SURVEY AND SUMMARY

Functional regulation of FEN1 nuclease and its link to cancer

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ABSTRACT

Flap endonuclease-1 (FEN1) is a member of the Rad2 structure-specific nuclease family. FEN1 possesses FEN, 5′-exonuclease and gap-endonuclease activities. The multiple nuclease activities of FEN1 allow it to participate in numerous DNA metabolic pathways, including Okazaki fragment maturation, stalled replication fork rescue, telomere mainten-ance, long-patch base excision repair and apoptotic DNA fragmentation. Here, we summarize the distinct roles of the different nuclease activities of FEN1 in these pathways. Recent biochemical and genetic studies indicate that FEN1 interacts with more than 30 proteins and undergoes post-translational modi-fications. We discuss how FEN1 is regulated via these mechanisms. Moreover, FEN1 interacts with five distinct groups of DNA metabolic proteins, allowing the nuclease to be recruited to a specific DNA metabolic complex, such as the DNA replication machinery for RNA primer removal or the DNA degradosome for apoptotic DNA fragmentation. Some FEN1 interaction partners also stimulate FEN1 nuclease activities to further ensure efficient action in processing of different DNA structures. Post-translational modifications, on the other hand, may be critical to regulate protein–protein inter-actions and cellular localizations of FEN1. Lastly, we also review the biological significance of FEN1 as a tumor suppressor, with an emphasis on studies of human mutations and mouse models.

INTRODUCTION

DNA replication, recombination and repair are essential for maintaining the integrity of genomes. These DNA metabolic pathways create various DNA intermediates that must also be efficiently processed, otherwise severe genomic instability will result. Many of the DNA inter-mediates formed are bifurcated nucleic acid structures that require nucleases to recognize the structure of the inter-mediate and to only cleave the appropriate phosphate diester, regardless of sequence. A nuclease that typifies these qualities is flap endonuclease-1 (FEN1), which is the archetypal member of the Rad2 nuclease family (1,2). FEN1 is a structure-specific metallonuclease best known for its essential roles in the penultimate steps of Okazaki fragment maturation and long-patch base excision repair (BER) (1,3,4). Furthermore, FEN1 has also been implicated in other major DNA metabolic pathways, including resolution of tri-nucleotide repeat sequence-derived secondary structures, rescue of stalled DNA replication forks, maintenance of telomere stability and apoptotic fragmentation of DNA (5–10).

How FEN1 is involved in multiple pathways that are seemingly contradictory (i.e. genome maintenance versus apoptotic DNA fragmentation) has been of interest to our lab and others. The multiple functions of FEN1 may be regulated via three interplaying mechanisms: (i) formation of complexes with different protein partners, (ii) cellular compartmentalization and (iii) post-translational modifi-cations. First, FEN1 is known to rely on interactions with other DNA metabolic proteins for recruitment to different machineries for DNA replication, repair or degradation. Some of these interaction partners of FEN1 also stimulate its nuclease activities to facilitate efficient processing of

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various bifurcated DNA intermediates. To date, 34 proteins from various DNA metabolic pathways have been identified as interacting with FEN1. Second, FEN1 was shown to localize to the nucleus in a DNA damage and cell cycle-dependent manner (11), implicating the dynamic nuclear localization of FEN1 as playing an important role in mediating its functions in DNA replication and repair. More recently, FEN1 was shown to super-accumulate in the nucleolus, where it is thought to maintain the stability of tandem repeats of ribosomal DNA (12). Furthermore, FEN1 was also shown to localize to the mitochondrion, where it plays an important role in mitochondrial DNA (mtDNA) replication and repair (13). Last, FEN1 can be acetylated, phosphorylated and methylated, and these post-translational modifications may be important for the regulation of the nuclease activities, protein partner selection and/or subcellular compartmentalization (14–16).

FEN1 displays a severe mutator phenotype in yeast when deleted (17), and therefore, functional deficiency of *FEN1* has been suggested to cause genomic instability and predisposition to cancer in mammals. Recently, a group of *FEN1* mutations was identified in human cancer specimens (9). Most of these mutations abrogated the ability of FEN1 to cleave model exonuclease (EXO) and gap-endonuclease substrates *in vitro*, while retaining the ability to cleave FEN substrates (9). To demonstrate the etiological significance of these somatic mutations, a mouse line that harbors an E160D Fen1 point mutation was generated to represent the mutations identified in human cancers (9). Selective elimination of the EXO and GEN hydrolytic specificities led to frequent spontaneous mutations and accumulation of incompletely digested DNA fragments in apoptotic cells. In addition, the mutant mice were predisposed to autoimmunity, chronic inflammation and cancers. Thus, the mutator phenotype results in the initiation of cancer, whereas chronic inflammation promotes cancer progression (9).

MULTIPLE STRUCTURE-SPECIFIC NUCLEASE ACTIVITIES AND THEIR BIOLOGICAL FUNCTIONS

Mammalian FEN1 was first characterized as DNase IV, an obligate 5′–3′ dsDNA exonuclease, during a survey of nucleases from rabbit tissues (18). After bacterial 5′–3′ exonucleases in eubacteria were discovered to be flap structure-specific nucleases (19), a mammalian FEN1 homolog was reported and determined to be the same as DNase IV, discovered >20 years earlier (20). Since then, much work to determine how FEN1 recognizes its substrates has been conducted (21–31). The predominantly accepted model for mammalian FEN1 has been the track-down model (27,32). Based on studies in which access to the 5′-end of the flap was blocked by streptavidin, FEN1 was postulated to initially recognize the free 5′-end of the flap structure and then track down the 5′-flap until reaching the double-stranded DNA (dsDNA) region where it then cleaved the scissile phosphate (27,32). Despite the popularity of the track-down model, more recent biochemical evidence suggests that

mammalian FEN1 first recognizes the dsDNA two-way junction, and then places the scissile phosphate in the active site by threading or clamping the single-stranded DNA (ssDNA) flap (22,33). The latter mechanism, first proposed by Joyce and co-workers for eubacterial FEN1 homologs (31), is more consistent with the ability of FEN1 to perform exonucleolytic cleavage and is likely the best description of the protein's substrate recognition mechanism *in vitro*. Furthermore, the latter model is also consistent with the notion that Rad2 nucleases are a family of DNA junction recognition enzymes (34).

In contrast to previous reports that the presence of dsDNA in the flap strand prevented FEN1 from cleaving (27,35), two separate studies determined that FEN1 has GEN activity, which cleaves single-stranded regions with gaps of four or more nucleotides (6,10). CRN-1, the FEN1 homolog in *Caenorhabditis elegans*, was shown to cleave the template strand of a gapped DNA duplex that resembled an intermediate DNA structure generated during apoptotic DNA fragmentation (6), whereas human FEN1 was found to cleave the template strand of gapped DNA fork and bubble substrates that mimic a stalled DNA replication fork. Furthermore, the presence of intrinsic FEN1 GEN activity was supported by mutational analysis. FEN1 mutations such as R70A, R326A/R327A, K244A/R245A at the dsDNA binding region abolished the EXO and/or GEN activities, but had little effect on the FEN activity (26). The E178A mutation, which occurs near the active center and the dsDNA binding region, was shown to eliminate 95% of the wild-type GEN activity, but retained most of its 5′-FEN activity (10). In contrast, another mutant, Q112R, identified in human cancer cells and located in the helical loop involved in ssDNA binding, was observed to increase GEN activity by 3-fold while retaining wild-type levels of the other two activities (9).

