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Functional analysis of human FEN1 in *Saccharomyces cerevisiae* and its role in genome stability

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The flap endonuclease, FEN1, is an evolutionarily conserved component of DNA replication from archaebacteria to humans. Based on *in vitro* results, it processes Okazaki fragments during replication and is involved in base excision repair. FEN1 removes the last primer ribonucleotide on the lagging strand and it cleaves a 5′ flap that may result from strand displacement during replication or during base excision repair. Its biological importance has been revealed largely through studies in the yeast *Saccharomyces cerevisiae* where deletion of the homologous gene *RAD27* results in genome instability and mutagen sensitivity. While the *in vivo* function of Rad27 has been well characterized through genetic and biochemical approaches, little is understood about the *in vivo* functions of human FEN1. Guided by our recent results with yeast *RAD27*, we explored the function of human FEN1 in yeast. We found that the human FEN1 protein complements a yeast *rad27* null mutant for a variety of defects including mutagen sensitivity, genetic instability and the synthetic lethal interactions of a *rad27 rad51* and a *rad27 pol3-01* mutant. Furthermore, a mutant form of FEN1 lacking nuclease function exhibits dominant-negative effects on cell growth and genome instability similar to those seen with the homologous yeast *rad27* mutation. This genetic impact is stronger when the human and yeast PCNA-binding domains are exchanged. These data indicate that the human FEN1 and yeast Rad27 proteins act on the same substrate *in vivo*. Our study defines a sensitive yeast system for the identification and characterization of mutations in FEN1.

INTRODUCTION

Genome integrity is maintained by a combination of proteins associated with DNA replication, recombination and repair. These proteins act together, sometimes with overlapping function, to prevent and/or repair errors in DNA metabolism. Since failure of these systems can result in genome instability that leads to a variety of diseases, including cancer, it is important to understand the *in vivo* roles of DNA metabolic proteins and the consequences of mutations in these proteins.

In addition to DNA synthesis during S phase of the cell cycle, replication proteins are required during DNA recombination, DNA repair and correction of replication errors. To maintain genomic stability, many DNA polymerases, including DNA polymerase δ (Polδ), possess an intrinsic proofreading exonuclease function that reduces the level of errors during replication. In addition, there are many DNA endo- and exonucleases implicated in correction of replication errors, removal of DNA damage and recombination. The human 5′ flap endonuclease, FEN1, has been proposed to function in all of these processes based on *in vitro* results (1–5).

During semi-conservative replication, DNA synthesis is initiated at an RNA primer that is synthesized by the primase working in complex with DNA polymerase α (Polα). DNA synthesis is not complete until the RNA primers are removed by a combination of RNase H and FEN1 or by FEN1 alone. FEN1 can also remove the displaced flap that may be generated during lagging strand replication when the 3′ end of a newly synthesized Okazaki fragment meets the 5′ end of a previously synthesized fragment (6–9). The ability of FEN1 endonuclease to remove such flap structures also accounts for its role in long patch base excision repair (BER) (1,10–13). In this process, following incision at the 5′ side of the abasic site, repair synthesis displaces the DNA strand creating a flap structure that is removed by a flap endonuclease.

These *in vitro* observations are supported by biochemical and genetic results with microorganisms, in particular the yeast *Saccharomyces cerevisiae*, whose Rad27/Rth1/YLK510 protein (2,3,14) exhibits 61% amino acid identity with human FEN1. Biochemically, the Rad27 nuclease is comparable with FEN1 in that it is a structure-specific nuclease and the yeast protein can act on flap structures *in vitro* like the human protein (15). Defects in various nucleases can lead to elevated recombination and mutation rates and characteristic mutation spectra (16–22). The genetic consequences of a *rad27* deletion (*rad27*-Δ) are dramatic, with a 50-fold increase in forward mutations inactivating the *CAN1* gene (19) and up to a 280-fold increase in mutations within simple repetitive DNA (23). Many of the forward mutations, that arise by duplications involving small direct repeats, and microsatellite changes are due primarily to increases in repeat length (17,19,23–25). These results are in contrast to other nuclease mutants that display both insertions and deletions (e.g. *pol3-01*) (26) or

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mainly deletions (e.g. *exo1*) (22). A *rad27* mutant also results in greatly increased rates of recombination (15,19,27,28), reduced telomere stability (29) and increased UV sensitivity (30).

Recent work in yeast has shown that genome stability is adversely affected by synergistic mutations in the replication, recombination and repair pathways. Synthetic lethal interactions have been observed for a deletion mutation in *RAD27* (*rad27*-Δ) and mutations in other genes that function in replication and repair. Included among these mutants are a DNA helicase, *dna2-1* (31), Polδ with defective proofreading function, *pol3-01* (17), a replication factor, *rfc::Tn3* (32), mutants in all of the double-strand break repair genes, *rad50–rad59* (19,33), the 5′ exonuclease, *exo1* (19), and a double mutant of the cyclin cell cycle control genes (*cln1* and *cln2*) (28). Less dramatic results are seen with other repair mutants in combination with *rad27*-Δ. For example, the combination of a *rad27*-Δ and a defect in DNA polymerase ε proofreading, *pol2-4*, shows synergistically elevated mutation rates (15). Strong interactions can be detected not only with a null allele of *rad27*, but even with a point mutation in *rad27* whose defect is barely detectable, and DNA Polδ mutations (15). Thus, the Rad27/FEN1 protein can be pivotal in many aspects of chromosome biology.

Proteins that function in highly conserved metabolic processes are well represented among those human genes capable of complementing a defect in the homologous gene in yeast, e.g. *CCND1* (34), *HHR6A/B* (35) and *XPD* (36). Among these conserved processes are several genes responsible for DNA repair, e.g. *OGG1* (37), Ku 70 (38), *APE* (39) and *MGMT* (40). The FEN1 nuclease is evolutionarily conserved and has been isolated and characterized from many organisms including human, mouse, cow, *Xenopus*, *Schizosaccharomyces pombe*, *Saccharomyces cerevisiae* and the archaebacteria *Methanococcus jannaschii*, *Pyrococcus furiouis* and *Archaeglobus fulgidus* (2,10,41–50). *Xenopus* (GenBank accession no. AAD02814) and human (Genbank accession no. CAA54166) FEN1 proteins share 55.2 and 60.8% identity with the yeast Rad27 protein (GenBank accession no. P26793), respectively, using a pairwise amino acid sequence comparison (SIM at http://www.hgsc.bcm.tmc.edu/SearchLauncher/) (51). The function of these proteins appears to be conserved based on the *in vitro* binding data and the ability of *Xenopus* and human FEN1 proteins to complement the sensitivity of an *S.cerevisiae* *rad27*-Δ mutant to the DNA alkylating agent methyl methane sulfonate (MMS) (42,52). This suggests that heterologous FEN1 proteins can substitute for yeast Rad27 in BER. Prior to the present study, there was no information about the ability of heterologous FEN1 to function in other aspects of DNA metabolism.

