

Elg1 forms an alternative RFC complex important for DNA replication and genome integrity

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Genome-wide synthetic genetic interaction screens with mutants in the *mus81* and *mms4* replication fork-processing genes identified a novel replication factor C (RFC) homolog, Elg1, which forms an alternative RFC complex with Rfc2–5. This complex is distinct from the DNA replication RFC, the DNA damage checkpoint RFC and the sister chromatid cohesion RFC. As expected from its genetic interactions, *elg1* mutants are sensitive to DNA damage. Elg1 is redundant with Rad24 in the DNA damage response and contributes to activation of the checkpoint kinase Rad53. We find that *elg1* mutants display DNA replication defects and genome instability, including increased recombination and mutation frequencies, and minichromosome maintenance defects. Mutants in *elg1* show genetic interactions with pathways required for processing of stalled replication forks, and are defective in recovery from DNA damage during S phase. We propose that Elg1-RFC functions both in normal DNA replication and in the DNA damage response.

Keywords: cell cycle checkpoints/DNA damage/DNA replication/genome stability/replication factor C

Introduction

DNA replication is typically highly processive. Replication fork stalling or arrest can result when replication forks encounter damage in the DNA, and at naturally occurring sequences such as replication fork barriers and replication slow zones (Rothstein *et al.*, 2000; Cha and Kleckner, 2002). Several mechanisms by which replication forks can be restarted following arrest have been described in bacteria (reviewed in Michel, 2000; Michel *et al.*, 2001). Stalled forks are susceptible to breakage and to replication fork reversal, both of which generate a double-stranded DNA end. A replication fork can then be re-established by homologous recombination followed by Holliday junction resolution or by branch migration. By analogy, some of these same processes are believed to occur in eukaryotes. In addition to these pathways, which are principally involved in restarting what are presumably collapsed replication forks, recent

work has demonstrated that the S phase checkpoint pathway is responsible for stabilizing replication forks and preventing fork collapse and formation of DNA structures that are substrates for replication restart pathways (Lopes *et al.*, 2001; Tercero and Diffley, 2001; Sogo *et al.*, 2002).

Replication factor C (RFC) was first identified as a protein complex required for SV40 DNA replication *in vitro* (Tsurimoto and Stillman, 1989; Virshup and Kelly, 1989). RFC is a five-subunit complex that recognizes the primer terminus and catalyzes the loading of the sliding clamp proliferating cell nuclear antigen (PCNA; Hubscher, 1996; Mossi and Hubscher, 1998). PCNA acts as a processivity factor for DNA polymerase δ on the leading and lagging strands. At least two alternative forms of RFC have been identified recently in yeast and humans. In the first of these, the large subunit of RFC, Rfc1, is replaced by the Rad24 protein (Green *et al.*, 2000). Rad24-RFC loads a PCNA-like clamp consisting of Rad17, Ddc1 and Mec3 (Bermudez *et al.*, 2003; Majka and Burgers, 2003), is required for DNA damage checkpoint responses in G₁ and G₂, and contributes to S phase checkpoint responses. In the second alternative RFC, Rfc1 is replaced by Ctf18 (Hanna *et al.*, 2001; Mayer *et al.*, 2001; Naiki *et al.*, 2001). Mutants in *ctf18* have defects in sister chromatid cohesion (Hanna *et al.*, 2001; Mayer *et al.*, 2001), the process by which newly replicated chromatids remain physically associated until entry into anaphase. Ctf18-RFC also contains the Ctf8 and Dcc1 proteins, and mutations in *ctf8* or *dcc1* recapitulate the cohesion defects observed in *ctf18* mutants (Mayer *et al.*, 2001). There is evidence that like Rad24, Ctf18 also contributes to the S phase checkpoint response. *ctf18 rad24* mutants have a mild S–M checkpoint defect that is not evident in the single mutants, and have a Rad53 activation defect in S phase (Naiki *et al.*, 2001).

