.5 molecules of FEN1 could bind to the DF-6,1 substrate at 25 mM KCl; we estimated that a 1:1 binding regime was reached at nearly 500 nM FEN1 (Figure S1B).

The FEN1 interactions during the 1:1 regime were much stronger than at 100 mM KCl (compare Figures S1B and S1A). Calculating $K_{D-bending}$ of DF-6,1 using concentrations of FEN1 up to 500 nM resulted in a good fit with a value of 120 ± 10 nM (Figure S3A) that agrees with the $K_M$ value determined from bulk-phase endonuclease cleavage assays (Finger et al., 2009; Patel et al., 2012). The salt concentration had a minimal effect on $K_M$, because decreasing the KCl concentration from 100 to 50 mM reduced $K_M$ by only 50% (Finger et al., 2009; Patel et al., 2012). Our observation that $k_{bending}$ and $k_{unbending}$ both decreased upon increasing the length of the 5’ flap could explain why the $K_M$ did not change although FEN1 formed a much more stable bent conformation with a DF substrate bearing a longer 5’ flap.

5’ Flap Threading into the Capped-Helical Gateway
Is a Prerequisite for DNA Bending

We next investigated if 5’ flap threading into the capped-helical gateway is a prerequisite for DNA bending. Blocking the 5’ flap in the DF substrate impairs the incision activity of FEN1, providing direct evidence that incision requires threading of the 5’ flap into the capped-helical gateway (Patel et al., 2012). We introduced biotin on a 5’ flap hairpin structure separated from the junction by six nucleotides of ssDNA (termed DF-hairpin$\text{biotin}_{5^\prime}$), which upon immobilization on an NA-coated coverslip blocks the 5’ flap (the blocked substrate is termed DF-hairpin$\text{biotin}_{5^\prime}$/NA) as illustrated in Figure 2G. The $K_D$ for DF-hairpin$\text{biotin}_{5^\prime}$/NA,1 measured by SPR was equal to that for DF-6,1 and 2-fold smaller than for DF-hairpin,1 (compare Figures S1D and S1A), which is in agreement with previous reports (Gloor et al., 2010; Patel et al., 2012). However, FEN1 could not bend DF-hairpin$\text{biotin}_{5^\prime}$/NA even at a saturating FEN1 concentration (2,000 nM) (Figure 2G). Preincubating DF-hairpin$\text{biotin}_{5^\prime}$/1 with 2,000 nM FEN1 prior to its immobilization to block the 5’ flap end resulted in a 100% bent population (Figure 2H). This bent population was highly stable because it resisted extensive washing with protein-free buffer (Figure 2H) and its FRET efficiency time traces displayed a single low FRET state without fluctuations (Figure 2I). The requirement of 5’ flap threading in inducing DNA bending provided direct evidence that DNA bending occurs after 5’ flap threading. It also demonstrated the importance of binding to the downstream dsDNA with the 5’ flap occupying the capped helical-gateway prior to the engagement of the 3’ flap and the upstream dsDNA.

DNA Bending Requires Capping the Helical Gateway on ssDNA at the Base of the Flap Junction

The FEN1:SF-0,1 complex shows a capped-helical gateway structured to accommodate only ssDNA even in the absence of the 5’ flap (Tsutakawa et al., 2011). This suggests that binding at the base of the flap junction is sufficient to induce structuring of the capped-helical gateway. However, because 5’ flap threading is required to induce DNA bending (Figures 2G and 2H), and it can contain modestly bulky groups (Barnes et al., 1996; Bornarth et al., 1999; Liu et al., 2006), our results suggested that FEN1 must verify the 5’ flap structure before committing to DNA bending. The most likely mechanism to mediate this is the proposed transitioning of the capped-helical gateway from an unstructured to a structured form during 5’ flap threading (Patel et al., 2012). We found that FEN1 initially threaded the 5’ flap through the unstructured capped-helical gateway as evident by its ability to bend DF-hairpin,1 with similar extent to DF-6,1 (compare Figures 2J and 1C). However, because DF-hairpin,1 contains an extra 10-base pair duplex hairpin, the magnitude of its bending would be reduced should the comparison be made with DF substrate bearing an equally long ssDNA 5’ flap. Indeed, having a gapped-DNA structure on the 5’ flap has been shown to decrease the $K_M$ by ~4-28-fold and the $k_{cat}$ by 2-fold (Finger et al., 2009; Liu et al., 2006). In a control experiment, SPR showed that the $K_D$ for DF-hairpin,1 was 2-fold less than for the DF-6,1 substrate (compare Figures S1D and 1A).