Characterization of the hydrolytic specificities of FEN1 has provided mechanistic insights into how a nuclease with a single active site can perform three separate activities. Kinetic analysis revealed that FEN1 displays its highest catalytic efficiency on the so-called double flap substrate, which is characterized by having a 5′-ssDNA flap of any length and a single nucleotide 3′-flap (26). Consistent with this, equilibrating flap substrates, which can adopt a double flap conformation, are thought to be the *in vivo* FEN1 substrate (21,25). In comparison to the catalytic efficiency on double flap substrates, the k_{cat}/K_M value for the EXO activity on a nick substrate (i.e. no 5′- or 3′-flap) was ~15-fold less than the FEN activity, whereas the efficiencies of the GEN activity on gapped-fork and gapped-duplex substrates were 6- and 250-fold less than the FEN activity, respectively (26). Despite the wide range of cleavage efficiencies, these three manners of hydrolyzation (FEN, EXO and GEN) all share a commonality: predominant incision 1 nt into the downstream dsDNA with minor cleavages 5′ or 3′ of this site. Because structural studies of FEN1 revealed that FEN1 contains two dsDNA-binding regions and a helical arch associated with ssDNA-binding (36), the ability of FEN1 to hydrolyze a wide range of substrates using the same active center was explained by

differences in the manner that FEN1 binds to various substrates (26). Moreover, cleavage patterns can be explained if the downstream dsDNA region (i.e. dsDNA that is incised) is bound by the H3TH motif with the active site in proximity to the scissile phosphate and subsequent placement of other substrate structural elements with the upstream dsDNA binding region and/or helical arch. In addition, the efficiency of cleavage could also be partially rationalized using this model (26). These findings support the concept that FEN1 employs distinct DNA substrate-binding modes to interact with different DNA substrates.

The ability of FEN1 to cleave nick and gapped-fork substrates is greatly enhanced by the addition of a 1 nt 3′-flap, similar to that observed with flap substrates (22). Recently, we and others have conducted more detailed kinetic studies with various FEN1 substrates with a 3′-flap. These studies suggest that the paramount structural feature of substrates recognized by FEN1 is a 3′-flap (22,30) rather than a 5′-flap (27). Although it was previously known that substrates bearing a 3′-flap were cleaved at a faster rate by FEN1 (37), the presence of the 3′-flap was further shown to increase single-turnover rates of catalysis in addition to its role in increasing enzyme-substrate affinity (22,30,37). Thus, occupation of 3′-flap binding pocket is not only critical for initial substrate capture, but for increasing subsequent first order rates of reaction after formation of the enzyme substrate complex by an unknown mechanism. With an increase in k_{cat} and concomitant decrease in K_M observed with double flap substrates, it is not surprising that the second order rate constants of reaction with double flap substrates approached levels suggesting that encounter of substrate and enzyme is rate-limiting.

FEN activity is critical for RNA primer removal and BER pathways

FEN activity, the most dominant activity of FEN1, is considered to play a major role in RNA primer removal during Okazaki fragment maturation of lagging strand DNA replication, as well as in removal of flap structures formed during long-patch BER. Details of the role of FEN1 in these two classical pathways were previously reviewed (1,4,38,39). It should be noted that the mechanisms of RNA primer removal in human and yeast are likely different. During lagging strand DNA synthesis in eukaryotic cells, Polα creates RNA/DNA primers containing ~10 nt of RNA and 10–20 nt of DNA to initiate synthesis of Okazaki fragments. In human and other mammalian cells, Polδ displaces the RNA/DNA primer to create a flap for FEN1 to cleave; however, the size of the flap generated by strand-displacement synthesis in mammals is still unknown. The essential role of FEN1 in RNA/DNA primer removal in mammalian cells has been validated by several *in vivo* studies using mouse models that showed that FEN1 deficiency causes defects in DNA replication, failure of cell proliferation and embryonic lethality (40–42). In contrast, deletion of *RAD27* (FEN1 yeast homolog) is tolerated in yeast, implicating the involvement of other nucleases in this process (43,44). Furthermore, recent studies suggest that long flap structures occur frequently during Okazaki fragment maturation in yeast. The displacement of flaps longer than 30 nt during Okazaki fragment maturation in yeast is envisioned to attract RPA, which inhibits FEN1, but recruits Dna2 endonuclease to cleave a large portion of the primer, as Dna2 can efficiently clamp onto the site. The cleavage of the long flap structure by the Dna2/RPA complex generates a short flap (∼5–7 nt) that resists binding and cleavage by RPA and Dna2, respectively (45). The FEN1 nuclease can then precisely remove the remaining flap to produce a substrate for ligation. However, the RPA-governed sequential actions of Dna2 and FEN1 for Okazaki fragment processing may only occur in yeast, and not higher eukaryotes. In mammalian cells, DNA2 predominantly migrates into mitochondria (46), and the residual nuclear DNA2 is not associated with DNA replication foci (46,47), suggesting that DNA2 is not a primary component in the DNA replication machinery. These findings support the hypothesis that FEN1 may be the primary nuclease in nuclear RNA primer removal in mammals, whereas coordinated actions of FEN1 and Dna2 are important in this process in yeast.

The FEN activity is also necessary for repairing DNA lesions that have an oxidatively-damaged sugar moiety, which cannot be removed by the dRP lyase of Polβ (13,48). In such a case, a few nucleotides are added by polymerase β or δ to produce a short flap, which is subsequently removed by FEN1 in complex with the processivity factor proliferating cell nuclear antigen (PCNA) (49). This alternative FEN1/PCNA-dependant BER pathway is termed long-patch BER (LP-BER) (49). A recent study by Wilson's group further suggested that the FEN and EXO activities of FEN1 might work together because the Polβ activity is only active on DNA duplexes that contain a gap, but not a nick. Therefore, the EXO activity of FEN1 may be important for cleaving the nick substrate and generating an optimal gap intermediate for Polβ during LP-BER (50). The short flap resulting from displacement DNA synthesis would then be removed by the FEN activity. In support of this model, the E160D FEN1 mutation, which specifically eliminates the EXO activity, causes mouse embryonic fibroblasts to be sensitive to DNA damaging agents such as methylmethane sulfonate (9).

Concerted action of EXO and GEN activities for resolution of DNA hairpin structures and apoptotic DNA fragmentation

The two other 'minor' activities of FEN1 (EXO and GEN) may be important for other DNA metabolic pathways, including resolution of tri-nucleotide repeat (TNR) sequence-derived secondary structures and apoptotic DNA fragmentation. Much evidence indicates that FEN1 efficiently cleaves ssDNA flaps, but inefficiently processes flaps that adopt secondary structures and lack a 3′-flap (27,51). However, absence of Fen1 in yeast results in a significant increase in TNR expansion (51). Furthermore, the expression level of FEN1 has recently

been shown to correlate with TNR expansion in mice (52). Therefore, three possibilities exist: (i) TNRs do not always form stable secondary structures, (ii) FEN1 uses an alternative approach to resolve the secondary structures (5,8) or (iii) increased unequal sister chromatid exchange, a phenotype associated with FEN1 deletion (53), causes TNR contraction and expansion. We have tested the hypothesis that concerted action of the EXO and GEN activities of FEN1 plays a role in resolution of secondary structures formed by $(CTG)_n$ and $(GAA)_n$ repeats (8). Using a yeast FEN1 point mutant, E176A, which is deficient in EXO and GEN activities but retains almost all of its FEN activity, we showed severe defects in the ability of this mutant to cleave various TNR intermediate substrates (8). Precise knock-in of the E176A point mutation in yeast caused an increase in both the expansion and fragility of a $(CTG)_n$ tract *in vivo* (8). Taken together, the combined biochemical and genetic analyses suggested that although FEN activity is important for ssDNA-flap processing, the EXO and GEN activities may also contribute to the suppression of TNR contraction and expansion. Therefore, we proposed the following model: in the presence of TNRs, which can base pair and fold back to form hairpin structures or loops, FEN1 endonucleolytic cleavage is suppressed. This increases the half-life of the flap, which can form an internal loop and ligate into the genome, resulting in TNR expansion. However, as a back-up approach, the EXO activity of FEN1 can remove the hairpin nucleotides in a gap or ssDNA/dsDNA junction, on which GEN cleavage can occur (Figure 1).