Given the central role of FEN1/Rad27 in many aspects of chromosome biology, we have explored the ability of human *FEN1* to complement a variety of defects in chromosome metabolism associated with a *rad27*-Δ mutant. The human FEN1 protein is not only capable of complementing the sensitivity to MMS, as was shown by Frank *et al.* (52), but it also complements the synthetic lethality between *rad27* and repair or replication mutants. Moreover, human FEN1 compensates for the *rad27*-Δ defect that leads to genetic instabilities, including forward mutations, microsatellite instability and elevated recombination rates. We also discovered that both yeast Rad27 and human FEN1 proteins can be genotoxic when they carry a mutation eliminating their nuclease activity. This genotoxic effect was observed even in the presence of wild-type *RAD27*. We found that the proliferating cell nuclear antigen (PCNA)-binding domain is important for the negative impact of the nuclease mutant based on domain swapping with the yeast protein. The yeast systems developed provide for the functional analysis of FEN1 and characterization of mutants, including the identification of dominant, genotoxic alleles of this protein.

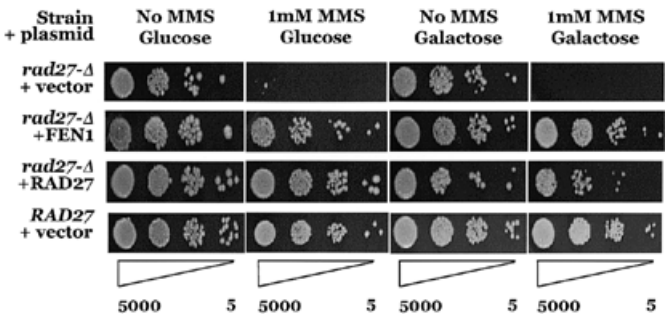

Figure 1. Human *FEN1* complements the MMS sensitivity of a *rad27* null strain. Ten-fold serial dilutions of cultures of a *rad27* mutant containing a *URA3* plasmid with a *RAD27* or *FEN1* were plated to media as indicated.

RESULTS

Human FEN1 complements the MMS sensitivity of a yeast *rad27*-Δ mutant

There are many examples in which human genes associated with conserved DNA metabolic systems can complement the corresponding defect in yeast. We examined the ability of human *FEN1* to complement the sensitivity of a *rad27*-Δ strain to the DNA alkylating agent MMS (gene and protein designations are summarized in Materials and Methods). As shown in Figure 1, the yeast *rad27*-Δ mutant is extremely sensitive to a low dose of MMS. This defect is fully complemented when human *FEN1* is highly expressed by induction with galactose, similar to the observations of Frank *et al.* (52). We found that survival on MMS is increased ~1000-fold when there is reduced expression of human FEN1 or Rad27 (i.e. growth on glucose media, which represses the *GAL1* promoter) (Fig. 1). Interestingly, high levels of FEN1 expression (on galactose) increased survival to that of a wild-type *RAD27* strain while high levels of Rad27 caused a reproducible reduction in viability and a 2- to 3-fold increase in MMS sensitivity. This indicates that although the FEN1 protein can substitute for Rad27, it is not functionally identical (see below and Discussion).

FEN1 complements *rad27* synthetic lethality with *rad51* or *pol3-01*

As noted in the Introduction, several of the genetic interactions of *rad27* have been identified by the synthetic lethal phenotype of double mutants. In order to assess the ability of *FEN1* to replace multiple functions of yeast *RAD27*, we tested it for complementation of the synthetic lethality phenotype

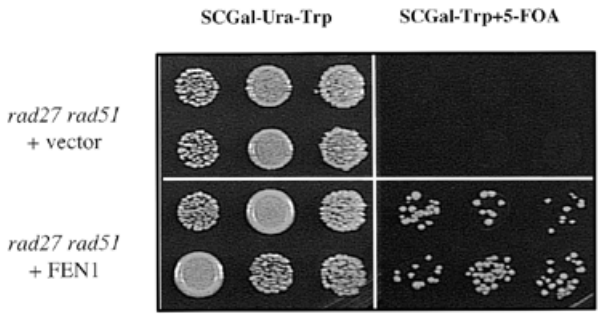

Figure 2. Human *FEN1* complements the synthetic lethality of a *rad27 rad51* strain. A *rad27 rad51* strain with a *URA3* plasmid containing the yeast *RAD51* gene was transformed with either a *TRP1* control vector or a *TRP1* plasmid containing human *FEN1*. Twelve independent transformants from each transformation were plated to medium selecting for (SC +Gal –Ura –Trp) and against (SC +Gal –Trp +5-FOA) the yeast *RAD51* plasmid. Approximately $3–5 \times 10^4$ cells were plated for each transformant. Six transformants for each transformation are shown in the figure.

displayed in a *rad27 rad51* and a *rad27 pol3-01* strain. Tishkoff *et al.* (19) proposed that lesions arising in a *rad27* strain are repaired by recombination utilizing *RAD51* functions. The mutation in *pol3-01* is in the 3′→5′ exonuclease domain and eliminates proofreading function of this polymerase (53). The synthetic lethality of a *pol3-01 rad27*-Δ strain could be due to excessive replication errors and/or the presence of abnormal structures during lagging strand replication (15,17).

