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Comprehensive mapping of the C-terminus of flap endonuclease-1 reveals distinct interaction sites for five proteins that represent different DNA replication and repair pathways

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Abstract

Flap endonuclease 1 (FEN-1) is a multifunctional and structure-specific nuclease that plays a critical role in maintaining human genome stability through RNA primer removal, long-patch base excision repair, resolution of DNA secondary structures and stalled DNA replication forks, and apoptotic DNA fragmentation. How FEN-1 is involved in multiple pathways, some of which are seemingly contradictory, is of considerable interest. To date, at least twenty proteins are known to interact with FEN-1; some form distinct complexes that affect one or more FEN-1 activities presumably to direct FEN-1 to a particular DNA metabolic pathway. FEN-1 consists of a nuclease core domain and a C-terminal extension. While the core domain harbors the nuclease activity, the C-terminal extension may be important for protein-protein interactions. Here, we have truncated or mutated the C-terminus of FEN-1 to identify amino acid residues that are critical for interaction with five proteins representing roles in different DNA replication and repair pathways. We found with all five proteins that the C-terminus is important for binding and that each protein uses a subset of amino acid residues. Replacement of one or more residues with an alanine in many cases leads to the complete loss of interaction, which may consequently lead to severe biological defects in mammals.

Keywords

FEN-1; C-terminus; protein-protein interaction

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Introduction

Nucleases that cleave DNA are essential for many aspects of DNA replication and repair. This group of enzymes can be broadly categorized according to whether they cleave exo- or endo-nucleolytically and sequence- or structure-specifically. Sequence-specific nucleases (e.g., restriction endonucleases) are regulated by the presence or absence of a sequence motif. The structure-specific nucleases bind unique DNA structures (e.g., Holliday junctions, replication forks, and flaps) that form during DNA replication and cleave regardless of sequence. Structure specific nucleases also have the ability to cleave non-optimal structures as well, but with varying efficiency. One such structure-specific nuclease is flap endonuclease-1 (FEN-1), well known for its involvement in Okazaki fragment maturation and long patch base excision repair (BER).[1,2,3] In both pathways, a DNA flap structure is formed, and kinetic analyses have shown that the DNA flap structure is the optimal substrate for FEN-1 *in vitro*. In addition, proteins involved in both pathways like proliferating cell nuclear antigen (PCNA) and apurinic/apyrimidinic endonuclease-1 (APE-1) are known to increase the efficiency of FEN-1 mediated flap cleavage *in vitro*.[4,5,6,7] More recently, FEN-1 has also been implicated to play a role in apoptosis, break-induced repair of stalled replication forks, and resolution of DNA secondary structures formed in repeat sequences, despite the fact that these structures are sub-optimal substrates for FEN-1 activity *in vitro*.[8,9,10] However, proteins involved in these processes, such as Endonuclease G (EndoG), and Werner syndrome protein (WRN), are known to interact with FEN-1 and increase the efficiency of FEN-1 cleavage on these suboptimal substrates. Thus, the ability of FEN-1 to participate in various pathways with different substrates may depend on the proteins with which it associates *in vivo*.

Thus far, twenty proteins have been reported to interact with FEN-1. Each of the known interacting proteins can be categorized based on the role in which it assists FEN-1. The first category includes protein partners assisting FEN-1 in RNA primer removal during DNA replication.[3] When a portion of the RNA-DNA primer is displaced to form a 5′-flap structure during Okazaki fragment maturation, it needs to be removed prior to ligating the remaining DNA segments. Consistent with the role of FEN-1 in Okazaki fragment maturation, it interacts with PCNA, DNA polymerase δ, replication protein A (RPA) and DNA ligase I to perform these activities.[11]

A second major category of FEN-1 interacting proteins includes those involved in DNA repair. The role of FEN-1 in long patch BER is regulated and coordinated by physical interaction with BER components such as polymerase β, APE-1 and PCNA.[6,12,13] Furthermore, the WRN helicase which is known to interact with and stimulate FEN-1, has also been shown *in vitro* to participate in long patch BER.[14] Therefore, WRN/FEN-1 association may also be important for efficient 5′-flap removal in long patch BER. Another DNA repair protein with which FEN-1 interacts is the Rad9–Rad1–Hus1 checkpoint complex.[15] The Rad9-Rad1-Hus1 complex is a heterotrimeric protein recruited to DNA damage sites *in vivo*.[16] Similar to PCNA, Rad9-Rad1-Hus1 can stimulate FEN activity on flap substrates *in vitro*.[15] The role of this interaction *in vivo* remains to be shown, but because Rad9-Rad1-Hus1 accumulates at sites of DNA damage, its interaction with FEN-1 is likely important to stimulate FEN-1 activity for DNA repair.[15]

More recent findings have suggested that there are additional FEN-1 interacting protein partners. We have demonstrated that CRN-1, the *Caenorhabditis elegans* FEN-1 homologue, physically interacts with CPS-6 (*C. elegans* EndoG homologue). This interaction, which only occurs when CPS-6 is released from the mitochondria, mediates stepwise DNA degradation during apoptosis.[8] Although it was shown that CRN-1 stimulates CPS-6 activity, the effect of CPS-6 association with CRN-1 on the nuclease activity of CRN-1 has not been clearly demonstrated. In human cell lines, hFEN-1 has been shown to interact with hEndoG via immunoprecipitation, however the effect of EndoG on FEN-1 stimulation has not been

demonstrated.[17] Previous studies also show that EndoG possesses DNase as well as RNase activities, creating a nick at double strand DNA (dG)n and (dC)n tracts and single stranded $(dC)_n$ tracts.[18–21]

Taken together, evidence is available to support the concept that one possible manner in which FEN-1's multiple functions are precisely regulated is through protein-protein interaction. Genetic dissection of the role of FEN-1 in these pathways would strengthen the notion that the protein interaction partners of FEN-1 regulate its activity. However, whether these protein partners utilize the same interaction surfaces remains to be shown. In addition, precise genetic dissection of the multiple roles of FEN-1 would require information regarding a single or multiple amino acid residue mutation that affects only one of the known FEN-1 interactions, while leaving the others relatively undisturbed. Based on sequence alignment and biochemical analyses, the two major conserved motifs, the N-terminal and intermediate domains, were found to be essential for FEN-1 catalytic activity whereas the C-terminal motif was shown to be involved in substrate binding *in vitro*.[22] The C-terminus of eukaryotic FEN-1 was shown to consist of two functionally distinct regions that may together create an important regulatory domain, including substrate binding.[23] Comparison of prokaryotic and eukaryotic FEN-1 homologues showed that eukaryotic FEN-1s have an extended C-terminal motif thought to be important because it contains a bipartite nuclear localization signal.[24] Via peptide mapping and protein truncation analysis, regions of the C terminus have also been shown to be crucial for protein-protein interaction.[25–27] For example, the ^{337}QGRLDDFFK^{345} motif is necessary for the high-affinity interactions with PCNA in prokaryotes and eukaryotes.[25,26] Furthermore, amino acid residues 363–380 of the FEN-1 C-terminus were demonstrated to be important for the binding of WRN.[27] Considering the role of the C-terminus in protein-protein interaction thus far, we rationalized that screening the C-terminus by site-directed mutagenesis might yield information regarding the importance of single or multiple amino acids for the interaction with various proteins. Here, we present a compilation of mutants that abrogate one or more of the interactions with five proteins that are representative of various DNA metabolic pathways. We found that all five proteins interact with the C-terminus of FEN-1 using similar amino acids, but there are residues that when mutated, affect only one interaction out of the five proteins. The amino acids unique to one protein interaction partner may be important for one aspect of DNA metabolism and thus, allow one to genetically dissect the role of FEN-1 in a pathway in yeast or mouse model studies.