Here we describe a functional genomics approach to identify previously uncharacterized factors required for the DNA damage response, particularly those involved in replication fork progression. To this end, we conducted genome-wide synthetic lethality screens with deletion mutants in *mus81* and *mms4*. Mus81 and Mms4 are subunits of an endonuclease with a preference for branched DNA structures (Boddy *et al.*, 2001; Chen *et al.*, 2001; Kaliraman *et al.*, 2001). In addition to this substrate preference, several lines of evidence connect this enzyme to the processing of stalled DNA replication forks (Haber and Heyer, 2001; Kaliraman *et al.*, 2001; Mullen *et al.*, 2001).

We identified a previously uncharacterized RFC homolog, *ELG1*. We find that Elg1 forms an RFC-like complex with the Rfc2–5 proteins, but not with Rfc1 or its homologs Rad24 and Ctf18. *ELG1* is functionally redundant with *RAD24* in the DNA damage response, yet does

not share its primary role in checkpoint activation. Of particular significance, *elg1* mutants display defects in DNA replication in both the presence and absence of DNA damage, suggesting that Elg1 functions directly in DNA replication. Cells lacking *elg1* require the intra-S phase checkpoint, homologous recombination proteins and pathways involved in replication restart following replication fork stalling for wild-type growth, and are defective in recovery from DNA damage in S phase. We propose that Elg1-RFC functions in lagging strand DNA synthesis to prevent replication fork stalling and to facilitate re-start of stalled replication forks.

Results

Genome-wide synthetic lethal screens with *mus81Δ* and *mms4Δ* identify *ELG1*

In order to identify novel genes that function to stabilize replication forks *in vivo*, we conducted genome-wide synthetic genetic interaction screens (Tong *et al.*, 2001) with strains carrying deletions of *MUS81* or *MMS4*. Several lines of evidence suggest that Mus81 and Mms4 are involved in the processing of stalled replication forks (Haber and Heyer, 2001; Kaliraman *et al.*, 2001; Mullen *et al.*, 2001). In these screens, the query mutant was crossed with the ~4600 strains that make up the complete set of *Saccharomyces cerevisiae* viable haploid deletion mutants. Double mutants that show reduced fitness compared with single mutants, as evidenced by lack of growth or slow growth, were scored as positive. Each screen was performed three times and interactions that were scored at least twice were confirmed by tetrad analysis. The result of these screens is presented as a genetic interaction network in Figure 1A, with tetrad analysis of the *elg1Δ* interactions shown in Figure 1B. Each line on the network represents a synthetic lethal or synthetic sick (slow growth) interaction between the linked genes. Consistent with models in which Mus81 and Mms4 function exclusively as a heterodimer (Boddy *et al.*, 2001; Kaliraman *et al.*, 2001), the screen with *mus81Δ* identified the same set of seven genes as the screen with *mms4Δ*. We found strong genetic interactions between *mus81Δ* and *mms4Δ* and deletions of either *sgs1* or *top3*. These interactions have been described previously and are believed to reflect the redundant roles of the Sgs1/Top3 and Mus81/Mms4 pathways in the repair of stalled replication forks and/or the resolution of recombination intermediates (Mullen *et al.*, 2001). *YLR235C* overlaps the *TOP3* gene and so was probably identified due to its effect on Top3 function. In addition to these known interactions, we identified four novel interactions, with *elg1Δ*, *esc2Δ*, *nce4Δ* and *vid22Δ*. We also found that *elg1Δ* is synthetic lethal with *mec2-1*, a checkpoint-defective allele of *RAD53* (Figure 1B), which indicates that *elg1Δ* mutant cells require a Rad53-dependent checkpoint for viability. Together, these data suggest that Elg1 is important for the integrity of DNA replication forks *in vivo*.

ELG1 is a member of the replication factor C family

ELG1 (enhanced level of genome instability) was first identified in a screen for increased Ty transposon mobility, and *elg1* mutants have been reported to confer an increase