A plasmid shuffle approach (54) was utilized to exchange a plasmid containing *RAD51* expressed from a *GAL1* promoter (pGALRAD51) with a plasmid containing human *FEN1* (YEp112SpGALFEN1) also expressed from a *GAL1* promoter, in a *rad27 rad51* strain. pGALRAD51 contains the *URA3* gene, which allows this strain to grow on medium lacking uracil. The *rad27 rad51* (pGALRAD51) strain was transformed with YEp112SpGALFEN1, which contains a *TRP1* selectable marker, or YEp112SpGAL, a control plasmid lacking *FEN1*. Twelve independent transformants from each plasmid were grown on two types of medium. The first medium selected for both plasmids (SC +Gal –Ura –Trp) and the second selected for the *FEN1*-containing plasmid but had counter-selection against the yeast *RAD51*-containing plasmid [SC +Gal –Trp +5-fluoro-orotic acid (5-FOA)]. Cells that are Ura^+ are poisoned by 5-FOA so that the SC +Gal –Trp +5-FOA medium selects for the growth of cells that have lost the *URA3*-containing plasmid. Survival of cells on this medium would indicate the ability of the *TRP1*-containing plasmid (either YEp112SpGALFEN1 or YEp112SpGAL) to complement function in the *rad27 rad51* double mutant. The transformants containing the *TRP1* vector control plasmid required the *RAD51* gene based on the lack of colonies on SC +Gal –Trp +5-FOA (Fig. 2). The transformants containing human *FEN1* could give rise to colonies on the YEp112SpGAL medium, which selects against the *URA3* plasmid containing *RAD51* (Fig. 2). This demonstrates that the human FEN1 protein can complement the synthetic lethality of a *rad27 rad51* double mutation.

A plasmid loss assay, similar to that of Gary *et al.* (15), was employed to assess *FEN1* complementation of the *rad27 pol3-01* synthetic lethality. A *rad27 pol3-01* strain containing *RAD27* on a *TRP1*-based plasmid (LC-80B) and regulated by its natural promoter was transformed with *URA3* plasmids that contain one of the following inserts under *GAL1* control: *RAD27*, human *FEN1*, a *FEN1/RAD27* chimera (FEN1-Ch) (Fig. 3, see Materials and Methods) or no additional gene. We assessed the loss of the *RAD27-TRP1* plasmid after >20 generations of growth in SC +Gal –Ura, which selects only for the presence of the inducible *FEN1* or *RAD27* construct. The human FEN1 protein expressed from the *GAL* plasmid was capable of restoring viability to the *rad27 pol3-01* mutant as well as Rad27, as demonstrated by the presence of Trp^- colonies after the non-selective growth (Table 1).

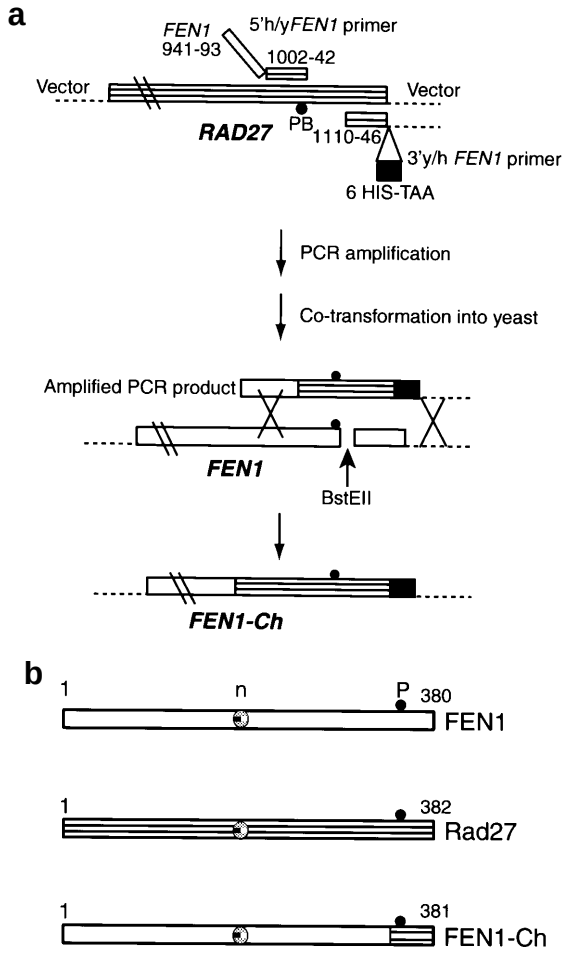

Figure 3. Construction of the human–yeast chimeric *FEN1* gene. (**a**) The C-terminal end of *RAD27*, including the conserved PCNA-binding sequence (PB), was amplified with PCR primers as indicated in the diagram. This PCR product was then used to repair *Bst*EII-digested YEp195SpGALFEN1 by homologous recombination in yeast following co-transformation. The resulting plasmid, YEp195SpGALFEN1-Ch, encodes a chimeric protein with the first 331 amino acids from FEN1 and the last 48 amino acids from Rad27. The sequences in this figure are not drawn to scale. The 6-His tag identified as an insert at the top of (a) is shown as a small black box in the lower portions of (b). (**b**) Diagrams of the FEN1, Rad27 and FEN1-Ch proteins are shown. The numbers refer to the number of amino acids, the closed circle designates the location of the conserved PCNA-binding sequence and the hatched oval represents the location of the nuclease mutation used in this study.

Table 1. Ability of various mutants to lose a *TRP1* plasmid, containing the wild-type *RAD27* gene (LC-80B)

Strain genotype (plasmid)	No. of isolates	Loss of *TRP1* marker linked to *RAD27* (%)[a]	Ability to compensate synthetic lethality
rad27 pol3-01 (LC-80B + vector)	5	0	NA
rad27 pol3-01(LC-80B + RAD27)	6	58–85	+
rad27 pol3-01 (LC-80B + FEN1)	5	44–62	+
rad27 pol3-01 (LC-80B + FEN1-Ch)	5	28–51	+

NA, not applicable.
[a]Minimal and maximal values of frequencies of the *TRP1* loss are shown.