Results

Involvement of the C-terminus of FEN-1 in Multiple Protein-Protein Interactions

Based on three-dimensional structures and interaction site mapping available for PCNA and WRN, we rationalized that the FEN-1 C-terminus, which is thought to be unstructured in the absence of a protein binding partner,[28] is important for various protein-protein interactions (Figure 1A). Previous studies have shown that the extended C-terminus, which contains several arginine and lysine residues, is important for nuclease activity through involvement in substrate binding.[23] Because the extended C-terminus is absent from archaeal Fen1s,[29] contains a nuclear localization signal,[30] and is involved in interaction with protein partners, it is likely that it performs other functions *in vivo* other than substrate binding. Protein-protein interactions are often mediated by intrinsically disordered regions of proteins because it allows flexibility to form intricate binding surfaces with multiple proteins that can be regulated by post-translational modifications.[31] Well known examples of this principle are the disordered N-terminal tails of histones[32] and the disordered C-terminus of the τ subunit of *Escherichia coli* DNA polymerase III.[33] Our goal is to identify the residues important for interaction with various proteins that represent different DNA metabolic pathways. To test our hypothesis, we truncated the C-terminus at several locations, referred to as DC1, DC2, DC3, and DC4 (Figure

1B), to determine if removal of these segments affect the relative ability of PCNA, APE-1, WRN, EndoG, and Rad9-Rad1-Hus1 to pull-down each truncation (Figure 2).

To validate our methodology, we first assayed the truncation mutants with PCNA because the interaction site is well-described biochemically and structurally.[28,30] The truncation mutants remove portions of the protein that are known to be unstructured. Therefore, these truncation mutants are not expected to affect the stability of the nuclease core domain or its folding. In support of this, SDS-PAGE analysis shows bands of the expected sizes after purification and nuclease activity can be detected with the truncation mutants (Supplementary Data Figure 1). The DC1 truncation showed little to no change in its ability to bind PCNA under our conditions. The crystal structure of the FEN-1/PCNA complex was reported to lack electron density in this region of FEN-1 suggesting that it is unstructured and does not interact with PCNA.[28] Thus, the fact that the DC1 truncation did not affect binding with PCNA is consistent with the crystal structure. The DC2 truncation decreased PCNA binding as demonstrated by the loss of more than 50% of relative binding (defined by normalizing wild type FEN-1 binding to 100%). The effect of the DC2 mutation is likely due to the loss of three hydrogen bonds and one salt bridge formed by amino acid residues K354, R355, and K356 with PCNA.[28] The DC3 mutant showed a drastic decrease in its ability to bind PCNA (Figure 2). The amino acid residues removed by this truncation were shown in the crystal structure to form a β-zipper with a strand of the interdomain connector loop of PCNA.[28] Therefore, the severe effect that the DC3 truncation has on binding is consistent with the structure. When the C-terminus was completely eliminated as in the DC4 mutant, the assay showed a higher relative binding in comparison to DC3. The ability to bind PCNA in the absence of the C-terminus is consistent with the fact that PCNA and the core nuclease domain interact.[28,34] However, the decrease in binding of DC3 compared to the complete truncation (DC4) of the C-terminus is unexpected because DC4 removes the well-characterized QxxLxDFF PCNA interaction motif. This will be addressed in more detail in the discussion, as this appears to be the case with several of the proteins.

Like PCNA, information concerning residues at the C-terminus of FEN-1 necessary for WRN interaction has been reported.[35] In that study, the residues responsible for interaction with FEN-1 have been localized to amino acid residues 363–380. For our assays, we used the region of WRN known for its ability to bind FEN-1 (WRNC, amino acids 949–1432).[35] Consistent with the previous study, the DC1 truncation mutant (amino acid residues 368–380) showed a relative binding percentage of less than 50%. Continued deletions of the C-terminus (DC2, DC3, and DC4) further decreased observable binding and appeared to eradicate binding completely (Figure 2). This suggested that other amino acids in the C-terminus also contribute to interaction with WRN.

For APE-1 and EndoG, relative binding was significantly reduced to 50% or less in the pull-down assays with all four of the truncation mutants. These results suggested that unlike PCNA, all four regions of the C-terminus are necessary for APE-1 and EndoG interaction. Like PCNA, a complete loss of observable binding was seen with the DC3 mutant, whereas a miniscule amount of binding was detected with the DC4 and DC2 mutants (Figure 2). Taken together, these results suggested that the amino acids most critical for interaction of FEN-1 with APE-1 and EndoG reside in the C-terminus upstream of amino acid A368.

The findings for each of the proteins in the Rad9-Rad1-Hus1 complex were similar to one another. A reduction in relative binding with all of the truncation mutants was observed. Moreover, Rad9 and Rad1 appeared to have identical results with undetectable interaction with DC3 and relative binding levels below 25% for DC1 and DC2. A slight increase in binding was seen with DC4, similar to that observed with PCNA. Binding with Hus1, was not detected for the DC2 and DC3 truncations, while DC4 and DC1 were observed to have relative binding

levels of 25% or below (Figure 2). Using the Rad9-Rad1-Hus1 complex, we found that the relative binding for the trimeric complex is below 50% for each of the truncations.

Overall, the results from the FEN-1 truncations indicated that all five proteins, which are involved in different DNA metabolic pathways, interact with the C-terminus of FEN-1. However, this analysis showed that the four regions of the C-terminus affect binding to varying degrees, suggesting that different regions of the C-terminus vary in importance with respect to the binding partner. Our ultimate goal is to find single or multiple amino acid mutants that affect only one protein interaction. Therefore, after determining that the C-terminus is important for all five interactions, we performed comprehensive site-directed mutagenesis scanning on the FEN-1 C-terminus as discussed below.

PCNA interaction on the FEN-1 C-terminus

Although several reports exist in the literature demonstrating the role of the ^{337}QGRLDDFFK^{345} motif in the C-terminal region of FEN-1 for the interaction of PCNA,[25,26] the co-crystal structure of FEN-1/hPCNA shows that additional amino acids in the FEN-1 C-terminus are involved in the PCNA interaction.[28] Using comprehensive alanine mutagenesis, multiple point and single point mutations were made to screen the entire C-terminus of FEN-1. Like the truncation mutations, the single-, and multiple-point mutations will likely not affect the stability and folding of the core nuclease domain because it is already unstructured in the absence of protein interaction partners. Further support for this comes from SDS-PAGE analyses, which show bands of the expected sizes after purification, and the presence of the nuclease activity in these mutant proteins. Performing pull-down assays for each mutation, we found several amino acids important for the interaction of PCNA with FEN-1 (Figure 3). In agreement with previous findings, most of the amino acids in the Q-x-x-[L/I] – x-x-F-F PCNA interaction motif affected the level of observable binding, with the exception of mutants 2 and 4 (residues R339 and D341, respectively).[24] In addition, residues D342, V346, S349, L350, S351, and S352 (single point mutants 5, 11, 17, 18, respectively) were shown to be important for binding as well. The loss of the hydrophobic side chains of V346 and L350 upon mutation to alanine may decrease binding due to the loss of the van der Waal contacts made by these two residues with L126 and a small hydrophobic pocket (C27/A67) in PCNA, respectively. A similar explanation can be employed for S352, because its hydroxyl group hydrogen bonds with the backbone amide of L121 of PCNA. The effect on binding observed with the D342A, S359A, and S351A mutations is not as easily explained as these residues according to the crystal structure only contact PCNA via hydrogen bonds using the backbone amide moiety, which should be insensitive to changes of side chain identity. These residues are hydrophilic, and mutation thereof may change the properties of the FEN-1 C-terminus in ways that prevent the formation of the β-zipper with PCNA.

WRN interaction on the FEN-1 C-terminus

The WRN protein has been shown to stimulate FEN-1's activities *in vitro*[9,35] and to interact with FEN-1 *in vivo*.[35] Here, we used the same alanine mutagenesis screening to analyze the entire C-terminus of FEN-1 (Figure 4). Our results not only confirmed previous findings,[27] but also pointed out specific amino acids within the defined region that were important points for interaction in the pull-down assays. Multiple point mutations in mutants 23 (E357/P358/E359), 33 (K365/K366/K367), and 42 (K377/R378/K380) showed a binding affinity less than 50%. Within the stretch of amino acids residues 363–380, mutants 35 (K366), 36 (K367), 40 (K375), 44 (R378), and 45 (K380) were shown to be important for interaction with FEN-1 as single residues. Just upstream of the previously identified interaction site, we found that there are several more amino acid residues involved in binding to WRN as noted with mutant 23.