We next investigated if structuring the capped-helical gateway concludes the 5’ flap verification process by extending the 5’ flap hairpin to fully duplex DNA (termed DF-hairpin$\text{duplex}_{5^\prime}$). We observed 33.5- and 18-fold reductions in DNA bending at 1,000 and 2,000 nM FEN1 in comparison with the DF-hairpin,1 at same FEN1 concentrations (compare Figures 2K and 2J). This decrease is consistent with the marked reduction of FEN1 activity on substrates bearing a fully duplex 5’ flap (Harrington and Lieber, 1994). The deficiency in bending is not due to its interaction with DNA because the $K_D$ for DF-hairpin$\text{duplex}_{5^\prime}$ was 2-fold lower than for DF-hairpin,1 (Figure S1D). This experiment also indicated that in the presence of the 5’ flap, the proper engagement of the 3’ flap and the upstream dsDNA could require the structuring of the capped-helical gateway, suggesting even more stringent requirement to verify the 5’ flap structure in the initial steps of substrate recognition.

DNA Bending Is Encoded by Binding at the Base of the Flap Junction

The dependence of DNA bending on 5’ flap threading and proper structuring of the capped-helical gateway prompted us to investigate the importance of the 3’ flap in inducing DNA bending. The presence of the 3’ flap enhanced the $K_M$ and $k_{cat}$ of FEN1 endonuclease activity on the DF substrate by ~4-10 and up to 8-fold, respectively (Finger et al., 2009; Friedrich-Heineken et al., 2003; Liu et al., 2006). The FEN1:SF-0,1 complex showed extensive interaction with the 3’ flap that comprised one-eighth of the overall FEN1:DNA binding surface (Tsutakawa et al., 2011). The removal of the 3’ flap from the DF-6,1 substrate resulted in 19- and 21-fold reduction in bending at 1,000 and 2,000 nM FEN1 in comparison with DF-6,1 (compare Figures 3A and 1C). In a control experiment, SPR showed that FEN1 bound SF-6,0 with a $K_D$ that was 1.5-fold lower than for DF-6,1 (compare Figures S1D and S1A). In addition to showing the importance of the 3’ flap in inducing DNA bending, this experiment demonstrated
that binding to the upstream dsDNA occurs after the binding to the 3' flap.

Our results underscore the importance of both 5' and 3' flaps in inducing DNA bending. However, the DNA is bent in the FEN1:SF-0,1 structure in the absence of the 5' flap (Tsutakawa et al., 2011). Therefore, we investigated the bending kinetics of SF-0,1 under dynamic conditions to characterize the contribution of the 5' flap to the interactions that induce DNA bending at the base of the flap junction. We initially used the SF-0,1 substrate labeled in a similar manner to the standard scheme and did not observe bending (Figure S4A). We anticipated that this might be due to the close proximity of Cy3 to the capped-helical gateway. Therefore, we used an acceptor (Alexa Fluor 647) attached to the nitrogenous base of the 3' flap and an internal donor (Cy3) in the downstream primer. In a control experiment, we observed a 4-fold reduction in DNA bending of internally labeled DF-6,1 substrate (termed IDF-6,1) in comparison with DF-6,1 (compare Figures 3C and 1C). SPR showed that FEN1 bound IDF with a \( K_D \) that was 2.5-fold less than DF (Figure S1D). Both \( K_M \) and \( k_{cat} \) of the FEN1 5'–3' exonuclease activity on SF-0,1 were reduced by 2-fold in comparison with the DF substrate (Finger et al., 2009). These results showed that the bending mechanism is fully encoded by binding at the base of the flap junction. However, in the presence of the 5' flap, DNA bending becomes coordinated with 5' flap threading and structuring of the capped-helical gateway.