Table 2. Rates[a] ($\times 10^7$) of interchromosomal recombination, microsatellite instability and mutation in *rad27*-Δ and *RAD27* strains expressing (on galactose) wild-type, nuclease-defective and chimeric *FEN1* constructs

Strain + plasmid	Recombination	Mutations in microsatellite[b]	Forward mutation at *CAN1*
rad27-Δ + vector	9.4	18.6	141
	(6.6–11.7)[c]	(12–23)	(115–215)
rad27-Δ + RAD27	2.3	18.5	11.4
	(1.9–2.7)	(17–21)	(7.8–17.1)
rad27-Δ + FEN1	2.3	2.3	20.6
	(1.6–5.7)	(1.4–11.9)	(4.9–56)
rad27-Δ + FEN1-Ch	2.8	4.9	16.3
	(1.7–4.2)	(3.7–10.9)	(5.6–58)
RAD27 + vector	0.9	0.9	3.0
	(0.7–1.8)	(0.7–1.3)	(2.1–3.6)
RAD27 + RAD27	2.2	18.5	14.5
	(1.7–2.8)	(13–31)	(11–25)
RAD27 + FEN1	0.8	1.4	2.9
	(0.7–1.0)	(1.0–3.0)	(2.1–6.8)
RAD27 + FEN1-Ch	0.8	5.1	3.3
	(0.6–0.9)	(3.1–6.3)	(2.4–4.1)
RAD27 + rad27-n	21	88	35
	(18–51)	(51–119)	(21–51)
RAD27 + FEN1-n	2.2	6.1	5.5
	(1.8–3.8)	(3.8–7.0)	(5.2–13.1)
RAD27 + FEN1-n,Ch	5.6	20	12.6
	(4.5–8.4)	(16–32)	(11–20)
RAD27 + rad27-p	0.7	1.2	1.0
	(0.6–3.2)	(0.8–1.7)	(0.1–2.9)
RAD27 + rad27-n,p	2.1	5.3	6.0
	(1.0–4.2)	(3.8–6.7)	(4.5–8.3)

[a]Rates were determined from the frequencies of at least 10 cultures using the method of the median.
[b]A +1 'frameshift' within a homonucleotide run of 12 A residues in the *LYS2* gene results in a Lys^+ phenotype.
[c]95% confidence interval in parentheses.

FEN1 complements genetic defects of a *rad27* mutant

A yeast *rad27* mutant exhibits genomic instability, including high rates of recombination and mutation (15,17,19,23). Mutants exhibit preferential increases in the size of microsatellite DNAs (17,19,24,25), suggesting that they might provide insight into several diseases that arise by microsatellite expansions (e.g. Huntington's disease). We addressed the ability of human *FEN1* to complement the genomic instability of a yeast *rad27* strain using three genetic assays. The first measures homologous recombination between *LYS2* alleles on chromosomes II and III. The second assay detects inactivating mutations in *CAN1* that result in canavanine resistance (Can^R). Finally, microsatellite instability was examined in an A_{12} homonucleotide run within the *LYS2* gene where revertants

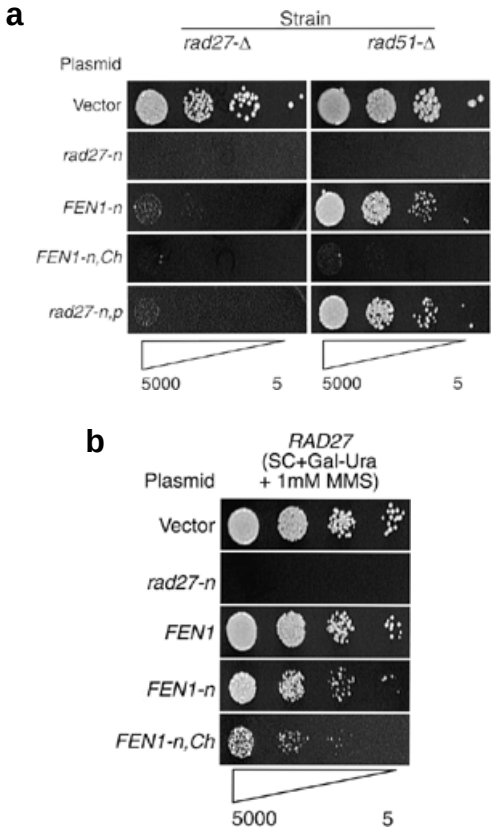

Figure 4. The impact of expressed human *FEN1* and *rad27* nuclease mutants on growth of *rad51* and *rad27* strains. (**a**) A *FEN1-n* mutant causes similar growth defects to those seen with a *rad27-n* strain. The impact of a PCNA-binding mutation on a nuclease-defective Rad27 protein is also presented. Ten-fold serial dilutions of transformants are pictured for each *RAD27*- or *FEN1*-containing plasmid. The replacement of the yeast C-terminus with a *FEN1-n* construct (FEN1-n,Ch) increases the negative impact of the FEN1-n protein. (**b**) The impact of expressed (galactose) Rad27-n, FEN1-n and FEN1-n,Ch on the response of a *RAD27* strain to 1 mM MMS. Shown are 1/10 serial dilutions.

have been demonstrated to occur predominantly by a +1 frameshift mutation (26).

The expression of FEN1 in a *rad27*-Δ mutant reduced recombination between *lys2* alleles and mutation at the homonucleotide run to nearly the level of a wild-type *RAD27* strain, and the *CAN1* forward mutation rate was also reduced 7-fold (Table 2). This indicates that the human FEN1 protein is capable of replacing most of the functions of Rad27 in DNA replication. It reverses the genome instability of a *rad27* mutant, in addition to complementing its MMS sensitivity and synthetic lethality. Taken together, these data indicate that the human FEN1 protein can replace both the BER and the replication functions of the yeast Rad27 protein in yeast.

It is interesting that overexpressed *RAD27* fails to reduce the level of microsatellite +1 mutations of a *rad27*-Δ strain and it increases the instability in a Rad$^+$ strain. In contrast, highly expressed FEN1 complements the mutator phenotype of a *rad27*-Δ strain and does not show any mutator effect in a RAD$^+$ strain (Table 2). In addition to reduced MMS resistance (Fig. 1), this is the second deleterious effect observed with overexpressed Rad27 protein that is not seen with overexpressed FEN1 protein.

A *FEN1* mutant lacking nuclease activity causes growth inhibition

The human FEN1 D181A mutant protein (FEN1-n) completely lacks nuclease activity *in vitro* but is still able to bind a flap substrate (55,56). Both *rad27* and *rad51* deletion strains have severe growth defects in response to expression of the homologous mutant protein, Rad27-n (Fig. 4a) (15). The expression of Rad27-n in a Rad$^+$ strain results in a 10 000-fold increase in sensitivity to 1 mM MMS and greatly increased genetic instability (Fig. 4b, Table 2). Since the wild-type FEN1 can complement several features of a *rad27*-Δ mutant, we compared the consequences of a nuclease mutation of FEN1 protein with its homologous yeast *rad27-n* mutation.