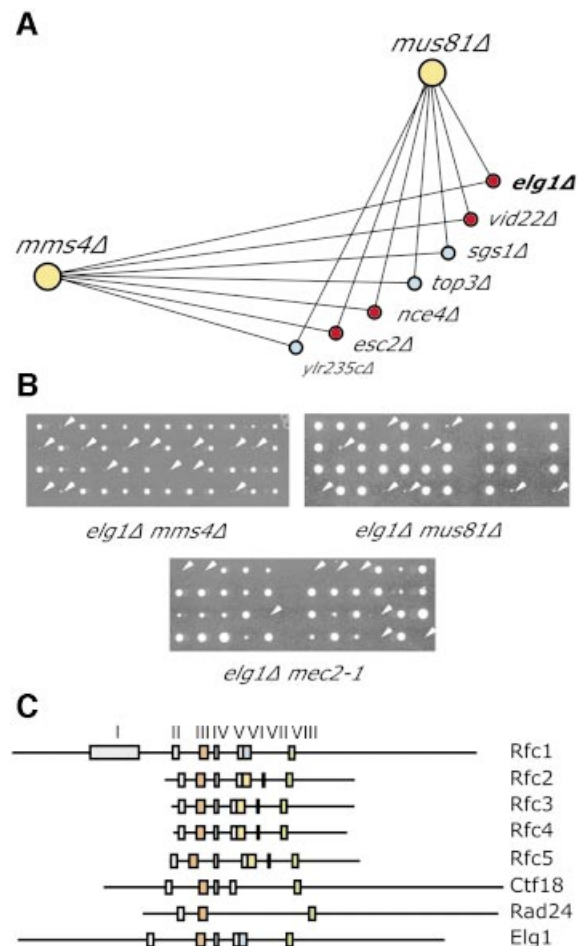


Fig. 1. Genome-wide synthetic lethal screens with *mus81Δ* and *mms4Δ* identify the RFC homolog Elg1. (A) The results from synthetic genetic array analysis with *mus81Δ* and *mms4Δ* presented as a genetic interaction map. Lines connecting genes represent synthetic lethality or synthetic slow growth. Red circles indicate novel genetic interactions. (B) Tetrad confirmation of the *elg1Δ* crosses. Each column represents the four spores from a single ascus. Double mutant colonies, as detected by selection for the dominant selectable marker linked to each gene, are indicated by white arrowheads. (C) Schematic representation of the conserved sequence blocks in the *S. cerevisiae* RFC family genes. Elg1 contains six of the seven RFC boxes found in Rfc1.

in direct repeat recombination (Scholes *et al.*, 2001). Using a *LEU2* direct repeat assay (Smith and Rothstein, 1999), we confirmed that deletion of *elg1* causes a 7-fold increase in recombination rate (data not shown). Detailed examination of the hypothetical translation product of *ELG1* revealed extensive similarity to the RFC-like protein family. Eight regions of sequence similarity have been defined in RFC proteins (Cullmann *et al.*, 1995). As indicated in Figure 1C and in the Supplementary data (available at *The EMBO Journal* Online), Elg1 contains all of the RFC boxes present in Rfc1 with the exception of the ligase homology region, RFC box I. The Rfc-specific boxes II, IV, VI and VIII are present in Elg1, as is the ATP-binding motif contained in boxes III and V. RFC box VII is present only in the small Rfc subunits, Rfc2–5, and is absent from Elg1. The RFC box VI in Elg1 bears greater similarity to box VIa, found in the large Rfc subunits, than it does to box VIb, found in the small subunits. Like the