Identification of FEN-1 C-terminus interaction sites for APE-1

Although the role of the FEN-1/APE-1 interaction has been studied *in vitro*,[6,7] no role for this interaction has been determined *in* due to the lack of information regarding the binding site. To determine which amino acids in the C-terminus of FEN-1 are involved in APE-1 interaction, we used pull-down assays with the series of C-terminal mutants described above. The multiple point mutants 6 (F343/F344), 13 (S349/L350), 16 (S351/S352), 33 (K365/K366/K367) showed a decrease in observable binding (Figure 5). Mutants 6 and 13 had the lowest relative binding within the context of a multi–residue mutation group, suggesting that these amino acids are important for interaction. The single point mutations F343A (7), F344A (8), S349A (14), K374A (40), and F375A (41) had the most significant decrease in relative binding. Interestingly, despite the fact that a double point mutation in mutant 16 and triple point mutation in mutant 33 showed decreased binding affinity, the amino acids within these groups did not exhibit a decrease when mutated alone. This observation illustrates that the combined loss of amino acids that are unessential for binding when mutated alone can have a synergistic affect on the relative binding ability.

Identification of FEN-1 C-terminus interaction sites for EndoG

Similar to APE-1, we performed the analysis as described above for the EndoG protein (Figure 6). The results from the multiple point mutations in mutants 13 (S349/L350), 23 (E357/P358/E359), and 33 (K365/K366/K367) showed that at least some of the EndoG interaction sites on FEN-1 lie within these regions. Consistent with these findings, the single point mutations in mutants 14 (S349), 15 (L350), 24 (E357), 25 (P358), 26 (E359), 34 (K365), 35 (K366), 36 (K367) also showed a substantial decrease in relative binding. Mutant 21 (R355) showed a decrease in binding as well, however the decrease is not as considerable as those single point mutations listed above. The interesting finding in the search for interaction sites of EndoG was that several of the amino acids on the FEN-1 C-terminus result in a complete, or very near complete, loss of binding when mutated to alanines. Our results demonstrated that amino acids S349/L350, E357/P358/E359, and K365/K366/K367 are crucial for interaction either in sets as multiple point mutations or as single point mutations.

Identification of FEN-1 C-terminus interaction sites for Rad9-Rad1-Hus1

The Rad9-Rad1-Hus1 complex is a heterotrimeric toroidal molecule, structurally similar to the homotrimeric PCNA, and is thought to be involved in DNA repair as a DNA damage sensor.[36,37,38] To identify where the physical interaction of the Rad9-Rad1-Hus1 occurs on FEN-1, the complex was also tested in the various mutants with Rad9, Hus1, and Rad1 individually and the complex as a whole. For Rad9 alone, relative binding affinity dropped below 50% for single point mutants 1 (Q337), 5 (D342), 8 (F344), 35 (K366), 36 (K367), 43 (K377), and 44 (R378) (Supplementary Data Figure 2). Multiple point mutants 6 (F344/F344), 33(K365/K366/K367), and 42 (K377/R378/K380) corresponding to the affected single point mutations as well as mutants 19 (K354/R355/K356) and 23 (E357/P358/E359) also exhibited a binding affinity below 50%. Rad1 assayed individually exhibited similar results. Single point mutants 5 (D342), 8 (F344), 34 (K365), 35 (K366), 36 (K367), 44 (R378), and 45 (K380) yielded a significant decrease in binding affinity. Multiple point mutants 6, 33, and 42 were also found to be important for Rad1 binding to FEN-1 (Supplementary Data Figure 3). Not surprisingly, Hus1 demonstrated a lowered binding affinity with multiple point mutants 6, 33, 42 just as Rad9 and Rad1 (Supplementary Data Figure 4). Also, single point mutants 36 (K367), 44 (K378), and 45 (K380) revealed a relative binding affinity lower than 25%. This suggested that Hus1 most strongly interacts with the downstream region of the FEN-1 C-terminus, while Rad9 and Rad1 interaction appeared to involve other regions of amino acids in the C-terminus.

As a complex, Rad9-Rad1-Hus1 used to pull-down FEN-1 (Figure 7) revealed that the relative binding was not as drastically decreased. However, relative binding affinity was decreased at

similar amino acid residues, reflected in mutants 23, 33, 36, and 42. The decreased binding affinity of the multi-point mutant 23 was only observed to decrease when using Rad9 alone in the pull down, while the results for mutants 33, 36, and 42 correspond to each of Rad9-Rad1-Hus1 complex proteins when assayed individually.

Stimulation and Base Excision Repair

To further investigate the role of the C-terminal amino acid residues, we assayed for the ability of WRNC to stimulate FEN-1 wild type, truncation mutants, and point mutants found to play a role in the binding of FEN-1 to WRNC (Figure 9). Assay of the truncation mutants showed that the ability of WRNC to stimulate FEN-1 truncations in comparison to wild-type decreases as progressively more amino acid residues of the FEN-1 C-terminus are removed. The DC1 truncation includes the removal of amino acid residues 368–380, which include residues K375, R378, and K380, that were identified as key amino acid residues important for binding (mutants 40, 42, 44, and 45). The results for truncation mutants DC2, DC3, and DC4 also showed a decrease in the ability to be stimulated by WRNC. The particular truncations are missing the identified amino acid residues important for WRNC binding. Thus, the data show that a decrease in WRNC/FEN-1 interaction corresponds to a decrease in WRNC mediated stimulation of FEN-1 flap endonuclease cleavage.

Similar to the truncation mutants, the results of the WRNC stimulation assays for the multiple point mutant FEN-1s that were shown to decrease binding (mutants 23, 33, and 42), also showed a decrease in WRNC mediated stimulation of flap endonuclease activity. In comparison to the multiple point mutants, the single point mutations (35, 36, 44, and 45) did not result in a drastic decrease in the ability to be stimulated by WRNC. This suggests that the synergistic loss in the ability of WRNC and FEN-1 to interact results in a corresponding loss in WRNC mediated FEN-1 stimulation.

Finally, we asked whether these mutants that were shown to be important for binding and stimulation could affect FEN-1's flap endonuclease activity in a reconstituted system. We used mutant 23 (E357/P358/E359) as an example to test the role of the FEN-1 C-terminus in a long patch BER reconstitution assay[39] in the presence of WRNC (Figure 10). In this assay, we compared the ability of wild type and mutant 23 FEN-1 proteins to be stimulated by WRNC to complete long patch BER reactions. In the presence of increasing amounts of WRNC (10–200 fmoles), the mutant 23 FEN-1 protein demonstrates a lower efficiency in the completion of long patch BER. In fact, the efficacy of wild type FEN-1 in the reconstitution assays is two-fold greater than that of the mutant protein in the presence of 100 or 200 fmoles of WRNC. Thus, the reduced binding capacity of the FEN-1 E357A/P358A/E359A mutant appears to lead to a reduction in FEN-1's ability to remove the flap generated as a long patch BER intermediate.