We found that similarly to Rad27-n, overexpressed FEN1-n reduces the viability of a *rad27* strain 100-fold. However, contrary to the effect seen with Rad27-n, it has little effect on the viability of a *rad51* strain (Fig. 4b, Table 2). Furthermore, expression of FEN1-n in a *RAD27* wild-type strain led to only a small increase in MMS sensitivity and genetic instability (Fig. 4b). Thus, whereas the impact of overexpressed human FEN1-n is less, the pattern is similar to that of overexpressed Rad27-n.

The FEN1 and Rad27-p proteins have similar genetic consequences

Since FEN1 interacts with PCNA and there is divergence between yeast and human 5′ flap endonucleases, it is possible that the human FEN1-binding site does not interact with yeast PCNA. To examine this, we compared the results of our genetic tests for yeast RAD27 with those of Rad27-p, a PCNA binding mutant, and FEN1. High levels of Rad27 caused genetic instability in both wild-type and *rad27* strains (Table 2) and viability was reduced to 24 and 31%, respectively. However, expression of Rad27-p did not reduce viability in either strain and showed no significant effect in our genetic assays. Similarly to the Rad27-p mutant, overexpression of FEN1 does not reduce viability nor does it reduce genetic stability (Table 2).

Expression of FEN1-n reduces viability of a *rad27* strain 100-fold but does not affect growth of a *rad51* mutant (Fig. 4a). These results with expressed FEN1-n in *rad27* and *rad51* mutants are more reflective of the phenotype seen with overexpression of a Rad27 nuclease-defective protein that also lacks PCNA binding (*rad27-n,p*) than of Rad27-n expression (Fig. 4a) (15, this study). Viability was reduced >5000-fold in a *rad27* mutant expressing Rad27-n, whereas Rad27-n,p and FEN1-n showed a 100-fold reduction (Fig. 4a). There was no loss of viability in *rad51* strains expressing FEN1-n or Rad27-n,p, in contrast to the inviability resulting from Rad27-n overexpression (Fig. 4a).

These results suggest that the human FEN1 and FEN1-n proteins function in a manner more similar to *rad27-p* and *rad27-n,p* mutants, respectively. It is possible that the interaction between FEN1 and yeast PCNA *in vivo* does not stabilize the FEN1 protein at the flap as well as the interaction between Rad27 and yeast PCNA. This is supported by the *in vitro* data from Wu *et al.* (5) which showed that human FEN1 nuclease activity was not stimulated by yeast PCNA. Therefore, in a wild-type strain background, the yeast Rad27 protein could readily displace FEN1-n, thereby eliminating the nega-

Table 3. Conserved motifs in Rad27/FEN1 proteins

Species	Nuclease motif[a]	PCNA-binding motif[b]
Homo sapiens	AAATEDMDCL	QGRLDDFF
Saccharomyces cerevisiae	AAASEDMDTL	QGRLDGFF
Pyrococcus furiosis	ASASQDYDSL	QSTLESWF

[a]Conserved sequence in the region of aspartic acid residues (underlined) required for metal ion positioning. The 10 amino acids of *H.sapiens* correspond to positions 174–183.
[b]Conserved sequence defined in Warbrick (57) and the homologous sequence in *S.cerevisiae* and *P.furiosis*.

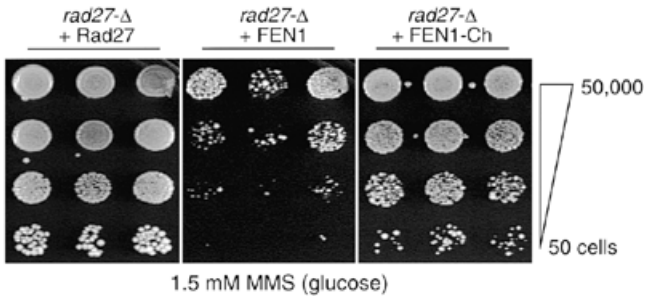

Figure 5. A chimeric FEN1 containing the C-terminal region of yeast Rad27 complements the MMS sensitivity of a *rad27*-Δ mutant. Ten-fold serial dilutions of *rad27*-Δ mutants expressing Rad27, FEN1 or FEN1-Ch proteins on glucose plates containing 1.5 mM MMS. Each vertical series corresponds to a different isolate.

tive impact seen in a *rad27*-Δ strain. We therefore attempted to modify FEN1 to make it more yeast-like.

A FEN1/Rad27 chimeric protein enhances FEN1 function in yeast

Since the human and yeast FEN1/Rad27 proteins are 39% diverged and the yeast and human PCNA molecules are 65% diverged, we created chimeras between the human and yeast FEN1/Rad27 proteins in the PCNA-binding regions (Fig. 3, see Materials and Methods). Based on *in vitro* data from other species (57,58) and the crystal structure of the highly conserved *Pyrococcus furiosis* FEN1 (45), the C-terminal region of the FEN1 protein is a separate domain and is likely to interact with PCNA. The chimera, FEN1-Ch, was comprised of the 331 amino acid FEN1 N-terminus, containing the catalytic regions, and the yeast C-terminus. This FEN1-Ch arrangement leaves both the DNA flap-binding region and catalytic domains unaltered, while the basic C-terminal tail, which associates with PCNA, is replaced with yeast sequence (the last 49 amino acids of human FEN1 were replaced by the last 48 amino acids from the yeast protein) (Fig. 3b, Table 3).

Both overexpressed FEN1-Ch and wild-type FEN1 complement most genetic defects of a *rad27*-Δ strain (Table 2, data not shown). Under low expression conditions and at MMS levels 50% higher than those shown previously (Fig. 1), FEN1 complementation was deficient (Fig. 5). However, FEN1-Ch, unlike FEN1, was comparable with Rad27 in its ability to complement the MMS sensitivity of the *rad27*-Δ mutant on medium containing 1.5 mM MMS and glucose as a sugar source (Fig. 5). This subtle difference between FEN1 and the FEN1-Ch in their ability to complement, along with the improved rescue of a *pol3-01 rad27* mutant on glucose (data not shown), demonstrates that the human–yeast FEN1-Ch is functionally improved over FEN1.