The finding that FEN1 plays an important role in suppression of TNR expansion in yeast has stirred great interest in exploring whether FEN1 is critical in destabilization of the TNR hairpin structures and whether deficiency of FEN1 in humans contributes to TNR-related genetic diseases, including Huntington disease (HD) (51,54). However, whether FEN1 deficiency plays a role in development of HD or other TNR-related diseases remains controversial. A survey for FEN1 mutations in human HD patients did not identify any mutations in this subset of patients (55). More recently, another study

showed that haploinsufficiency of FEN1 did not accelerate the expansion of $(CTG)n$ $(CAG)n$ (n: 105–110) repeats, which resemble the myotonic dystrophy type 1 disease locus, in mice (129Ola genetic background) (56). On the other hand, Yang and Freudenreich (57) showed that haploinsufficiency of FEN1 promotes expansion of $(CAG)_{120}$ repeats that mimic the human HD locus in mice (CBAxC57BL/6 genetic background. We note that these two TNR mouse models have different genetic backgrounds, which may contribute to the discrepancies in TNR phenotypes. It is likely that FEN1 mutations on their own may not significantly promote TNR expansions or contractions due to redundant pathways in maintenance of TNR stability, but in combination with other genetic mutations, FEN1 deficiency may contribute to TNR instabilities and related diseases in humans. Supporting this view, a previous study showed that microsatellite instabilities were not promoted in mice heterozygous for FEN1 knockout, but haploinsufficiency of FEN1 in a genetic background with an adenomatous polyposis coli (APC) gene mutation did significantly increase microsatellite instabilities (40).

The concerted action of the EXO and GEN activities of FEN1 may also play a role in oligonucleosomal fragmentation of chromosomes in apoptotic cells (58). In collaboration with the Xue group, we have shown that crn-1, a *C. elegans* homolog of human FEN1, is important for apoptosis (6). Reduction of CRN-1 activity by RNA interference resulted in cell death phenotypes similar to those displayed by a mutant lacking the mitochondrial endonuclease CPS-6/endonuclease G (Endo G). We proposed that CRN-1 associates and cooperates with CPS-6 to promote stepwise DNA fragmentation, whereby CPS-6 nicks the dsDNA and the EXO activity of CRN-1 subsequently removes a few nucleotides from the 5′-end to generate a gap. The GEN activity of CRN-1 then cuts the template strand at the DNA gap, leading to DNA fragmentation (Figure 1). Supporting this hypothesis, the EXO- and GEN-deficient FEN1 mutation, E160D, retards the disposal of apoptotic DNA and results in anti-dsDNA circulating in the blood system of mutant mice (9).
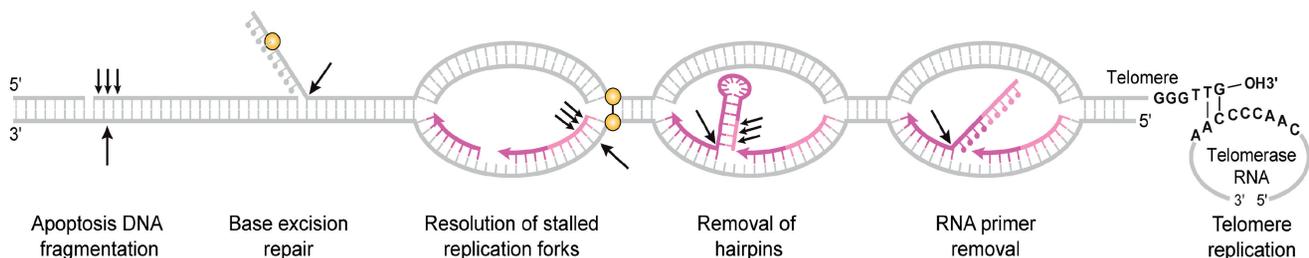
Figure 1. DNA metabolic pathways and corresponding biochemical activities of FEN1. FEN1 is implicated in six DNA metabolic pathways. Its FEN activity is essential for cleaving the single-stranded flap resulting from displacement DNA synthesis during RNA primer removal and in long-patch base excision repair. The 5′-EXO activity of FEN1 removes a few nucleotides from 5′-blunt ends or nicks to generate a gap, which can be cut by the gap-endonuclease (GEN) activity of FEN1 at the opposite template strand. The concerted action of the EXO and GEN activities produces DNA double strand breaks, which are important for removal of hairpin structures, rescue of stalled replication forks and apoptotic DNA fragmentation. FEN1 activities may also be important for maintenance of the telomere, but how they act at the chromosome end is unknown. Arrows: FEN1 nuclease cleavage sites; Red: newly synthesized RNA and DNA; yellow circles: damaged DNA or cross-links.

FEN1 functions in other DNA metabolic pathways

FEN1 has been implicated in the rescue of stalled replication forks caused by exogenous physical and chemical insults. FEN1 forms a complex with WRN in response to DNA damage that arrests replication forks (10,59), suggesting a role of FEN1 in resolution of stalled replication forks. Results from the Brosh group suggest that the FEN activity may be important for cleaving regressed replication forks to resolve the chicken foot structure (59). Alternatively, stalled replication forks may be restarted via the break-induced recombination pathway, in which a DNA double-stranded break (DSB) is created on the stalled replication fork to initiate downstream recombination cascades (60). ssDNA gaps that accumulate on stalled replication forks are potential targets for endonucleases to generate DSBs. In such a case, the GEN activity, which is dramatically stimulated by WRN, cleaves the fork to initiate the break-induced recombination (10). Human FEN1, but not the GEN-deficient mutant, E178A, is capable of rescuing yeast *fen1* null mutants from UV- and campothecin-induced DNA damage, indicating the importance of the GEN activity of FEN1 in restarting stalled replication forks. Furthermore, we have recently shown that deficiency in FEN1 nuclease activities in yeast also causes defects in replication of rDNA, in which DNA replication forks are frequently stalled by naturally occurring fork barriers such as DNA-binding proteins (12).

Several lines of biochemical and genetic evidence have suggested that FEN1 plays a role in maintenance of the integrity of GC-rich repeat DNA sequences, including CEB-1 minisatellites, rDNA regions and telomeres. Deletion of Rad27 in yeast causes instabilities of the human minisatellite locus CEB1 inserted into the yeast genome (61,62). Two models have been proposed to explain how FEN1 deficiency causes minisatellite expansions or contractions. In the first model, deficiency in the FEN activity leads to unprocessed 5′-flap structures. The GC-rich minisatellites, such as CEB1 sequences, that are prone to forming secondary structures, fold back and anneal to the template strand (61,62), although such secondary structure can be processed by the concerted action of EXO and GEN activities of FEN1 (8,26). Alternatively, the non-cleaved DNA flap may result in DNA DSBs and trigger improper DNA recombination events, leading to expansions or contractions (17,62).