Discussion

The multi-functional roles of FEN-1 in a variety of pathways is hypothesized to be a result of FEN-1's ability to interact with a multitude of proteins, each interaction consisting of a unique signature of amino acid residues. Here we were able to show the importance of the C-terminus for interaction with several proteins and to identify amino acid residues within the C-terminus specific for particular interactions (Figure 8). In addition to indicating a region of the C-terminus important for binding of a particular partner, several of the truncations demonstrated the possibility of interaction sites on FEN-1 other than the C-terminus. The DC4 mutant, a truncation of the entire C-terminus, was able to bind to PCNA, and the Rad9, Hus1, Rad1 proteins (both individually and as a complex) suggesting that interaction sites within the nuclease core domain are present. In fact, two reports provide structural evidence to indicate that PCNA can also interact with FEN-1 on its nuclease core domain in addition to its C-terminal domain.[28,34] Thus, the notion of interaction sites for the proteins of the Rad9-Rad1-

Hus1 complex within the nuclease domain of FEN-1 is possible. Interestingly, the DC4 mutant was capable of interacting better with PCNA, Rad9, Rad1, and Hus1 compared to the DC3 mutant. This observation suggests that residues 337 to 344 may weakly compete with other proteins for a binding site in the nuclease core domain. Possibly upon interaction of residues 337 to 344 of FEN-1 with PCNA or the Rad9-Rad1-Hus1 complex, the competition is alleviated allowing the nuclease core domain to interact with the protein partner. This notion is supported by the observation above that extension of the intact FEN-1 C-terminus weakens the interaction of proteins with the nuclease core (i.e., the DC3 mutation binds more weakly than the DC4 truncation).

To further evaluate the role of the specific interaction sites on FEN-1 we performed a set of stimulation assays. Using FEN-1 mutants that abrogated interaction, we tested the ability of WRNC to stimulate FEN-1 activity. In accordance with previous findings, amino acid residues important for interaction may not be critical for stimulation of FEN-1 activity *in vitro*,[28, 30, 34] however they do have an affect in the protein's ability to be stimulated as shown here with WRNC. The lack of complete loss of ability to be stimulated may be due to the possible presence of weaker interaction sites on the FEN-1 nuclease core domain (as discussed above in the case of PCNA). Furthermore, because WRNC and FEN-1 interact with DNA, the presence of DNA may bridge the weak interactions of the nuclease domain and the protein partner, thereby allowing stimulation to occur, albeit weakly, in the absence of detectable binding. For instance, Gomes and Burger have clearly illustrated two amino acids in the nuclease core domain are necessary for stimulation by PCNA, while another two are essential for high affinity interaction.[40] Thus, residues important for high affinity interaction in non-equilibrium assays such as the pull-down assay are not necessarily important for stimulation and vice versa. Further work must be completed in this area to determine whether there are specific amino acid residues on the FEN-1 protein important for stimulation by the other proteins in this study that are distinct from the residues important for interaction.

We recently evaluated the role of the PCNA/FEN-1 interaction in a mammalian model by knock-in of the F343A/F344A double mutant into the mouse S129 background (FFAA mice). Our assessment of the FFAA mutation on the C-terminus of FEN-1 revealed that disruption of the FEN-1/PCNA interaction results in DNA replication defects *in vitro* and severe phenotypic defects in mice. The FFAA mice died at birth, most likely due to pulmonary hypoplasia and pancytopenia suggesting that the interruption of an interaction at the C-terminus of FEN-1 disrupted a critical pathway in which FEN-1 is involved – DNA replication.[41] Because it is known that murine Fen-1 harboring the FFAA mutation can be stimulated by PCNA *in vitro*, loss of the ability to stimulate cannot explain the DNA replication defects *in vivo*. We were, however, able to show that the FFAA mutation impairs recruitment of FEN-1 to replication foci.[41] In agreement with the aforementioned observation, the C-terminus, which is larger in eukaryotic FEN-1s than prokaryotes and is involved in nuclear localization,[3] may be important for recruitment to sites outside the nucleus. Thus, the extended C-terminus of FEN-1 in eukaryotes may play a role in sub-nuclear localization in addition to nuclear localization as previously described.[24] Disruption of other FEN-1 pathways using amino acid residues specific for a particular pathway partner of FEN-1 described here may lead to further understanding of the roles of these interactions in sub-nuclear localization and/or processing of DNA intermediates.

Materials and Methods

Reagents

Cyanogen bromide-activated Sepharose (CNBr-Sepharose) was purchased from Amersham Biosciences (Piscataway, NJ). Anti-FEN-1 mouse monoclonal antibody (GTX70185, FEN-1-4E7) was purchased from Genetex Company (San Antonio, TX). Anti-mouse IgG/HRP

was purchased from Santa Cruz Biotechnology (Santa Cruz, CA). PVDF membrane was purchased from Bio-Rad Laboratories (Hercules, CA). Enhanced chemiluminescence substrate (SuperSignal) was obtained from Pierce Chemical (Rockford, IL).

Proteins

Recombinant human EndoG and Rad9-Rad1-Hus1 complex were purchased from Axxora LLC (San Diego, CA). The WRNC construct was expressed from a pET41b vector originally inserted using the *Nde*I and *Xho*l restriction sites. WRNC was purified by immobilized metal affinity and cation exchange chromatography. Wild type human FEN-1 and its truncations were purified as previously described.[42] Briefly, FEN-1 and its truncations were expressed in BL21 (DE3) from a pET28b vector encoding for wild type and truncated FEN-1s possessing a C-terminal His_6-tag. The His-tagged proteins were then purified using immobilized metal affinity chromatography (IMAC) Prepease® columns (USB Corporation, Cleveland, OH) per manufacturer's instructions. Multiple- and single-point human FEN-1 mutations were introduced into the pET28b vector containing the wt FEN-1 described above using the QuikChange Site-Directed Mutagenesis Kit (Stratagene, La Jolla, CA) and the appropriate primers, which are listed in the supplementary data (Table 1). Similar to FEN-1, PCNA and APE-1 (clone obtained from laboratory of T. O'Connor[43]) were over-expressed in *E. coli* BL21DE3 and purified using the IMAC Prepease columns (USB Corporation, Cleveland, OH) as with wt FEN-1. The Rad9-Rad1-Hus1 complex is a heterotrimer containing three subunits called Rad9, Hus1 and Rad1, respectively. The proteins were independently expressed and purified from *E. coli*. Proteins were quantified by the Bradford assay kit (Bio-Rad, Hercules, CA).

Immobilization of proteins on CNBr-Sepharose beads

Immobilization was performed according to the manufacturer's instructions. Briefly, the beads were swelled in 1 mM HCl. Proteins to be immobilized (PCNA, WRN, APE1, EndoG, and 9-1-1 subunits) were re-suspended in coupling buffer (0.1 M $NaHCO_3$, 0.5 M NaCl, pH 8.3) and added to the swelled-beads with rotation overnight at 4°C. The beads were then blocked with 0.1 M Tris, pH 8.0, for 2 h at room temperature. Free protein was washed away with PBS, followed by three wash cycles of alternating pH (0.1 M acetate buffers, pH 4.0 and 0.1 M Tris buffer, pH8.0). The protein density on beads is about100 ng/μl.

Pull down assay

For the pull-down assay, 10 μl of protein-coated beads were incubated with 100 ng human FEN-1 (wild type or mutant) overnight at 4°C in pull-down buffer (50 mM HEPES, 100 mM NaCl, 1% BSA, 1mM DTT, 10% glycerol, pH 7.5). After washing four times with PBS-0.1% tween-20, the beads were boiled for 5 min at 95°C in 30 μl SDS-PAGE sample buffer. The supernatants were analyzed by Western blotting.

Western blotting and data analysis

Boiled samples were spun down. 10 μl of supernatant was subjected to SDS-PAGE, followed by transferring to PVDF membrane. The membrane was then blocked and detected using mouse monoclonal anti-FEN-1 antibody and goat anti-mouse IgG/HRP. Results were scanned and quantified using ImageJ 1.37 software. Data are expressed as the means ± S.D. of triplicate results. Asterisks denote statistically significant data (*, $p < 0.05$; **, $p < 0.01$). Statistical analysis was achieved by using a student *t-test*.