As noted above, the impact of a Rad27 nuclease mutation on genetic stability (Table 2) and MMS sensitivity (Fig. 4b) in a Rad^+ strain was greater than for FEN1-n. The growth inhibition in a *rad51* background by a *rad27-n* mutant shows dependence on the presence of a functional yeast PCNA-binding domain (Fig. 4a) (15). If the difference between Rad27-n and FEN1-n phenotypes were due to a poor interaction between FEN1 and PCNA, then the chimera may increase the effect of a *FEN1-n* mutant in yeast. Relative to FEN1-n, overexpression of FEN1-n,Ch in a *RAD27* strain increases mutation rates 2.3-fold, increases recombination 2.5-fold and causes a slight increase in sensitivity to MMS (Table 2, Fig. 4b). In contrast to non-chimeric FEN1-n, expression of FEN1-n,Ch inhibits growth of a *rad51* mutant to nearly the extent of Rad27-n (Fig. 4a). Thus, by exchanging the terminal region of FEN1 with the corresponding region of Rad27, their functions appear comparable.

DISCUSSION

Although human FEN1 has been characterized *in vitro*, the *in vivo* functions of this nuclease are less clear. Since the FEN1 and Rad27 proteins have comparable enzymatic activities and share 61% amino acid identity, it was anticipated that the cellular role of FEN1 function could be examined in yeast. We have found that at the functional level, FEN1 and Rad27 are highly related so that yeast provides an excellent opportunity to study the human protein and its mutants. Specifically, FEN1 can restore genome stability and DNA damage resistance to a *rad27* mutant (Fig. 1, Table 2) and it can replace the requirement for *RAD27* when cells are defective in other aspects of DNA repair (*rad51*) and replication (*pol3-01*) (Fig. 2, Table 1). In addition, the similar dominant-negative response of a flap-binding mutant and the consequences of changing the PCNA-binding region further support the use of yeast to examine human *FEN1* function. As discussed below, comparisons between overexpressed wild-type and altered Rad27 and FEN1 proteins suggest similar mechanisms of *in vivo* function.

Functional complementation of a *rad27*-Δ mutant by *FEN1*

The role of FEN1 in maintaining genome stability was investigated by evaluating its ability to decrease the high levels of spontaneous mutation, microsatellite instability and recombination when expressed in a *rad27* mutant. Nearly full complementation was detected for all three genetic end points (Table 2). Similarly to a previous report, we found that *FEN1* was also able to complement MMS sensitivity (Fig. 1) (52). Surprisingly, even the low levels of expression from a repressed *GAL1* promoter (e.g. on glucose medium) resulted in high survival on MMS (Fig. 1). These results, along with the ability to complement *rad27 rad51* and *rad27 pol3-01* double mutants, suggest that FEN1 protein is able to act on the same structural intermediates as Rad27. This leads us to propose that FEN1 may have a functional role similar to Rad27 on

comparable intermediates within human cells. Thus, observations in yeast, with human *FEN1* mutants or polymorphisms that may be identified, could have direct implications for human health.

Similarities and differences between Rad27 and FEN1 overexpression and the impact of PCNA binding

Although it is clear that human FEN1 is capable of complementing a *rad27* mutant in a manner similar to that of Rad27, there are several phenotypic differences between overexpressed wild-type FEN1 and Rad27 or respective mutants. First, overexpression of yeast Rad27 failed to reduce the microsatellite instability in a *rad27*-Δ strain, whereas there was an 8-fold reduction when FEN1 was overexpressed (Table 2). Second, in a wild-type strain, there were increases in microsatellite instability (4.8-fold) and forward mutation at *CAN1* (20-fold) with high levels of Rad27 expression but not with high levels of FEN1 expression (Table 2). Third, low level expression of FEN1 and Rad27 reveals a subtle difference in the ability to rescue a *rad27* strain exposed to 1.5 mM MMS (Fig. 5). Fourth, the expression of the nuclease mutants in a wild-type background revealed a stronger genetic impact for all three end points (Table 2) and a 10 000-fold greater reduction of growth on MMS (Fig. 4b) with Rad27-n expression as compared with FEN1-n. Finally, the dominant-negative impact of Rad27-n on a *rad51* strain was >1000-fold greater than the impact with FEN1-n expression (Fig. 4a). Consistently, the yeast protein, whether wild-type or mutant, had dominant-negative effects when overexpressed that were either not seen or were seen to a lesser degree with FEN1 expression.

Given the bi-functional nature of the Rad27/FEN1 protein, overexpressed Rad27 could impact on PCNA interactions as well as flap nuclease processing. Among the proteins that bind PCNA are several critical to replication (DNA Polδ, DNA ligase and RFC), cell cycle regulation (human p21) and DNA repair (human XPG, MLH1 and MSH2) (59–66). It has been shown that p21 and FEN1 bind the same site on PCNA *in vitro*, and the interactions between PCNA and FEN1 or p21 are mutually exclusive (65,67). In addition, overexpression of p21 inhibits processivity of the DNA polymerase–PCNA complex (68), possibly by blocking normal DNA Polδ interactions with PCNA. This is consistent with the model that the sliding clamp function of PCNA plays an important role in providing stability to DNA polymerase.

We found a closer parallel between the phenotypic consequences of expressed FEN1 and the PCNA-binding mutant of *RAD27* than between FEN1 and Rad27 (Table 2). Likewise, Rad27-n,p mirrored the phenotype of FEN1-n better than Rad27-n in the wild-type strain (Table 2, Fig. 4a), whereas the FEN1-n,Ch resembled Rad27-n more than FEN1-n (e.g. survival of a rad51 strain) (Fig. 4a). Thus, the overall pattern of similarity of *RAD27* to *FEN1* is determined in part by the PCNA-binding capability. This leads us to propose that the C-terminal PCNA-interacting region of FEN1 may have species specificity for interaction with PCNA in spite of the highly related core sequences (Table 3). The subtle differences between overexpression of FEN1 and Rad27 could be due, in part, to disruption of normal yeast PCNA–protein interactions by Rad27. The lack of a negative effect with overexpressed FEN1 may indicate that FEN1 competes less effectively than Rad27 and other yeast PCNA-binding proteins for PCNA. Moreover, the reduced interaction between yeast PCNA and human FEN1 protein could lower the stability of the human protein at the flap, allowing other mechanisms of repair, including the Rad27 nuclease in a wild-type or *rad51* strain, to remove the flap in an unobstructed manner.