FEN1 selects for a DF substrate containing a one-nucleotide 3' flap through the acid block overseeing the 3' flap binding pocket (Tsutakawa et al., 2011). Because the presence of the 3' flap is critical for inducing DNA bending, we investigated if the high selectivity for a one-nucleotide 3' flap is encoded for in the mechanism of DNA bending. Consequently, we increased the length of the 3' flap in the standard DF-6,1 substrate by one nucleotide to create DF-6,2. We observed 13- and 8-fold reductions at ISF-0,1) at 2,000, 3,000, and 4,000 nM was, respectively, 9-, 5-, and 3.5-fold less in comparison with IDF-6,1 (compare Figures 3B and 3C). However, bending on ISF-0,1 was underestimated because SPR showed that the internal labeling scheme reduced the binding of FEN1 to SF by 2.5-fold more than DF (Figure S1D). Both \( K_M \) and \( k_{cat} \) of the FEN1 5'–3' exonuclease activity on SF-0,1 were reduced by 2-fold in comparison with the DF substrate (Finger et al., 2009). These results showed that the bending mechanism is fully encoded by binding at the base of the flap junction. However, in the presence of the 5' flap, DNA bending becomes coordinated with 5' flap threading and structuring of the capped-helical gateway.

![Figure 3. The Effect of 3' Flap on DF Bending by FEN1](image)

(A) The effect of removing the 3' flap. A schematic of SF-6,0 is shown, together with FRET efficiency histograms.
(B) The effect of removing the 5' flap. The SF substrate with the alternative internal labeling scheme is illustrated (ISF-0,1) with FRET efficiency histograms.
(C) The effect of internal labeling of DF-6,1. A schematic of IDF-6,1 and FRET efficiency histograms are shown.
(D) The effect of increasing the 3' flap length. A schematic of DF-6,2 and FRET efficiency histograms are depicted. See also Figure S1 for SPR binding study and Figure S4 for SF-0,1 histograms.
1,000 and 2,000 nM FEN1 in comparison with DF-6,1 (compare Figures 3D and 1C) consistent with its effect on FEN1 incision activity (Friedrich-Heineken et al., 2003). In a control experiment, SPR showed that FEN1 bound DF-6,2 with a value of $K_D$ that was 1.5-fold lower than with the DF-6,1 substrate (compare Figures S1D and S1A). These results also supported our conclusion that engagement of the 3′ flap occurs before binding to the upstream dsDNA.

**Engagement of Active-Site Metal Ions and Two-Nucleotide Unpairing Occur after DNA Bending**

The FEN1:SF-0,1 structure shows the 5′ flap paired at the nick junction and distant from the active-site metal ions (Tsutakawa et al., 2011). Consequently it has been proposed that prior to catalysis, if the 5′ flap were present, it would occupy the capped helical gateway without engaging the active-site metal ions. Indeed, we confirmed this proposition by showing that in the absence of metal ions and presence of EDTA that FEN1 is able to bend the DF-6,1 substrate (Figure 4A). However, the structure of the bent DNA and the kinetics of the DNA bending were very different than in the presence of metal ions. A fully bent DNA population could not be achieved even at the saturating FEN1 concentration (2,000 nM). Instead, we observed broadening in the FRET efficiency histograms (Figure 4A). The FRET time traces exhibited very rapid fluctuations between three different FRET states, ranging from E $\sim$0.4 (bent) to $\sim$0.6 (intermediary bent) and $\sim$0.8 (unbent) (Figure 4B). The percentage of the underlying populations of the three FRET states at FEN1 concentrations of 1,000 and 2,000 nM are shown in Figure 4A. The rate-limiting transition for DNA bending at 1,000 nM FEN1 was from the unbent to the intermediary bent state and for DNA unbending from the bent to the intermediary state (Figure 4C). The $k_{bending}$ and $k_{unbending}$ of the rate-limiting transitions were 5.5- and 10-fold faster than in the presence of metal ions, respectively (compare Figures 4C, 1H, and 1I). In a control experiment, we showed that catalytically incompetent Ca$^{2+}$ is a good mimic of Mg$^{2+}$ because FEN1 bent...
the DF-6,1 substrate containing a nonhydrolyzable phosphothioylated 5’ flap in the presence of Mg^{2+} with a $K_d^{bending}$ that is similar to that in the presence of Ca^{2+} (Figure S3B).