Stimulation assay

Reactions were carried out with the indicated amount of wild type or mutant FEN-1 (1.16 pmol), in the presence or absence of WRNC (900 fmol), and 100 fmol of flap substrate in

reaction buffer. The reaction buffer contained 30mM HEPES pH 7.6, 5% glycerol, 40 mM KCl, 0.1 mg/mL BSA, 8mM $MgCl_2$, based on previous evidence that it is the optimal buffer for stimulation of FEN-1 by WRN.[27,44] Each reaction was brought to a total volume of 20 μl with water. All reactions were incubated at 37°C for 30 min and terminated by adding an equal volume of stop solution (80% formamide, 10 mM EDTA, 1.0 mg/mL bromophenol blue, 1.0 mg/mL xylene cyanol). Reactions were analyzed on a 7 M urea, 15% denaturing PAGE in 1×TBE. Gels were dried and visualized by phosphorimager analysis (Amersham Biosciences, Piscataway, NJ) and quantified using Image Quant software.

Reconstitution assay

An in vitro reconstitution assay was performed based on the previously published protocol.[39] Briefly, in a 15 μL reaction, the indicated amounts of the BER protein components (see Figure 10 and its corresponding legend) were mixed with 1mmol ATP, a [α-^{32}-P]-dATP, and 25 pmol of the remaining three dNTPs, and a 52-nt duplex oligo containing tetrahydrofuran (THF) opposite the C at the 31st position for the substrate. The reaction mixture was incubated at 37°C for 30 minutes and stopped with formamide dye (80% formamide, 20 mM NaOH, 20 mM EDTA, 0.5% bromophenol blue and 0.05% xylene cyanol). The products were separated on a 20% polyacrylamide gel containing 8 M urea in 1X Tris/Borate-EDTA buffer, pH 8.4. The radioactivity was quantified using phosphorimager analysis and Image Quant software. The Sigma Plot software was used for data analysis.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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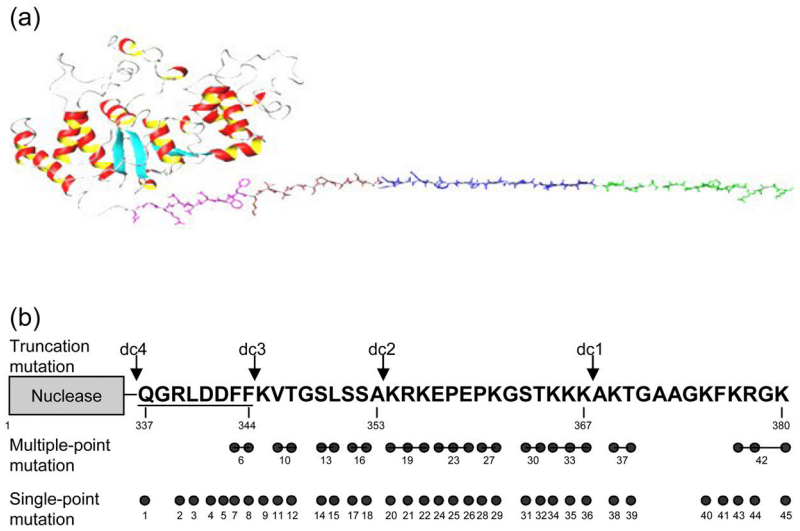

Figure 1.

Design of the FEN-1 C-terminal scanning mutations. A) Structural model of the human FEN-1 protein showing the core nuclease domain and an unstructured C-terminal tail. This model was generated by modifications to PDB structure 1UL1 to only show one molecule of FEN-1 (molecule z). Amino acid residues after K356 were not observed in this crystal structure; therefore, amino acid residues 357 through 380 were modeled in using the Swiss-PDB Viewer 3.7 software. In addition, the backbone angles of the entire C-terminus (a.a.337–380) were set to extended β-sheet values using the same program. Note, the C-terminus is modeled, and thus, the C-terminus is more likely a random coil in reality. The final ribbon diagram shown was generated using MolMol2K.2. B) Truncations, multiple-and single-point mutations used in this study. Arrows indicate the truncating sites; Circles indicate the mutation site of the corresponding amino acid; Multiple-point mutations are indicated by line-connected circles. Underlined residues indicate the PCNA binding motif.

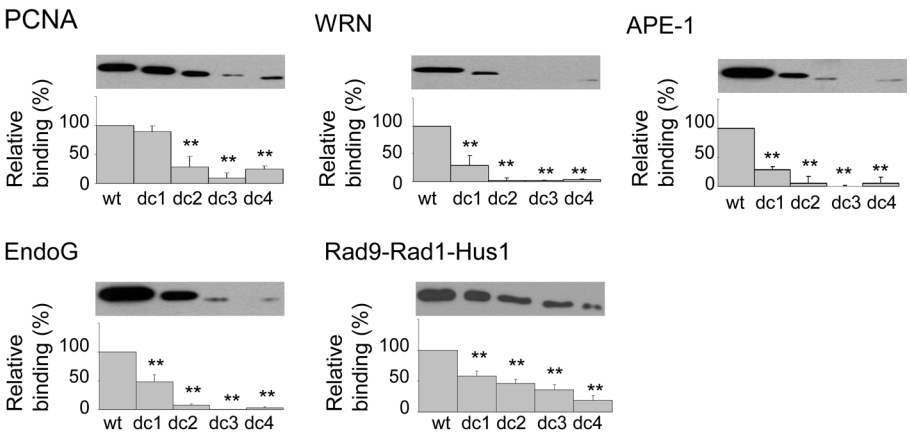

Figure 2.

Mapping of interaction regions of human FEN-1 with five proteins by pull-down assay (*, $p < 0.05$; **, $p < 0.01$). Proteins: PCNA, WRN, APE-1, EndoG, and the Rad9-Rad1-Hus1complex were immobilized on Sepharose beads as described in Material and Methods, followed by incubation with the human FEN-1 wild type protein or proteins with truncations of different length. The precipitated proteins were separated by a 4–15% gradient SDS-PAGE and immuno-detected with anti-human FEN-1 antibody.