Recently, FEN1 has also been suggested to contribute to telomere stability in yeast (63,64) and human cells (7,65,66). Abrogation of FEN1 function may influence the transformation of a cell into a cancer cell by compromising telomere stability and driving genomic instability. The Steward group analyzed the telomeres of human cancer cells after FEN1 depletion, and showed that FEN1 is required for telomere stability in cancer cells that rely on the alternative lengthening of telomere (ALT) mechanism (7,65). FEN1 depletion resulted in telomere dysfunction that was characterized by formation of telomere dysfunction-induced foci (TIFs) and end-to-end fusions in ALT-positive cells. In contrast, no telomere dysfunction was observed in telomerase-positive cells with FEN1 depletion, suggesting that ongoing telomerase activity protected telomeres. The FEN1 mutations affect telomere stability and genome fidelity by promoting telomere fusions and anaphase–bridge–breakage cycles, which further drive genome instability, thereby contributing to the transformation process (7,65). In addition, Chai and colleagues have shown complementary data indicating that FEN1 is in complex with telomerase *in vivo* (66), and have demonstrated that FEN1 deficiency in mouse embryonic fibroblasts leads to an increase in telomere end-to-end fusions. In cancer cells, FEN1 deficiency induces gradual shortening of telomeres, but does not alter the single-stranded G-overhangs (66). This is the first evidence that FEN1 and telomerase physically co-exist as a complex and that FEN1 can regulate telomerase activity at telomeres in mammalian cells (66). A recent study shows that FEN1, but not exonuclease 1, can cleave varying model substrates containing a telomeric G-quadruplex structure, suggesting that FEN1 may be important in resolution of G-quadruplex structures during replication of telomere sequences (67). Because FEN1 can cleave both DNA and RNA, we postulate that FEN1 may also function to remove the telomerase RNA template in the last cycle of telomere extension (Figure 1).

REGULATORY MECHANISMS

Localization as a major means of functional regulation of FEN1

Despite the wealth of information available on the biochemical functions of FEN1 and its roles in genome stability and cancer avoidance, cellular compartmentalization and dynamics that correspond to FEN1's involvement in various DNA metabolic pathways have not been fully elucidated. Previously, we demonstrated that FEN1 migrates into the nucleus in response to DNA damage and during S-phase of the cell cycle (Figure 2) (11). Furthermore, primary sequence analysis of eukaryotic FEN1 identified a bipartite C-terminal motif that is rich in positively-charged amino acid residues as a putative nuclear localization signal, which was validated by site-directed mutagenesis (11). More recently, we found that FEN1 super-accumulates in the nucleolus (Figure 2) and plays a role in the resolution of stalled DNA replication forks that form at sites of natural replication fork barriers (12).

High-resolution imaging of cells stained with anti-FEN1 antibodies revealed that FEN1 is also present in the mitochondria (Figure 2) (13,68). We conducted fluorescence microscopy on a C-myc-tagged FEN1 mutant protein ($Y^{83}H$) to clearly show that this mutant protein localized to mitochondria and not to nuclei (13). These data were complemented by cell fractionation experiments, which identified FEN1 in the mitochondrial extract. Further support for FEN1 migration into the mitochondrion comes from the work of Kalifa and Sia, demonstrating the mitochondrial localization of yeast FEN1 using immunohistochemical and genetic approaches (69).
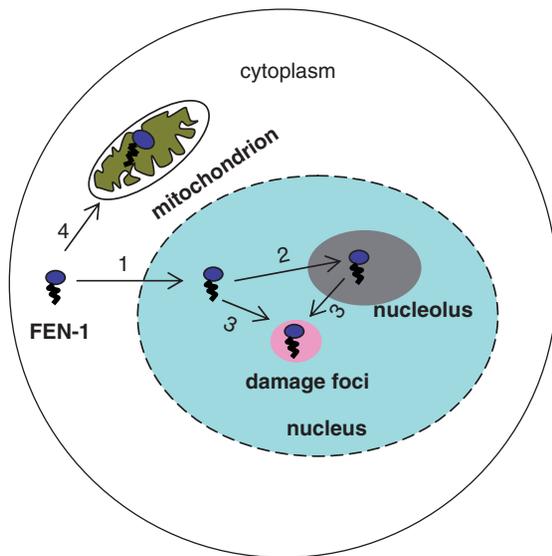
Figure 2. Dynamic subcellular localization of FEN1. FEN1 localizes to four distinct subcellular compartments in mammalian cells: cytoplasm, mitochondria, nuclear plasm and nucleolus. In response to cell cycle transitions and DNA damage, FEN1 dynamically migrates via four different routes: (i) nuclear translocation from the cytoplasm, (ii) accumulation in the nucleolus from the nuclear plasm; (iii) formation of replication/DNA damage foci from the nuclear plasm and nucleolus and (iv) mitochondrial import from the cytoplasm. The nuclease domain of FEN1 is represented by an oval, and the C-terminus is indicated as a zig-zag tail.

The replication and repair of mtDNA depends on many factors that are encoded in the nucleus, as the gene products of the mtDNA genome are limited to 22 tRNAs, 2 rRNA subunits and 13 proteins involved in energy production (70). All other genes required for mitochondrial function are encoded in the nuclear genome, and their gene products must be imported (70). Recently, other DNA replication and repair proteins encoded in the nuclear genome, in addition to FEN1, were shown to be localized in mitochondria in addition to the nucleus (13,71,72). Because reactive oxygen species are mainly generated in mitochondria, mtDNA is profoundly susceptible to oxidative damage, the accumulation of which is positively correlated with aging and age-related diseases like neurodegenerative disorders. Studies of mtDNA damage have shown that a significant portion of oxidative damage occurs on the deoxyribose moiety. Such damage results in the formation of deoxyribolactone, which if repaired by short-patch BER, gives rise to protein-DNA cross-links. Therefore, the majority of deoxyribolactone products are preferentially processed by LP-BER. More recently, we have shown that immunodepletion of FEN1 or DNA2 nuclease impairs the ability of mitochondrial extracts to process model substrates representing intermediate structures during RNA primer removal, suggesting that FEN1 plays a role in mtDNA replication as well. Because FEN1 is the nuclease responsible for flap cleavage in RNA primer removal and LP-BER, its presence in the mitochondria is not surprising. However, the mechanism by which it enters the mitochondrial matrix is unknown,

as it is devoid of known canonical mitochondrial targeting sequences. We suggest that FEN1, like DNA2 nuclease, may be imported into mitochondria via an internal mitochondrial targeting motif.

Protein–protein interaction: FEN1's functional complexes

The types of proteins with which FEN1 interacts suggest its pivotal role in several DNA metabolic pathways. We therefore carried out an extensive investigation of the network of all possible physical protein–protein interactions (PPIs) of human FEN1 and its homologs in *Saccharomyces cerevisiae* (Rad27) and *C. elegans* (CRN-1). Manual curation of PPIs from the literature began by species-specific identification of PPIs through the PPI database STRING (http://string-db.org). This method identified 105, 137 and 42 PPIs from human, *S. cerevisiae*, and *C. elegans*, respectively, with a default confidence of 0.4. We ultimately identified 34 PPI partners of FEN1 that were previously evaluated by various methods, including co-immunoprecipitation and yeast two hybrid. Based on the known effects of these interactions on the biochemical activities and biological functions of FEN1, its interacting partners can be grouped into four functional categories (Figure 3).