We next investigated if FEN1 required the binding of fully base-paired flap junctions prior to two-nucleotide unpairing by introducing two mismatched bases at the flanking positions of the scissile phosphate in the DF substrate (termed DF-8 mismatch,1) in anticipation that this would bypass the two-nucleotide unpairing step. However, we detected 22- and 8-fold reductions in DNA bending at 1,000 and 2,000 nM FEN1 in comparison with DF-8,1 (compare Figures 4D and 2B). In a control experiment, we showed that FEN1 bound DF-8 mismatch,1 substrate with a $K_d$ that was similar to DF-6,1 (compare Figures S1D and S1A). Interestingly, in the absence of metal ions, FEN1 even lost its ability to bend the DF-8 mismatch,1 substrate (Figure S4B). Introducing a single mismatch into the duplex region at the 5’ flap junction has been shown to reduce the activity of FEN1 by 30-fold and alter the specificity of the DNA cleavage site (Beddows et al., 2012). The reduction in DNA bending in DF-8 mismatch,1 indicated that FEN1 prefers to bind a substrate with a fully base paired double flap junction in a first step and then mediates the two-nucleotide unpairing. It also indicates that FEN1 bends the downstream dsDNA and threads the 5’ flap into the capped-helical gateway to precisely position the hydrophobic wedge and the 3’ flap binding pocket to induce DNA bending and then engages the upstream dsDNA.

**DISCUSSION**

In this study, we characterized and built the time line of key binding events in DF substrate recognition by FEN1. We demonstrate that DNA bending requires 5’ flap threading into the capped-helical gateway (Figures 2G and 2H) and is fully encoded by binding at the base of the flap junction (Figures 3A and 3B). The 5’ flap threading and DNA bending function in a highly coordinated manner. FEN1 threads DNA through an unstructured capped-helical gateway (Figure 2J) and positions itself properly at a fully base-paired flap junction (Figure 4D). This allows the hydrophobic wedge and the 3’ flap-binding pocket to precisely induce DNA bending in a mechanism that is coupled with the structuring of the capped-helical gateway (Figure 2K).

The timing of 5’ flap threading into the capped-helical gateway with respect to binding at the base of the flap junction remains elusive. We found a strong evidence that 5’ flap threading is a prerequisite for DNA bending. This suggests that threading occurs either before or after FEN1 binds the downstream dsDNA through the K^{+}-binding H2TH motif but before the binding of the hydrophobic wedge, 3’ flap binding pocket, and upstream dsDNA. The hydrophobic wedge and 3’ flap binding pocket will engage next but before binding to the upstream dsDNA. This is supported by the importance of the structural transition of the capped-helical gateway (Figure 2K), the precise positioning of FEN1 at the flap junction (Figure 4D), and the presence of a one-nucleotide 3’ flap (Figures 3A and 3D). We currently do not have the means to directly pin down the timing mechanism between threading and binding to the downstream dsDNA. We predict that if tracking occurred first, then a longer 5’ flap would enhance the interaction of FEN1 and $k_{bending}$. If binding to the downstream dsDNA occurred first, then a shorter 5’ flap would be more readily able to thread into the capped-helical gateway than would a longer one, and would therefore have faster $k_{bending}$. The incision reaction with both short and long 5’ flaps requires their threading (Patel et al., 2012). We observed a reduction in $k_{bending}$ upon increasing the 5’ flap length (Figures 1H and 2D–2F) and therefore favor the hypothesis that threading occurs after binding to the downstream dsDNA. This model is also applicable to other 5’ nucleases. The substrates of GEN1, XPG, and Exo1 all contain a downstream dsDNA that is equivalent to that seen in the DF substrate of FEN1 and these enzymes display a high degree of conservation in the K^{+}-binding H2TH motif (Grasby et al., 2012).