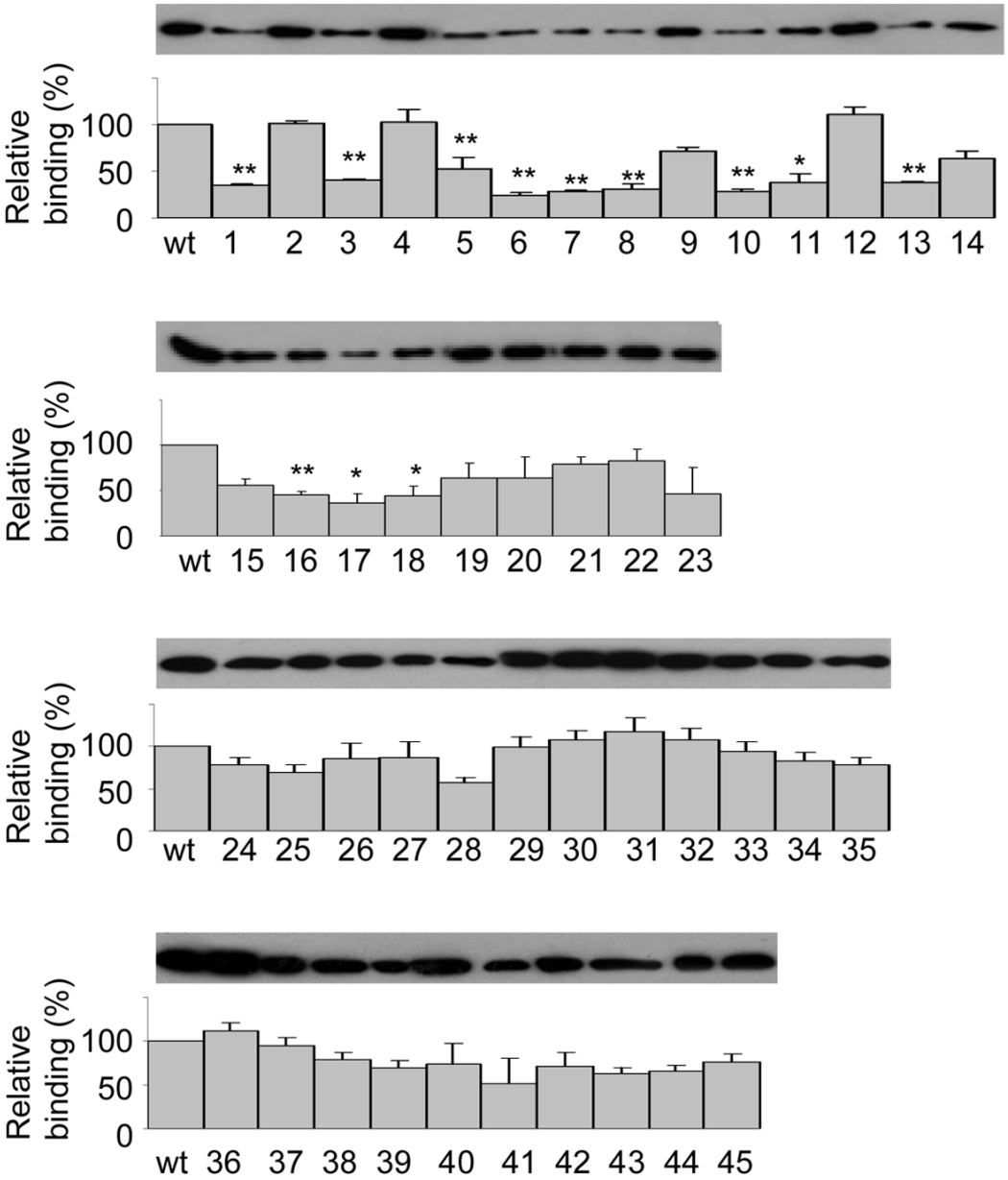

Figure 3.

Determination of interaction sites of human FEN-1 with PCNA by pull down assay (*, $p < 0.05$; **, $p < 0.01$). Purified PCNA protein was immobilized on Sepharose beads as described in Material and Methods, followed by incubation with the human FEN-1 wild type protein or proteins with different mutations. The precipitated proteins were separated by 4~15% gradient SDS-PAGE and immuno-detected with anti-human FEN-1 antibody.

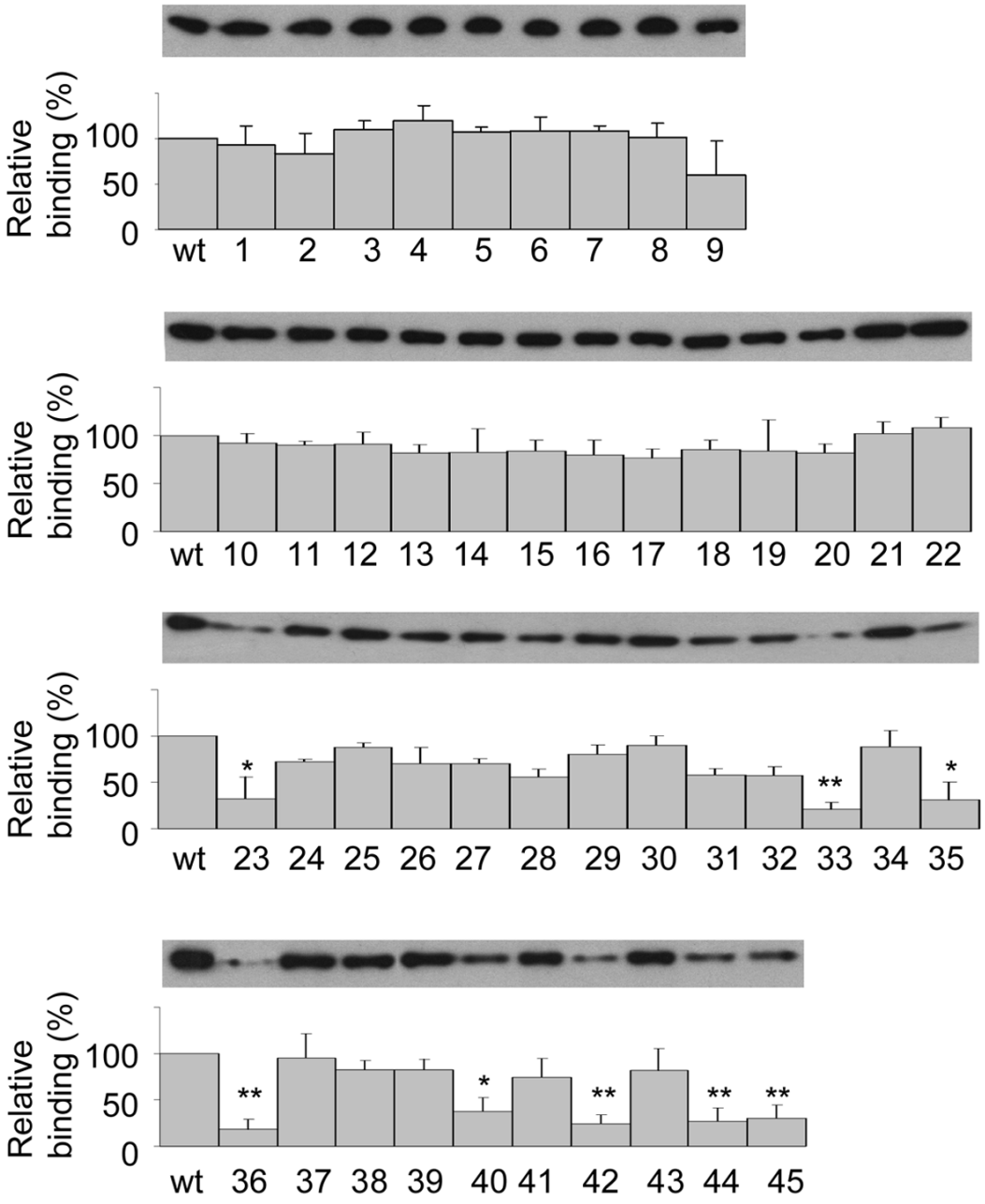

Figure 4.

Determination of interaction sites of human FEN-1 with WRN by pull down assay (*, $p < 0.05$; **, $p < 0.01$). Purified WRN protein was immobilized on Sepharose beads as described in Material and Methods, followed by incubation with the human FEN-1 wild type protein or proteins with different mutations. The precipitated proteins were separated by 4~15% gradient SDS-PAGE and immuno-detected with anti-human FEN-1 antibody.

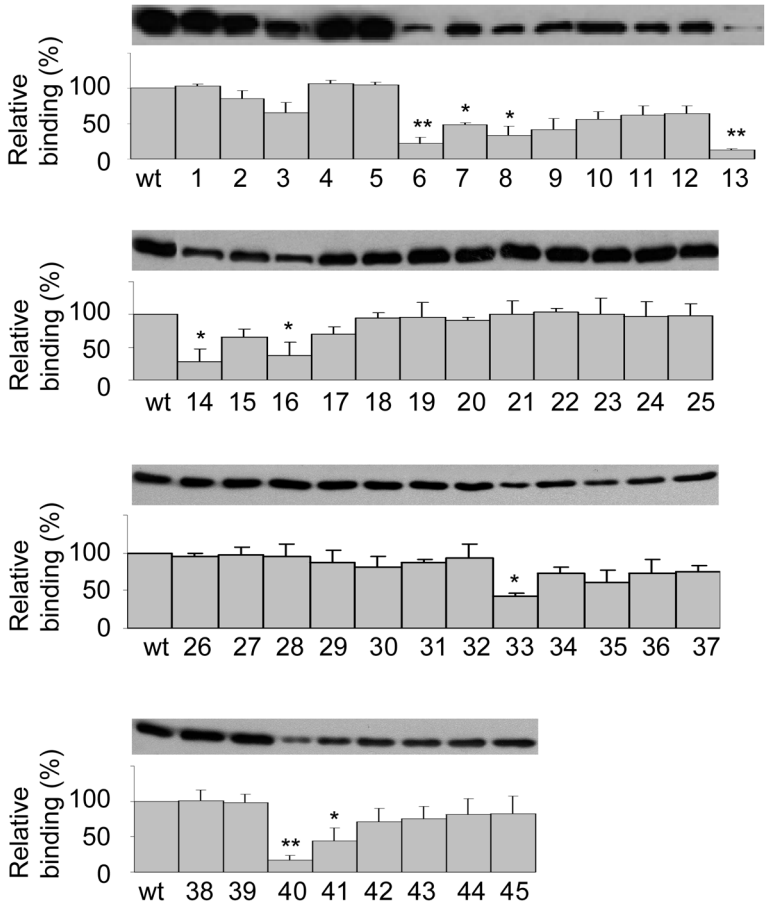

Figure 5.