The first category includes protein partners that assist FEN1 in RNA primer removal during DNA replication. In eukaryotic cells, DNA synthesis is primed by the primase/polymerase-α (primosome), which synthesizes the RNA/DNA initiator primer (73). After primer synthesis, replication factor C (RFC) loads the homotrimeric ring-shaped protein known as PCNA onto dsDNA. PCNA acts as a sliding clamp molecular adaptor that localizes bound proteins to DNA. When the processive replisome encounters the downstream Okazaki fragment, a portion of the RNA/DNA primer is displaced to form a 5′-flap structure, which needs to be removed prior to ligating the remaining DNA segments. FEN1 interacts with PCNA, Pol α/ε, RPA, hnRNP A1 and DNA ligase I for efficient Okazaki fragment processing (74–78). RPA has been reported to inhibit FEN1, based on the 'sequential nuclease cleavage' model in yeast cells, in which DNA2 and FEN1 sequentially cleave the RNA primers (45).

FEN1 also interacts with members of the RecQ helicase family, including WRN, BLM and RecQ5. The possible physiological roles of these interactions with FEN1 in DNA replication and replication fork rescue have been discussed in detail elsewhere (59,79). In contrast to PCNA, Wrn protein stimulates the GEN activity, thereby cleaving the ssDNA region in the duplex DNA molecule (10). In addition, there is evidence that formation of a functional FEN1/Wrn complex is important for resolving stalled DNA replication forks. Moreover, phenotypes associated with yeast DNA2 mutants can be rescued by expression of BLM or WRN, which stimulate FEN1 activity (59,80). Therefore, it is interesting to speculate that the complex formed between FEN1 and either RecQ helicase is the functional homolog of DNA2. Furthermore, Imamura and Campbell have postulated that a helicase such as BLM may be necessary to facilitate
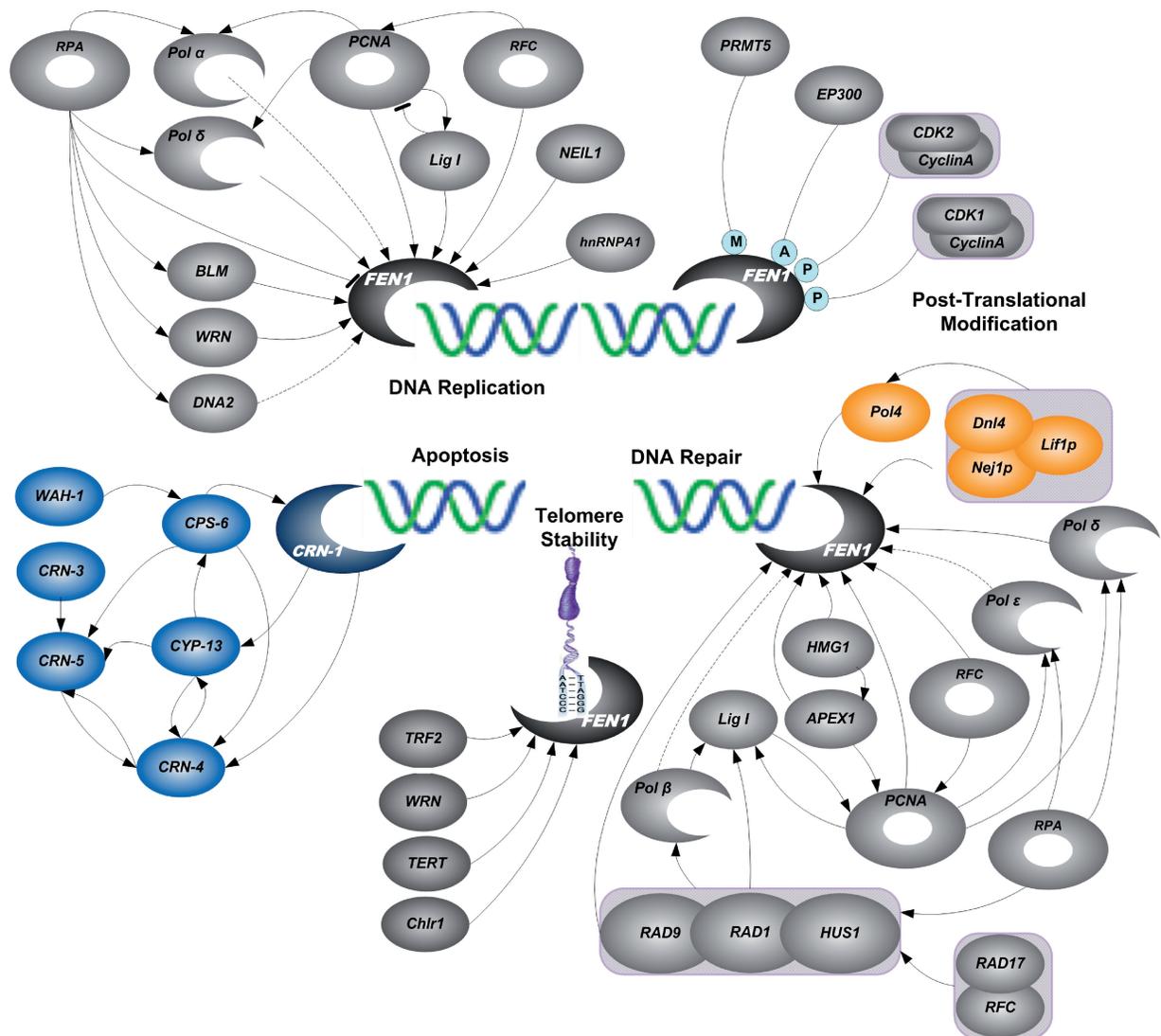
Figure 3. Protein–protein interactions mediate FEN1's actions in different DNA metabolic pathways. FEN1 interactive proteins were categorized into five functional groups: DNA replication, DNA repair, apoptotic DNA degradation, maintenance of telomere stabilities and post-translational modifications, based on the known biochemical activities and pathways of these proteins. Protein–protein interactions (PPIs) of FEN1 that occur in mammalian cells are colored in gray. The physical and functional interactions of FEN1 (Rad27) with Pol4 and FEN1 with Dnl4/Lif1, which are important for the processing of DNA ends in *S. cerevisiae* (91,117), are colored in yellow. Two nucleases, CPS-6 and CRN-1, the *C. elegans* homologs of EndoG and FEN1, respectively, have been implicated in mediating apoptotic DNA degradation in *C. elegans* (6,118) and are colored in blue. APEX1, AP endonuclease 1; BLM, bloom syndrome protein; CDK1, cyclin-dependent kinase 1; CDK2, cyclin-dependent kinase 2; CPS-6, ortholog of human mitochondrial endonuclease G (EndoG); CRN-1, cell-death-related nuclease family member (crn-1); CRN-3, cell-death-related nuclease family member (crn-3); CRN-4, cell-death-related nuclease family member (crn-4); CRN-5, cell-death-related nuclease family member (crn-5); Cyclin A, cyclin A2; CYP-13, cyclophilin homolog; DNA2L, DNA2-like helicase; Dnl4, DNA ligase IV homolog; EP300, E1A binding protein p300; FEN1, flap structure-specific endonuclease 1; hCHLR1, DEAD/H (Asp-Glu-Ala-Asp/His) box polypeptide 11; HMG1, high-mobility group box 1; HNRNPA1, heterogeneous nuclear ribonucleoprotein A1; Hus1, HUS1 checkpoint homolog; LIG1, DNA ligase 1; NEIL1, Nei endonuclease VIII-like 1; Nej1p, homologous to mammalian XRCC4 protein; PCNA, proliferating cell nuclear antigen; POL α, DNA polymerase α-catalytic subunit; POL β, DNA polymerase β; POL δ, DNA polymerase δ-catalytic subunit; POL ε, DNA polymerase ε, catalytic subunit A; Pol4, DNA polymerase IV; PRMT5, protein arginine methyltransferase 5; Rad1, DNA repair exonuclease rad1 homolog; Rad17, homolog of human and *S. pombe* Rad1 and *Ustilago maydis* Rec1 proteins; RAD27, 5′–3′ exonuclease, 5′-FEN; Rad9, DNA repair exonuclease rad9 homolog A; RFC, replication factor C; RPA, replication protein A; TERT, telomerase reverse transcriptase; TRF2, telomeric repeat binding factor 2; WAH-1, worm AIF homolog; WRN, Werner syndrome ATP-dependent helicase; Solid line, physical and functional interactions; dashed lines, genetic interactions; rightwards arrow: stimulatory to the enzyme activities; multimap, inhibitory to the enzyme activity; reverse assertion, post-translational modifications.