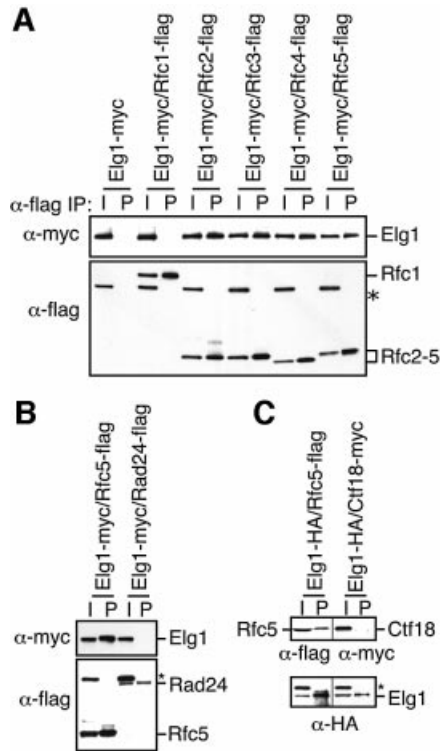


Fig. 2. Elg1 forms complexes with Rfc2, 3, 4 and 5, but not with Rfc1, Rad24 or Ctf18. **(A)** Extracts from yeast strains expressing the indicated epitope-tagged RFC proteins were immunoprecipitated with antibody against the flag epitope. Ten percent of the input extract (I) and the immunoprecipitate (P) were fractionated by SDS-PAGE. Immunoblots were probed with anti-flag antibody to detect Rfc1, Rfc2, Rfc3, Rfc4 and Rfc5, and with anti-myc antibody to detect Elg1. A non-specific cross-reacting polypeptide is indicated (*). **(B)** Extracts from strains expressing the indicated proteins were immunoprecipitated with anti-flag antibody to precipitate Rfc5 and Rad24. Immunoblots were probed with anti-flag antibody to detect Rfc5 and Rad24 and with anti-myc to detect Elg1. **(C)** Extracts were immunoprecipitated with anti-HA antibody to precipitate Elg1. Immunoblots were probed with anti-flag antibody to detect Rfc5, anti-myc to detect Ctf18 and anti-HA to detect Elg1.

Rfc1 homologs Rad24 and Ctf18, Elg1 is conserved throughout *Eucaryota*. Homologs of Elg1 are readily identifiable in *Schizosaccharomyces pombe* (NP_595265), *Drosophila melanogaster* (AAF49530), mouse (BAC39389.1) and human (CAC44537). Together, these data indicate that Elg1 is an Rfc1 homolog and, by analogy with the Rfc1 homologs Rad24 and Ctf18, suggests that Elg1 forms an alternative RFC complex with the small RFC subunits Rfc2–5.

Elg1 forms a novel RFC complex

High-throughput protein–protein interaction screens have detected Elg1 in complexes with Rfc2, Rfc4 and Rfc5 (Gavin *et al.*, 2002; Ho *et al.*, 2002). In order to test the possibility that Elg1 forms an RFC-like complex distinct from the canonical replication RFC, we conducted co-immunoprecipitation experiments with Elg1 and the five subunits of RFC. Elg1 protein was not detectable in Rfc1 immunoprecipitates (Figure 2A, lane 4). In contrast, immunoprecipitates of Rfc2, Rfc3, Rfc4 or Rfc5 all contained Elg1 (Figure 2A, lanes 6, 8, 10 and 12). Elg1 was not immunoprecipitated in the absence of RFC,

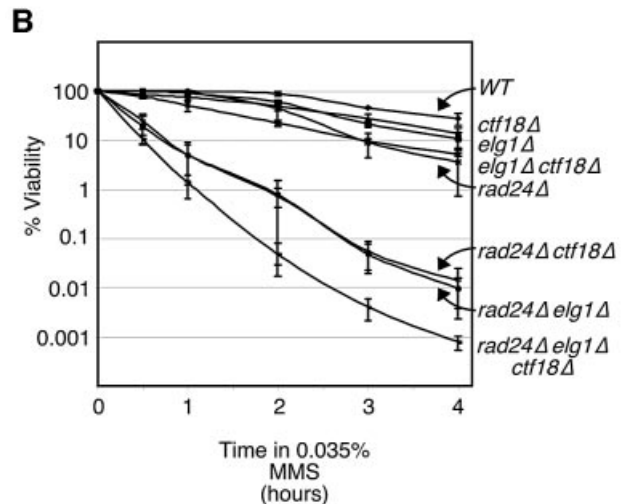
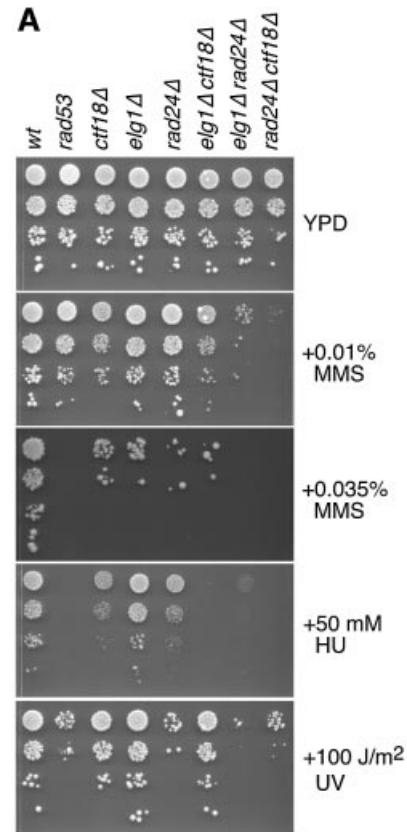