Although the absence of negative effects from overexpressed human *FEN1* is probably due to less efficient PCNA binding, it appears that efficient flap processing by FEN1 is able to occur *in vivo*. A similar conclusion had been drawn for PCNA-binding mutants of *RAD27* in yeast (15). Furthermore, Wu *et al*. (5) demonstrated that human FEN1 is not stimulated by yeast PCNA *in vitro*, yet it shows significant levels of nuclease activity.

The replacement of the C-terminal end of human FEN1 with yeast sequence may create a stronger interaction between the chimeric FEN1 and yeast PCNA *in vivo*. Increasing the interaction between FEN1 and PCNA may also stabilize FEN1-Ch or FEN1-n,Ch at a flap, producing phenotypes more similar to those of Rad27 and Rad27-n, respectively (Table 2, Figs 4a and 5). An alternative explanation for the effect of FEN1-Ch and its derivatives is that FEN1-Ch may be more capable than FEN1 of penetrating the yeast nucleus. Regardless of the mechanism, the chimeras are a convenient tool for the study of wild-type and mutant human FEN1 in yeast.

The dominant *FEN1* nuclease mutation and implications for double-strand break induction

Kim *et al*. (69) demonstrated using X-ray scattering that the FEN1 protein releases the DNA following cleavage at a flap *in vitro*. Disruption of the metal-binding center of FEN1 results in complete loss of the nucleolytic activity of the protein as seen with the FEN1-n mutant (D181A allele), but this protein still binds a DNA flap substrate *in vitro* (55,56). The corresponding *rad27-n* mutation causes genetic instability and sensitivity to MMS (Table 2, Fig. 4) (15). We found that the corresponding human *FEN1-n* mutant is also dominant for increasing genome instability in a yeast cell (Table 2, Fig. 4a). When the human FEN1 PCNA-binding region was replaced by that of Rad27 (i.e. FEN1-n,Ch), this dominant effect was enhanced further, resulting in a phenotype more like that of the *rad27* nuclease mutant (Table 2, Fig. 4a).

Results from *Escherichia coli* suggest that stalled replication forks may be sites of DNA double-strand breaks (70) which can be repaired by recombination. A nuclease-defective Rad27 or FEN1 protein may bind a flap and remain bound due to its inability to process the flap. This bound protein–DNA complex could block replication and lead to DNA double-strand breaks, which would require homologous recombination proteins such as Rad51 and Rad52 for efficient repair in yeast (19,33). Our data are consistent with replication blocks and subsequent DNA double-strand breaks being caused by the Rad27-n and FEN1n,Ch proteins. First, the recombination in a Rad^+ strain was elevated 23- and 6-fold with expression of *rad27-n* or the *FEN1-n,Ch*, respectively. Second, expression of either the *rad27-n* or the *FEN1-n,Ch* nuclease mutants (which fail to process flaps and therefore remain bound) inhibit growth of a *rad51* strain, whereas *rad27-n,p* and *FEN1-n* do not (Fig. 4a). Enhanced PCNA binding in the FEN1-n,Ch

construct could help to deliver or maintain the faulty protein at the flap, causing blocked or stalled DNA replication.

Utility of yeast for the identification of *FEN1* polymorphisms with negative effects on DNA metabolism and genome stability

Based on a variety of negative effects of the yeast *rad27-n* alone and in combination with other genetic defects (see Introduction), it is likely that mutations or polymorphisms in human *FEN1* could also lead to errors in human DNA metabolism. Such negative effects could result from the action of a *FEN1* mutant allele *per se*, such as the dominant allele of *FEN1-n* studied here, or from interaction with other genes and/or environmental factors. In yeast, synergistic negative effects were found not only for the null *rad27* allele, but also for the *rad27-p* allele where phenotypic consequences are barely detectable (15). FEN1 alleles with decreased capability to compensate for the absence of yeast *RAD27* would be candidates for such synergistic effects in combination with polymorphisms in other human genes. We have demonstrated that mutations that eliminate or reduce the nuclease activity of FEN1 appeared to be toxic and/or genotoxic even in the presence of wild-type Rad27 protein (i.e. act as dominant alleles). Nuclease-deficient alleles of *FEN1* can be identified based on their growth inhibition of genetically sensitized *rad27* and *rad51* yeast strains and based on their genotoxic effects in yeast. We discovered that exchange of the C-terminus of human FEN1 with the corresponding region of the yeast protein exacerbates the negative effects of such mutations *in vivo*. This phenotype enhancement approach with the human–yeast chimera can be directed to increase the sensitivity of yeast cells to FEN1 alterations. At present, there are no reports of attempts to identify *FEN1* polymorphisms. The systems we have developed could be used for the functional identification and characterization of *FEN1* mutations and polymorphisms. Variants of human FEN1 found to be partially functional, toxic or genotoxic in yeast would be good candidates to investigate in epidemiology studies.

MATERIALS AND METHODS

Gene and protein designations

The human flap endonuclease 1 protein is referred to as FEN1 and the gene as *FEN1*. The wild-type of the homologous yeast gene is *RAD27* and alleles are in lower case, while the protein is Rad27. Mutations in the human and yeast genes for the flap endonuclease 1 are designated *FEN1-n* and *rad27-n*, corresponding to nuclease-deficient mutations D181A in the human gene (55) and D179A in the yeast gene (15), respectively. The *rad27-p* mutation F346A/F347A prevents binding to PCNA (15) and the *rad27*-Δ null mutation is a deletion of the *RAD27* gene (27). FEN1-Ch is a chimeric protein comprised of the N-terminal 331 amino acids of FEN1 plus the C-terminal 48 amino acids of the yeast Rad27, thereby replacing the terminal 49 amino acids of FEN1. Double mutations are designated by a combination of symbols that designate the single mutations (e.g. FEN1-n,Ch, the chimeric protein carrying the D181A nuclease mutation in the N-terminal region of FEN1). The corresponding *FEN1* alleles are in italics.