strand displacement synthesis (80), which suggests that the RecQ helicases might coordinate formation and FEN1-mediated removal of 5′-flap structures during Okazaki fragment maturation. When dealing with the ends of chromosomes, FEN1 interacts with proteins involved in telomere stability, which include TRF2, Wrn and TERT (7,66). Furthermore, FEN1 has also been shown to interact with Chlr1, a member of the DEAD/DEAH helicases in the chromosome cohesion complex (81), which implies that FEN1 may play a role in

communicating between the DNA replication and chromosome segregation machinery.

The second category of FEN1-interacting proteins is DNA repair proteins. In mammalian cells, single base lesions are repaired by single-nucleotide BER or LP-BER. Apurinic endonuclease 1 (APE1) recognizes the apurinic/apyrimidinic residues (AP) and cleaves 5′ to the AP sites after the actions of glycosylases such as Neil1, thereby generating a nick on which Pol β is loaded (82,83). If the lyase activity of Pol β cannot remove the deoxyribose moiety after it has added the complementary nucleotide to the free 3′-hydroxyl, strand displacement synthesis occurs, thereby creating a small 5′-flap (2–10 nt) that must be cleaved by FEN1 with subsequent ligation of the nick to complete the repair (48). The role of FEN1 in LP-BER is regulated and coordinated by physical interaction with important BER components such as Pol β, APE1, Lig 1 and PCNA (82,84,85). The LP-BER protein Neil1 has also been reported to interact and stimulate FEN1 nuclease activities *in vitro* (83). Furthermore, WRN has recently been shown *in vitro* to participate in LP-BER by facilitating Pol β strand-displacement synthesis via its helicase activity (86). Therefore, WRN's interaction with and stimulation of FEN1 may also be important for efficient 5′-flap removal in LP-BER. More recently, the FEN1/WRN complex was implicated in replication fork rescue through break-induced repair (10). FEN1 was also shown to interact with the human Rad9-Rad1-Hus1 checkpoint complex (9-1-1 complex) (87–89), a heterotrimeric protein that is similar to the PCNA toroidal sliding-clamp. Formation of this complex is stimulated by Rad17/RFC in yeast in a p53-dependent manner (90). Although the 9-1-1 complex is unable to stimulate Pol δ synthesis like PCNA, it can stimulate the FEN activity of FEN1 on flap substrates (89). The role of this interaction *in vivo* remains to be shown. Because 9-1-1 accumulates at sites of DNA damage, the interaction of FEN1 with 9-1-1 is likely important for stimulating FEN-1 activity for DNA repair. Recently, *Sc*FEN1 (RAD27) was shown to interact with Pol4 and Dnl4/Lif1, which are components of the non-homologous end-joining dsDNA break repair pathway (91). The various functional interactions between FEN1 and proteins involved in different DNA repair pathways indicates its wide range of roles in DNA repair.

The third category consists of apoptotic proteins, and is exemplified by Endo G. Parrish *et al.* (6) demonstrated that CRN-1, the *C. elegans* FEN1 homolog, in association with CPS-6 (Endo G), mediates stepwise DNA degradation during apoptosis. This is the first example in a burgeoning field that is sure to lead to the identification of additional apoptotic FEN1-interacting proteins across many species. Furthermore, Parrish *et al.* (6) have identified seven novel nucleases that play a role in apoptotic DNA fragmentation in *C. elegans*. Most of these proteins form a complex called a 'degradosome' to efficiently break down the chromosomes and nucleic acids during development (6). Association of FEN1 with apoptotic proteins such as Endo G may be a key mechanism to switch FEN1's role from DNA replication and repair to apoptotic DNA fragmentation. FEN1 interaction with Endo G also greatly enhances its minor GEN and EXO activities, important for disposal of apoptotic DNA.

The final category of FEN1-interaction partners are those proteins that post-translationally modify FEN1, including p300, Cdk1-Cyclin A, Cdk2-Cyclin A and PRMT5 (14–16). These proteins and their involvement in FEN1 function are discussed in detail in the next section.

Despite the wide variety of proteins, many of these PPIs are predominantly mediated via FEN1's C-terminal extension (88). In the absence of a protein partner, the extended C-terminus is predicted to be intrinsically disordered (DisEMBL http://dis.embl.de and PONDR http://www.pondr.com); thus, FEN1 joins a growing list of proteins that are mostly folded, but have regions of local disorder that mediate PPIs (92,93). Locally disordered regions of proteins are known to undergo disorder-to-order transitions upon binding a target. This allows for the formation of complexes with high specificity and relatively low affinity, which is important for proteins that must initiate an action and then dissociate when finished with that action. The disordered region of a protein also confers the ability to interact with multiple protein partners and modifying enzymes thereof using the same motif (92,93). Thus, the nucleolytic action of FEN1 in the cell is likely directed by different protein partners by binding initially to the C-terminal region of FEN1 as a means to sequester the protein to specific sites in the nucleoplasm. Although high affinity interaction is mediated by the extended C-terminus, a crystal structure of FEN1 in complex with PCNA revealed that core nuclease domain interactions also exist (94), but are probably too weak to mediate interactions strong enough to be detected by non-equilibrium binding assays. Therefore, many of the proteins identified as FEN1 interaction partners probably utilize the extended C-terminus, but interactions are likely not exclusive to this region.

Recently, we have determined the residues critical for FEN1 interaction with its five different interactive partners (88). Consistent with the role of a disordered region, each interactive partner of FEN1 requires a distinct combination of extended C-terminal residues for binding. For instance, the Q337, L340, D342, F343, F344, V346, S353 and S352 residues within and next to the PIP box of FEN1 are essential for the FEN1/PCNA interaction, whereas the K366, K367, K375, R378 and K380 residues are required for the FEN1/WRN interaction (88). On the other hand, the lysine residues, including K375 and K380, of FEN1 have been shown to be acetylated by p300 (14). The distinct PPI signatures at the C-terminal region of FEN1, together with post-translational modifications, may provide a mechanism to allow FEN1 to interact with different proteins and be recruited to different DNA metabolic machineries in response to DNA damage.

Simple deletion of a gene is not a high-resolution approach to study the biological significance of a PPI, because a single protein may interact with multiple partners. In addition, the complete deletion of an essential gene is often embryonically lethal in animals. Therefore, to obtain high-resolution biological information for a specific interaction, we take an alternative approach of

knocking-in rather than knocking-out identified mutations that specifically disrupt one interaction. Phe343 and Phe344 of FEN1 are key residues that mediate the interaction between the PIP-box of FEN1 and the interdomain connector loop motif of PCNA (49,95) (Figure 4A). Substitution of these two residues with two Ala residues (FFAA) abolishes the high affinity physical interaction between FEN1 and PCNA (49,95). Disruption of this interaction does not affect the *in vitro* nuclease activity of FEN1. Instead, it disrupts precise PCNA-mediated coordination among the polymerase, nuclease and ligase during the processes of Okazaki fragment maturation and LP-BER (Figure 4B). As a consequence, the FFAA mutation causes failure of recruitment of FEN1 to the replication foci and inhibits processing of Okazaki fragments, even when present only in the heterozygous form. Furthermore, heterozygous FFAA mutant mice develop aneuploidy-associated cancers, similar to what has been observed in human cancers (L. Zheng *et al.* unpublished data).