Determination of interaction sites of human FEN-1 with APE-1 by pull down assay (*, $p < 0.05$; **, $p < 0.01$). Purified APE1 protein was immobilized on Sepharose beads as described in Material and Methods, followed by incubation with the human FEN-1 wild type protein or proteins with different mutations. The precipitated proteins were separated by 4~15% gradient SDS-PAGE and immuno-detected with anti-human FEN-1 antibody.

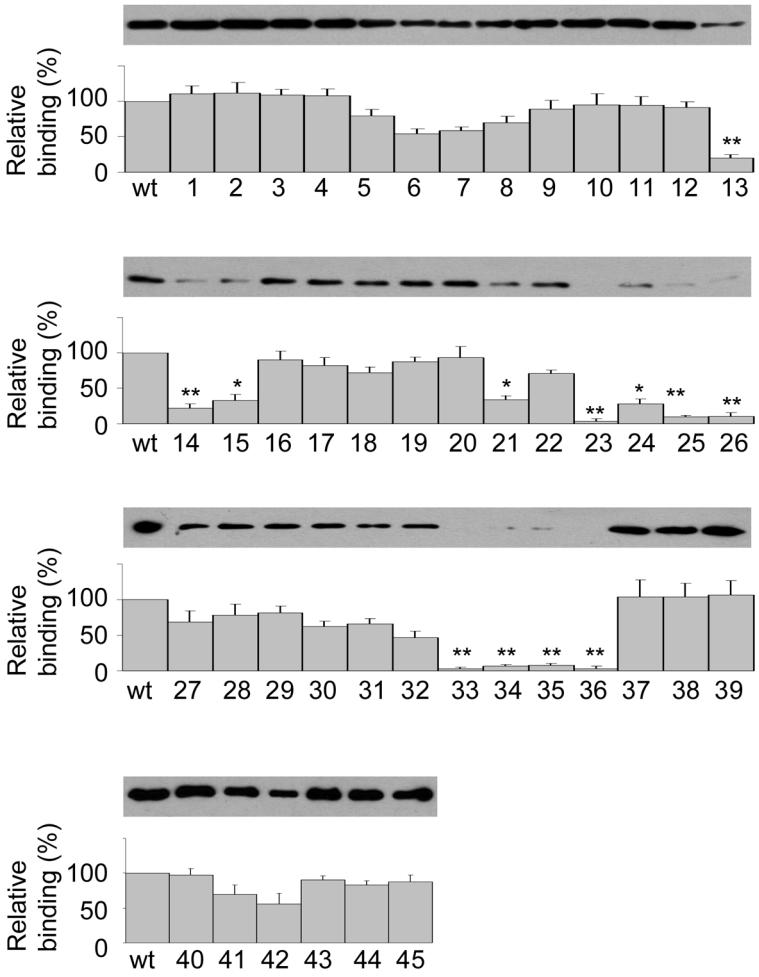

Figure 6.

Determination of interaction sites of human FEN-1 with EndoG by pull down assay (*, $p < 0.05$; **, $p < 0.01$). Purified EndoG protein was immobilized on Sepharose beads as described in Material and Methods, followed by incubation with the human FEN-1 wild type protein or proteins with different mutations. The precipitated proteins were separated by 4~15% gradient SDS-PAGE and immuno-detected with anti-human FEN-1 antibody.

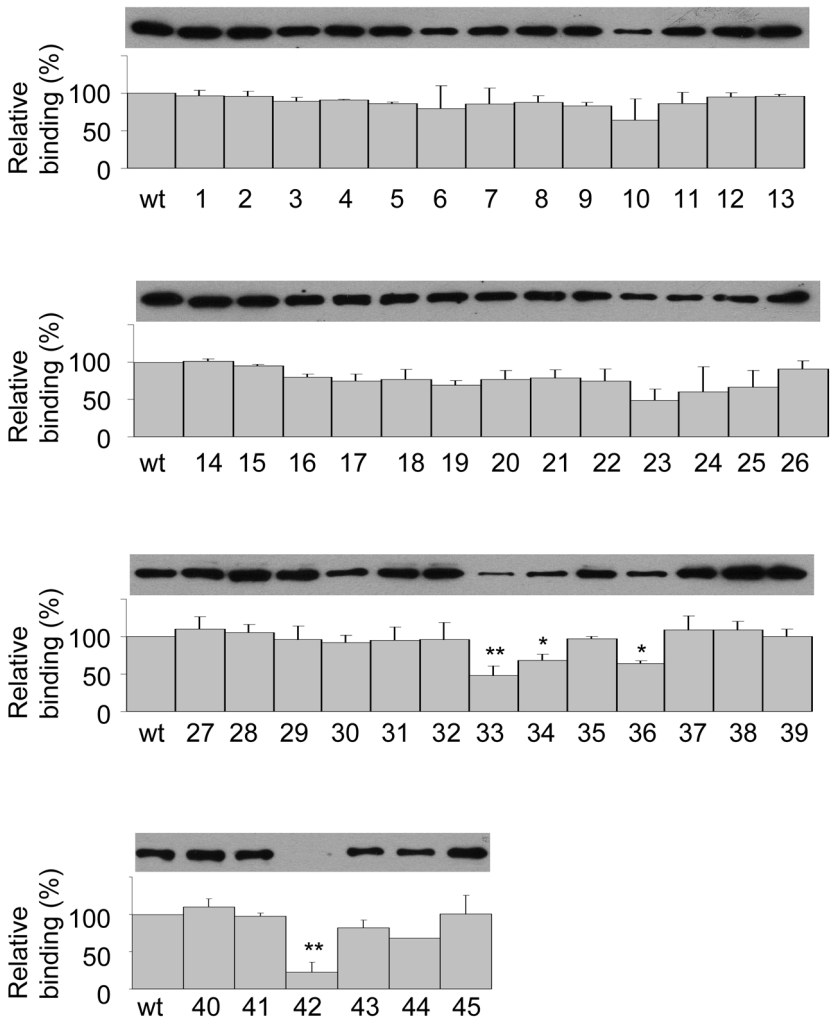

Figure 7.

Determination of interaction sites of human FEN-1 with the Rad9-Rad1-Hus1 complex by pull down assay (*, $p < 0.05$; **, $p < 0.01$). Purified Rad9-Rad1-Hus1 protein was immobilized on Sepharose beads as described in Material and Methods, followed by incubation with the human FEN-1 wild type protein or proteins with different mutations. The precipitated proteins were separated by 4~15% gradient SDS-PAGE and immuno-detected with anti-human FEN-1 antibody.