Structures of the substrate and product complexes of FEN1 suggest that engagement of the active-site metal ions and two-nucleotide unpairing occur after DNA bending (Tsutakawa et al., 2011). In support of this, we observed that FEN1 has an intrinsic ability to bend DF-6,1 in the absence of metal ions (Figure 4A). However, this bending displayed much faster on and off rates and occurred via an extra partially bent intermediary state (Figures 4A–4C). We anticipate that the 5’ flap still occupies the capped-helical gateway in the partially bent DNA conformation, because no bending was observed without threading. The partially bent step suggests a structural role for the active-site metal ions in the conformational transition that induces DNA bending in addition to their role in two-nucleotide unpairing. It also suggests that the transition might undergo multiple intermediary conformational steps. The fast rates of DNA bending and unbending are consistent with the observation that trapping threaded 5’ flaps using a similar scheme to that presented in Figure 2G is reduced by 4- to 5-fold in the absence of Ca^{2+} (Patel et al., 2012). The faster $k_{bending}$ in the absence of metal ions demonstrated that 5’ flap threading is the rate-limiting step in inducing DNA bending. FEN1 prefers to bind the DF substrate with a fully base-paired flap junction and then engages the active-site metal ions. This is evident by the severe reduction in DNA bending of the DF substrate when the two nucleotides flanking the scissile phosphate were premelted, and the loss of its intrinsic bending in the absence of metal ions (Figures 4D and S4B).

In conclusion, our findings support a model that although DNA bending and two-nucleotide unpairing are common intermediary steps in 5’ nucleases, members of this superfAMILY vary in their specific requirements for the induction of DNA bending. Therefore, although there are conserved structural features, such as hydrophobic wedges, H2TH motifs, helical gateways, and the active sites, unique motifs enable members of this family to select for and against other structural features and encode for all interactions required for substrate specificity by delaying the induction of the intermediary DNA bending step. This would provide a mechanism that terminates nonspecific actions of 5’ nucleases on junctions before committing further into catalysis and bend the DNA.

**EXPERIMENTAL PROCEDURES**

**Protein Expression and Purification**

Native FEN1 gene was cloned in plasmid pDest14 (Invitrogen), expressed in *E. coli* Rosetta 2 (DE3):pLysS and purified to homogeneity using multiple
chromatography steps as described in the Extended Experimental Procedures.

**smFRET**

DNA constructs (Figure S1), their constituent oligonucleotides, annealing procedure, purification, and smFRET experiments are discussed in detail in the Extended Experimental Procedures. The smFRET measurements were made using a custom-built objective-based total internal reflection fluorescence (TIRF) microscope (Sobhy et al., 2011). Cy3 and Alexa Fluor 647 on the surface immobilized DNA constructs were excited with 532 and 640 nm lasers, respectively. The emissions of the donor and the acceptor were split inside a Dualview. Only molecules detected in both emission channels were analyzed. The identification of FRET states and inference of idealized trajectories from smFRET time traces used the vFRET package implemented in MATLAB. The calculation of the TDP and exponential fitting used MATLAB code written by Jonghyun Park and kindly provided by Professor Jong-Bong Lee from POSTECH, Korea. Data fitting and other processing used Origin Pro software.

**SUPPLEMENTAL INFORMATION**

Supplemental Information includes Extended Experimental Procedures and four figures and can be found with this article online at http://dx.doi.org/10.1016/j.celrep.2013.05.001.

**LICENSING INFORMATION**

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**ACKNOWLEDGMENTS**

This work was funded by KAUST Baseline and Faculty Initiated Collaborative research funds. We thank Mostafa Zedan for providing some FEN1 protein, Dr. Jong-Bong Lee for his support, and Dr. Nicholas E. Dixon for his critical reading of the manuscript and valuable suggestions.

Received: December 11, 2012  
Revised: April 3, 2013  
Accepted: May 2, 2013  
Published: June 6, 2013

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