Fig. 3. Elg1 is required for the DNA damage response. **(A)** Ten-fold serial dilutions of cultures of the indicated mutants were spotted on YPD, YPD containing 0.01% (v/v) MMS, 0.035% (v/v) MMS, 50 mM HU, or on YPD that was subsequently exposed to 100 J/m² UV. Plates were incubated at 30°C for 2–3 days. **(B)** Logarithmically growing cultures of the indicated mutants were incubated in YPD containing 0.035% (v/v) MMS at 30°C. At the indicated times, samples were withdrawn and plated on medium lacking MMS to determine the number of viable cells. The percentage of viable cells relative to the number of viable cells at $t = 0$ is shown. Plots represent the average of three experiments, and error bars span 1 SD.

indicating that the immunoprecipitations were specific (Figure 2A, lane 2). Therefore, Elg1 forms a complex with Rfc2–5 but not with Rfc1. The simplest interpretation of

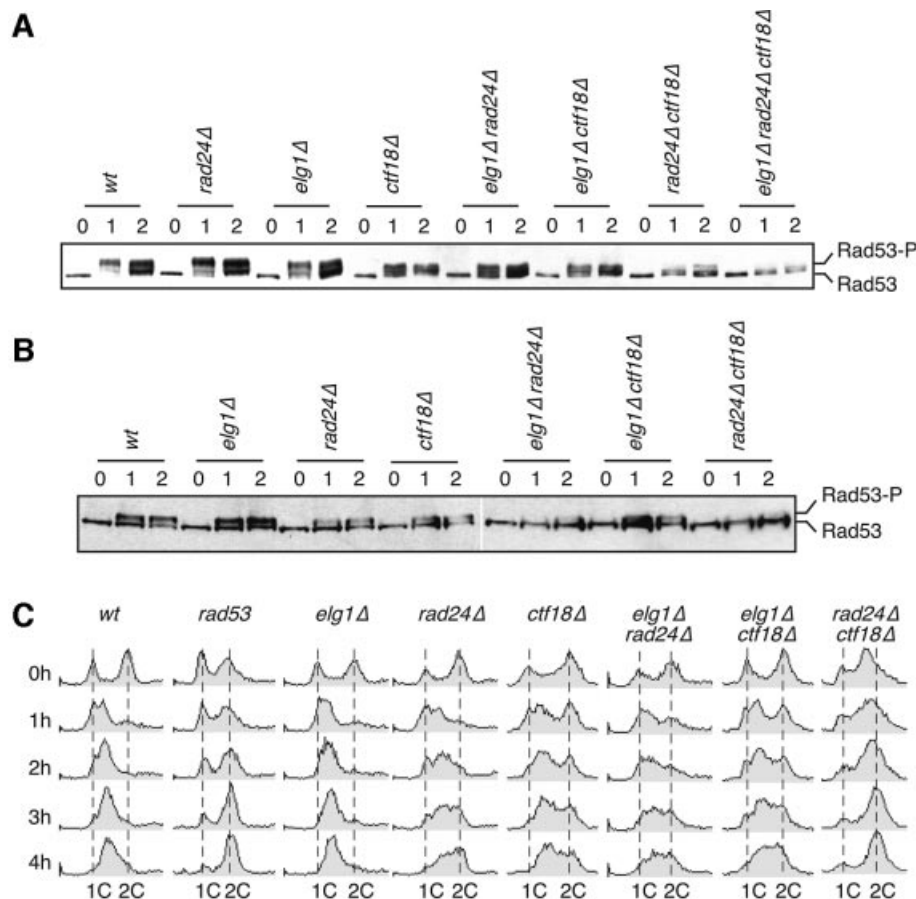


Fig. 4. Rad53 activation defects in *elg1Δ*. (A) S–M checkpoint. Logarithmically growing cultures were arrested in G₁ with α -factor and released into medium containing 200 mM HU. At the indicated times, samples were fixed and extracts fractionated by SDS–PAGE. Following transfer, the immunoblot was probed with anti-Rad53 antibody. Phosphorylation of Rad53 causes a shift in electrophoretic mobility (Rad53-P) and is a marker for checkpoint activation. (B) Intra-S phase checkpoint. Logarithmically growing cultures were treated with 0.035% (v/v) MMS. At the indicated times, samples were withdrawn and Rad53 activation was analyzed by immunoblotting. (C) Cell cycle progression in the presence of MMS was assessed by flow cytometry. Logarithmically growing cultures were treated with 0.035% (v/v) MMS. At the indicated times, samples were fixed and the DNA contents of cells in each sample were analyzed by flow cytometry. The positions of cells with 1C and 2C DNA contents are indicated on the histograms.

these data is that Elg1 forms an alternative RFC complex in which it substitutes for Rfc1 in binding to Rfc2–5.

Although genetic data suggest that Elg1 is functionally distinct from Ctf18 and Rad24 as neither *ctf18Δ* nor *rad24Δ* shows as strong a genetic interaction with *mus81Δ*, *mms4Δ* or *rad53* (data not shown), it remained formally possible that Elg1 is a member of the Rad24-RFC or the Ctf18-RFC complexes. To exclude these possibilities, we first immunoprecipitated Rad24 and assayed for the presence of Elg1 (Figure 2B). Elg1 was detected in Rfc5 complexes, but not in Rad24 complexes. We next immunoprecipitated Elg1 and assayed for the presence of Ctf18 (Figure 2C). Ctf18 was not present in Elg1 complexes, although again Rfc5 was present. We conclude that Elg1 forms a novel RFC complex with Rfc2–5.