Post-translational modifications: a primary event for functional regulation

FEN1 is a target for at least three post-translational modifications *in vivo*: phosphorylation (15), acetylation (14,96) and methylation (16). The Cdk1-cyclin A or Cdk2/cyclin E complex phosphorylates FEN1 at Ser187 in late S phase, and *in vitro* Cdk1-cyclin A phosphorylation of FEN1 reduces the endonuclease and exonuclease activities of FEN1 without affecting DNA binding (15). In addition, phosphorylation of FEN1 abolishes PCNA binding (15). Serine phosphorylation is hypothesized to be one of the cell cycle regulatory mechanisms of FEN1 activities, and Ser187 is the only FEN1 residue that has been shown to be phosphorylated (15). In late S phase,

phosphorylation may be used to block FEN1's nuclease activities and its recruitment to the DNA replication site by PCNA. Therefore, this phosphorylation may contribute to halting the replication machinery, thereby ensuring exit from S phase (15). In addition, phosphorylation of FEN1 was also shown to regulate its nucleolar localization and to influence the dynamic roles of FEN1 in ribosomal DNA replication and damage repair (12). In response to UV irradiation and upon phosphorylation, FEN1 migrated to the nucleoplasm to participate in the resolution of UV crosslinks on DNA, most likely through the concerted action of its EXO and GEN activities (12). Based on yeast complementation experiments, the Ser187Asp mutation, which mimics constitutive phosphorylation, abolishes nucleolar accumulation of FEN1. On the other hand, replacement of Ser187 by Ala, which eliminates the only phosphorylation site, causes retention of FEN1 in the nucleoli. Both mutations cause UV sensitivity, impair cellular UV damage repair capacity and reduce overall cellular survival (12).

Furthermore, the methylation and phosphorylation of FEN1 offer a possible answer to one critical question of how FEN1 can efficiently bind to PCNA and DNA substrates, and then dissociate once the nuclease reaction is complete to avoid blockage of Lig1 reaction during DNA replication in the S phase of the cell cycle. Our recent discovery sheds light on this puzzle. We demonstrated that FEN1 is methylated at the R192 residue, and this methylation prevents FEN1 phosphorylation at the S187 residue (16). The methylated form of FEN1 interacts with PCNA, but the phosphorylated form of FEN1 causes prompt dissociation from PCNA, providing a mechanism for the nuclease to dynamically associate with and dissociate from PCNA, and thus, the DNA substrate (16). Based on our data, we postulate that in the early stages of
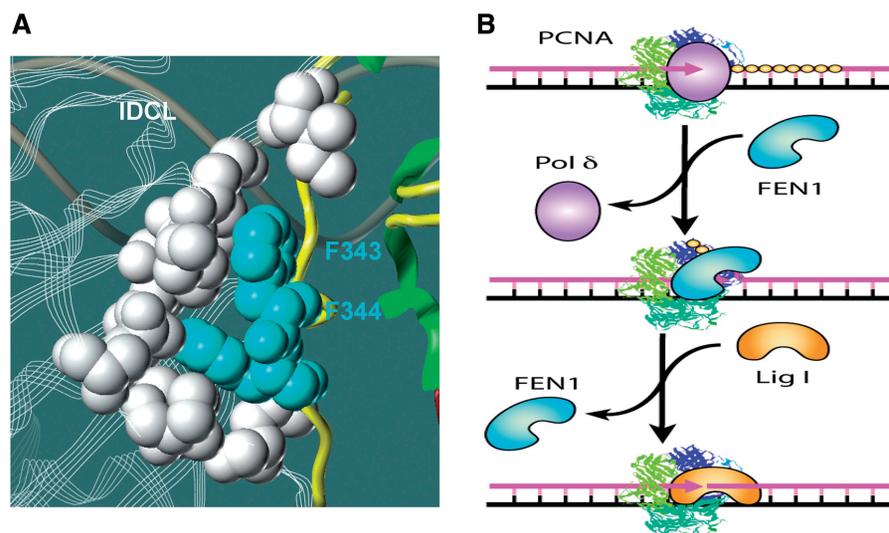
Figure 4. Model of the PCNA-mediated sequential actions of Pol δ, FEN1 and Lig I. (**A**) Structural basis for FEN1/PCNA interactions. Three dimensional structure demonstrating that F343 and F344 of FEN1 are key residues that mediate the physical interaction between FEN1 and PCNA. The structural model was based on crystal structures of the FEN1/PCNA complex (36,94). IDCL: interdomain connector loop. (**B**) Sequential recruitment of Pol δ, FEN1 and Lig 1 via PCNA interaction. The PCNA trimmer encircles the DNA duplex and serves as a platform for sequential recruitment of Pol δ, FEN1 and Lig I, which are key enzymes in Okazaki fragment maturation.

Okazaki fragment maturation, FEN1 exists in a methylated form, which enables it to replace Pol δ and access PCNA and the flap structure. Upon cleavage of the RNA primer flap, FEN1 is demethylated, thereby allowing the nuclease to be phosphorylated by a cell cycle-dependent kinase, cdk1 or cdk2. Phosphorylation of FEN1 causes the nuclease to fall off PCNA and the DNA nicks, leading to Lig1 recruitment and DNA ligation. However, the mechanism by which FEN1 is demethylated remains to be investigated.

Another intriguing question is how FEN1 phosphorylation influences the FEN1/PCNA interaction. Previous studies have demonstrated that phosphorylation may induce conformational changes and modulates protein–protein interactions (97–99). We suggest that phosphorylation of the S187 residue adds a negative charge to the embedded S187 residue, leading to exposure of the phosphorylated S187 residue. This may in turn stabilize a conformation whereby the positively-charged C-terminal random coil folds back to interact with the negatively-charged nuclease core domain, masking the interface for PCNA binding. As a consequence, phosphorylated FEN1 fails to interact with PCNA. This hypothesis is consistent with a previous study showing that phosphorylation of PTEN induced conformational changes that led to the masking of the PZD binding domain and disruption of the interaction between PTEN and PZD domain-containing proteins (98). In addition, the theory of phosphorylation-induced conformational change can also explain why FEN1 phosphorylation at the S187 residue abrogates FEN1 nucleolar localization. FEN1 co-localizes with WRN and nucleolin (C23) in nucleoli (12), and the interactions between FEN1 and nucleolar proteins such as WRN and nucleolin are important to retain the nuclease within nucleoli. However, because the C-terminal region is also the key element for interaction with WRN, folding back of the C-terminal region in phosphorylated FEN1 may also disrupt FEN1 interaction with WRN or other proteins, leading to migration out of nucleoli.