Plasmids and cloning methods

Multicopy, 2 μm plasmids YEp195SpGAL, YEp112SpGAL, and the gene disruption plasmids pR2.10 (*RAD27*) and pRad51Δ were provided by Ed Perkins and Alan Clark (NIEHS), Louise Prakash (University of Texas Medical Branch, Galveston, TX) and Nancy Kleckner (Harvard University, Boston, MA), respectively (27,71,72). pGALRAD51, isolated from a yeast cDNA library (73) by its ability to complement the MMS sensitivity of a *rad51* strain, was a gift from Kevin Lewis (NIEHS). Plasmids containing wild-type *FEN1*, pET-FCH and the *FEN1* allele lacking nuclease activity, pET-FCH-D181A, are described in Shen *et al.* (55,56). The plasmids carrying various alleles of yeast *RAD27* under a strong galactose-inducible promoter (*GAL1*p) used in this study were: pRG105A (*rad27-n*), pRG106A (*RAD27*), pRG107A (*rad27-p*) and pRG108A (*rad27-n,p*). These plasmids were described in Gary *et al.* (15). The human genes encoding wild-type *FEN1* and the *FEN1-D181A* mutant, which lacks nuclease activity, were isolated from pET-FCH or pET-FCH-D181A by enzymatic digestion with *Xba*I and *Hin*dIII and cloned into the *Xba*I–*Hin*dIII sites of YEp195SpGAL adjacent to the *GAL1* promoter, creating YEp195SpGALFEN1 (pFEN1) and YEp195SpGALFEN1-n (pFEN1-n), respectively. The base vector, YEp195SpGAL, is a multicopy plasmid containing a *URA3* selectable marker. YEp195SpGALFEN1-Ch (pFEN1-Ch) and YEp195SpGALFEN1-n,Ch (pFEN1-n,Ch) were constructed by PCR amplification and homologous recombination in yeast (Fig. 3). The following primers were used to amplify the C-terminus of pRG106: 5′h/yFEN1, agcagttctctgagg agcgaatccgcagtggggtcaagaggctgagtaagagcTTGAAATCTGGCATTCAGGGTAGGTTAGATGGGTTCTTCC; and 3′y/hFEN1, CTATGACGTCGCATGCACGCGTACGTAAGCTTAGTGATGATGATGATGATGtcttcttccctttgtgacttttattcttatttttgttc. The lower case letters represent the yeast *RAD27* DNA sequences, the upper case letters are human *FEN1* DNA sequence and the underlined sequences are from the vector YEp195SpGAL. The double underlined sequence is a 6-His tag that is located immediately upstream of the stop codon and can be used for protein purification. All sequences in the primers are in the correct reading frame for protein translation. The resulting product was co-transformed into yeast with either *Bst*EII-digested YEp195SpGALFEN1 or YEp195SpGALFEN1-n to produce YEp195SpGALFEN1-Ch and YEp195Sp-GALFEN1-n,Ch, respectively. The resulting chimera contains a unique *Bsm*I restriction site within the yeast sequence and the conserved PCNA-binding domain (Table 3). Constructs were verified by DNA sequence analysis. The exchanged amino acid sequences share 37% identity. Seven of the eight amino acids within the conserved PCNA-binding domain (57) are identical, and the C-terminal regions are of similar charge (Table 3). Sequence comparisons were accomplished using the Human Genome Center web site at: http://www.hgsc.bcm.tmc.edu/SearchLauncher/ (51).

Media and strains

All yeast strains were derived from CG379 (S1) and only haploid strains were used in this work. Strains with the A_{12} microsatellite in the *LYS2* gene were described in Tran *et al.* (26). The construction of the *rad27* and *rad51* mutants, the *pol3-01 rad27*-Δ double mutant as well as strains for the analysis of interchromosomal recombination, ALE100 and ALE101, have been described previously (15). The *rad27* null

was constructed by replacing bases +58 to +755 of the 1146 bp coding sequence by the *E.coli hisG* gene using the gene disruption plasmid pR2.10 (27). Construction of *rad51 rad27* (pRAD51) was accomplished in this study by deleting the *RAD51* locus from a *rad27* strain using pRad51Δ in the presence of the pGALRAD51 plasmid.

Genetic assays

The ability of the synthetic lethal strains to survive with FEN1 expression was assessed by either a plasmid shuffle (54) or a plasmid loss assay (15). After transforming in either the *FEN1-TRP1* plasmid, YEp112SpGALFEN1, or a *TRP1* vector control, YEp112SpGAL, into *rad51 rad27* (pGALRAD51), the strains were maintained on SC –Ura –Trp +Gal; this medium selects for pGALRAD51, which contains the *URA3* gene, and YEp112SpGAL. The strains subsequently were plated to medium selecting for and against the *RAD51* plasmid (SC –Ura –Trp +Gal and SC –Trp +Gal +5-FOA, respectively) to compare viability in the presence of Rad51 with the viability in its absence.

Plasmid loss was assessed in a *pol3-01 rad27* strain containing a CEN plasmid with both *RAD27* and *TRP1* genes, LC-80B (15). After transformation with each of the following constructs: RG106 (*RAD27* under *GAL1* control), YEp195SpGALFEN1, YEp195SpGALFEN1-Ch and YEp195SpGAL, the transformants were maintained on uracil-selective medium (SC +Gal –Ura) for ~20 generations then plated to SC +Gal –Ura medium. This medium selects for the *URA3*-based *FEN1* and *RAD27* plasmids, but there is no selection for the *TRP1*-based *RAD27* plasmid (LC80B). Colonies formed subsequently were replica plated to SC +Gal –Trp, YPG and SC +Gal –Ura media. The frequency of plasmid loss for the *TRP1*-linked plasmid was determined by dividing the number of colonies failing to form on the SC +Gal –Trp replica plate by the number of colonies formed on the SC +Gal –Ura plate (total number of colonies plated).

Rates were calculated from the frequency measurements of at least 10 cultures for each assay using the method of the median (74). Mutation rates in the microsatellite, A_{12} homonucleotide run at *LYS2*, and forward mutations in the *CAN1* gene were assessed as previously described in Tran *et al.* (22,26). Measurements of the rate of ectopic interchromosomal recombination between *lys2* alleles in chromosome II and chromosome III have been described (15,75). This assay selects for interchromosomal gene conversion events and crossovers that lead to chromosomal translocations.

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