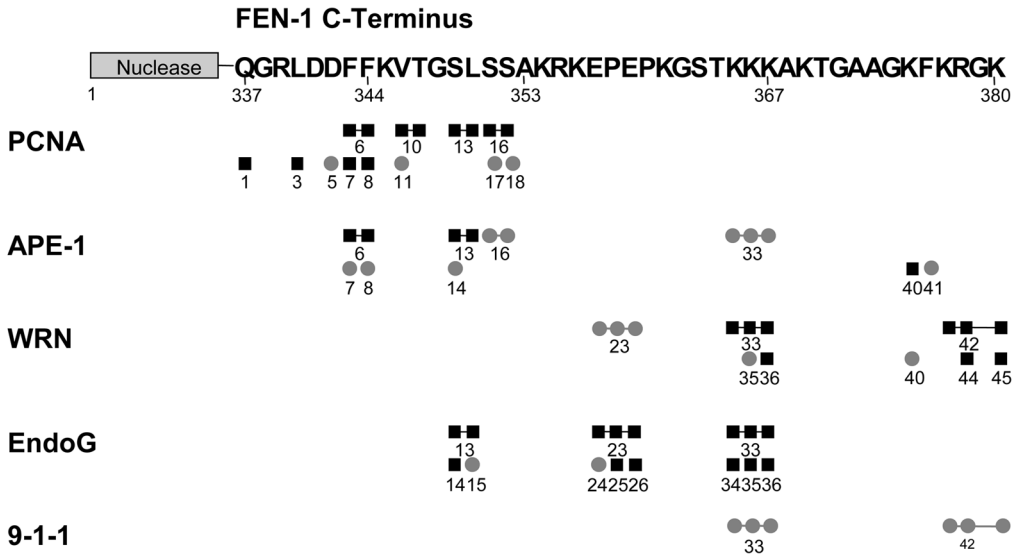

Figure 8.

Summary of interaction sites of human FEN-1 with different proteins. Circles and rectangles indicate the interaction sites of FEN-1 with different proteins. Circles indicate $p < 0.05$ while rectangles indicate $p < 0.01$.

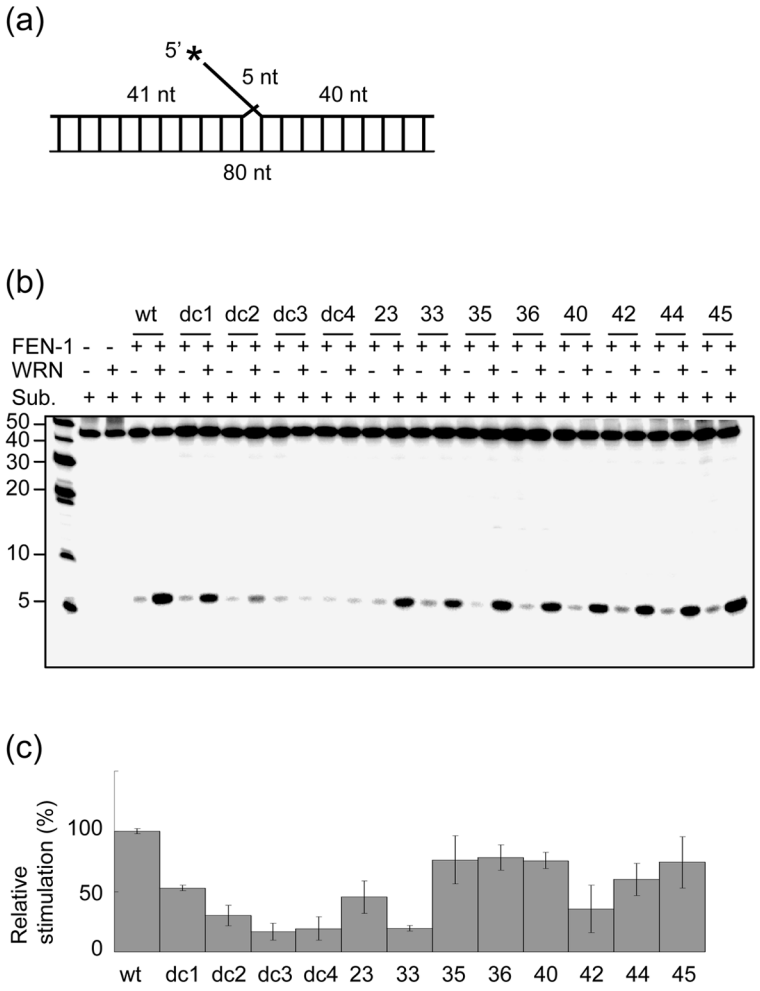

Figure 9.

Disruption of FEN-1/WRN protein interaction reduces the stimulation of FEN-1 flap activity by WRN. (a) Flap substrate for FEN-1 activity assay. The flap strand of the DNA substrate was ^{32}P radio-labeled at the 5′ end. (b) Stimulation assay of FEN-1 mutants by WRNC. (c) Relative stimulation based on the data presented in panel (b). The fold-increase of WRN stimulation of wild type FEN-1 flap activity was set arbitrarily at 100%.

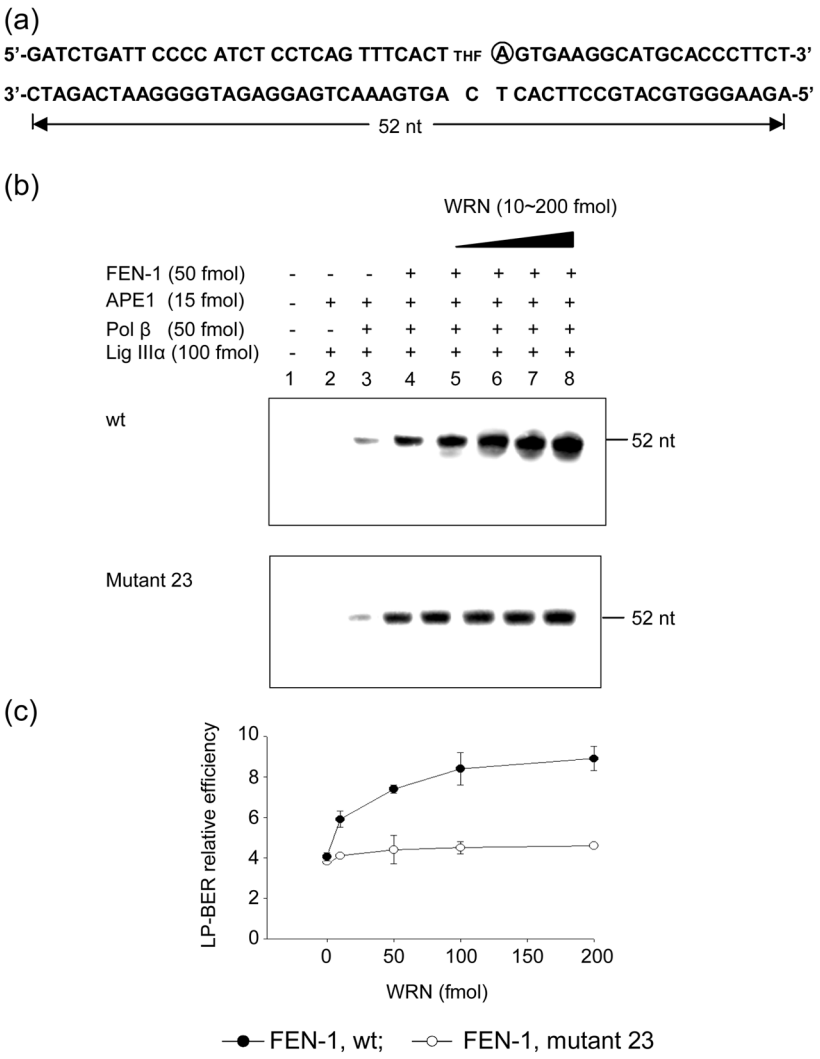

Figure 10.

Disruption of FEN-1/WRN protein interaction in FEN-1 mutant 23 (E357A/P358A/E359A) reduces the BER efficiency. (a) The long patch BER (LP-BER) substrate with tetrahydrofuran (THF) to mimic an abasic site. Ais the site for incorporation of radio-labeled nucleotide. The total length of the DNA oligonucleotide is 52 nucleotides. (b) BER reconstitution reactions with wt and mutant 23 FEN-1 proteins with increasing concentrations of WRNC. (c) Relative LP-BER efficiency based on the data presented in panel (b). The amount of BER product from the reaction without WRNC protein was arbitrarily set as 4.