FEN1 can also be post-translationally modified by acetylation (14,100). Hasan *et al.* (14) observed an acetylated form of FEN1 in HeLa cells, and the amount of this form was enhanced upon UV treatment. The p300 acetyltransferase interacts with FEN1 *in vitro* and *in vivo* and acetylates FEN1 *in vitro* (14). Mass spectrometry analysis of *in vitro* acetylated FEN1 revealed four lysine sites located the very C-terminus of FEN1 that could be acetylated (K354, K375, K377 and K380) (14). *In vitro* acetylated FEN1 had reduced PCNA-independent nuclease activity and DNA binding affinity, but intact PCNA-binding and stimulating capacity (14,96). The inhibitory effect of FEN1 acetylation on its enzymatic activity is puzzling, considering that the nuclease activities of FEN1 are important for processing different DNA intermediate structures in various DNA metabolic processes. Hubscher and colleagues suggested that FEN1 acetylation functions to avoid premature processing of Okazaki fragments (14). Inhibition of FEN1 would promote the formation of longer flaps that require the two-nuclease pathway (DNA2-FEN1) (14). In support of this, Bambara and colleagues showed that p300 acetylates Dna2, thereby stimulating its 5′–3′ endonuclease, helicase and DNA-dependent ATPase activities, and enhancing its binding with DNA substrates (35). More recently, three acetylation sites, K80, K267, K375, were identified in FEN1 purified from human cells (100). Among these three *in vivo* acetylation sites of FEN1, two are different from the *in vitro* identified sites. The functional roles of these new acetylation sites remain unknown. Because K267 is close to the downstream dsDNA binding region, it could regulate DNA binding. Like K267, K80 is solvent exposed, but on the opposite side of the DNA binding interface. Therefore, like acetylation of K375 in the extended C-terminus, these acetylations may regulate PPIs.

FEN1 MUTATIONS LINK THE MUTATOR GENE TO CANCER

Functional deficiency of *FEN1* has been suggested to cause genomic instability and cancer predisposition, and the importance of FEN1 in the prevention of oncogenesis has recently been studied (40). In mice, homozygous knockout of *FEN1* is embryonically lethal, consistent with the observation that *Fen1* null mouse blastocysts are arrested in S phase (41). *Fen1* heterozygous knockout mice are viable and appear to be free of disease. However, *FEN1* heterozygous knockout mice that are also heterozygous for the *APC* gene develop adenocarcinomas that result in decreased survival (40). These results suggest that FEN1 is a tumor suppressor gene (101).

The initiation and development of cancer is considered a microcosm of the evolutionary process, a hallmark of which is the accumulation of numerous genetic abnormalities in multiple genes (102,103). Inherited mutations of specific genes, such as *BRCA1* or *BRCA2*, and mismatch repair genes, which result in susceptibility to breast cancer and colorectal cancer, respectively, account for only 20% of cancers (104). In most cancers, genetic changes arise by somatic mutations (104). Based on our understanding of the FEN1 nuclease, we predict that there could be four classes of FEN1 mutations that may be identified in cancer cells, which might impair (i) the nuclease activities, (ii) compartmentalization, (iii) protein–protein interactions or (iv) post-translational modification sites. Thus far, we have identified FEN1 mutations that eliminate the EXO and GEN activities, but retain the FEN activity (9). To demonstrate the etiological significance of these somatic mutations, we established the first mouse line harboring the E160D mutation, which interferes with FEN1 binding to the magnesium ion responsible for substrate binding and specifically abrogates the EXO and GEN activities of FEN1 (9,105). This mouse line was used to model the FEN1 mutations identified in human cancers (9). Selective elimination of nuclease activities in this mouse line led to frequent spontaneous mutations and accumulation of incompletely digested DNA fragments in apoptotic cells. Two independent studies demonstrated that the E160D mutant mice are

susceptible to the development of cancers (9,106). We further revealed that the mutator phenotype results in the initiation of cancer, whereas chronic inflammation promotes cancer progression (9). These findings support the view that FEN1 is required for genome stability and can therefore be considered a tumor suppressor gene (101).

The onset of tumorigenesis is rooted in the accumulation of genetic aberrations, such as point mutations, frame shifts, chromosomal defects and microsatellite expansions and contractions. The sequential progression from normal to malignant cells is accompanied by an increase in these alterations in cells, which continues within the heterogeneic tumor cell population during tumor growth. The mechanisms by which these errors accumulate are thought to be due in part to damage acquired by genes involved in genomic stability. This instability would result in further mutations to regulatory genes. The involvement of FEN1 in controlling genomic stability through multiple metabolic actions suggests that functional loss of FEN1 through mutations would facilitate further tumor mutagenesis.

Deficiency in the activity of FEN1 nuclease to degrade apoptotic DNA, on the other hand, can result in production of anti-DNA auto-antibodies and subsequently lead to autoimmune-related chronic inflammation, which may promote cancer progression, possibly via activation of NF-kB (9). The FEN1 E160D mutant mouse model will be useful for resolving additional issues in cancer biology, including the identification of essential mediators of chronic inflammation in lung tissue; the determination of whether suppression of inflammatory responses by altering cytokine profiling with NF-kB inhibitors, or by anti-inflammatory agents, reduces cancer incidence; and the establishment of whether E160D mutant mice, deficient in DNA repair and apoptotic DNA degradation, are more susceptible to tobacco-induced tumorigenesis. Answering these questions will be important to designing new cancer therapeutic regimens that act by the suppression of specific inflammatory responses.

Contrary to FEN1's role as a tumor suppressor, several studies from our and other groups suggest that FEN1 may also be required to support the growth and progression of cancers. FEN1 is detectable in all proliferative tissues, but is at very low levels in quiescent cells (107). Furthermore, FEN1 expression is elevated in cultured lung cancer cells as compared to normal cells (108), and FEN1 is over-expressed in many cancer types (109–112). In addition, the level of FEN1 expression in tumor tissues has been correlated with increased tumor grade and aggressiveness (109). Because FEN1 plays an essential role in DNA replication, it is thought that high levels of FEN1 are required to support hyperproliferation of cancer cells. Consistent with FEN1 over-expression in cancer tissues, several breast cancer cell lines have increased FEN1 expression at the mRNA and protein levels that correlate with decreased CpG2 methylation levels in comparison to matched normal tissues (111). Whether the epigenetic changes in the promoter region of FEN1, as mentioned above, occur in cancers other than breast cancer remains to be determined.

CONCLUDING REMARKS

Compelling evidence from *in vitro* and *in vivo* studies indicates that FEN1 is a multifunctional nuclease that participates in distinct DNA metabolic pathways. Interestingly, FEN1 can promote cancer in two very different ways: (i) mutation of the gene can result in genomic instability and initiate malignant transformation and (ii) overexpression of the gene confers a growth advantage to tumors. In response to the latter way to promote cancer, FEN1 has been suggested as cancer therapeutic target (113–116). However, like other current chemotherapeutics, inhibitors of FEN1 will likely be fraught with side effects and have the potential to propagate or even initiate new cancers. Nonetheless, such work should continue to increase our clinical cancer arsenal, with eventual hopes of novel drug delivery mechanisms.

Despite our substantial knowledge of FEN1 structure, biochemical activities and biological functions, many questions remain to be addressed. For instance, how does FEN1 dynamically translocate from one location to another; and in particular, how is FEN1 recruited to replication and repair foci to execute flap cleavage and subsequently dissociate from DNA substrates to avoid interference with Lig1-mediated DNA ligation? What is the mechanism for phosphate diester cleavage? How FEN1 is demethylated? How does FEN1 avoid constant phosphorylation and failure to interact with PCNA? How does FEN1 migrate into the mitochondrion for its function? Do FEN1 mutations that abolish FEN1 post-translational modifications, localization or a specific protein–protein interaction cause cancer and/or other diseases? Future studies will fill in the knowledge gaps regarding this structure-specific nuclease.

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