Elg1 function in the DNA damage response is redundant with that of Rad24

Several lines of evidence have implicated RFC complexes in the checkpoint response to DNA damage, particularly during S phase. We tested the sensitivity of *elg1Δ*, *rad24Δ* and *ctf18Δ* mutants, as well as all pairwise double mutants, and the triple mutant, to the DNA alkylating agent

methyl methanesulfonate (MMS), the replication inhibitor hydroxyurea (HU) and UV radiation (Figure 3A). In addition, we measured mutant cell viability following exposure to 0.035% MMS in a 4 h time course (Figure 1B). First, we found that the *elg1Δ* mutant was only mildly sensitive to MMS, and was resistant to HU and UV. This contrasts with *rad24Δ*, which was significantly sensitive to all three agents, and with *ctf18Δ*, which displayed sensitivity to MMS and to HU, but was only modestly UV sensitive. These different sensitivities distinguish the Rfc1 homologs, and indicate that Elg1 is not a central player in DNA damage repair.

We next examined the sensitivity of each possible double mutant combination and the triple mutant. Both double mutants with *rad24Δ* were significantly more sensitive to all agents than any of the single mutants, whereas the *rad24Δ ctf18Δ elg1Δ* triple mutant conferred the greatest sensitivity to MMS. These findings indicate that the Rfc1 homologs are in partially redundant pathways for DNA damage resistance.

Elg1 contributes to Rad53 activation

Rad24 has a well-documented role in activation of the checkpoint kinase Rad53 (Shimomura *et al.*, 1998;

Pelliccioli *et al.*, 1999; Naiki *et al.*, 2000). Activation of Rad53 can be readily assessed by immunoblot detection of a phosphorylation-dependent shift in Rad53 mobility (Pelliccioli *et al.*, 1999). We first assayed Rad53 activation in HU-arrested cells to assess S–M checkpoint function in *elg1Δ* cells (Figure 4). *ELG1* was not required for Rad53 activation following HU arrest, as we observed wild-type levels of Rad53 phosphorylation in the *elg1Δ* mutant (Figure 4A). The *ctf18Δ rad24Δ* mutant displayed a clear defect in Rad53 activation, as previously reported (Naiki *et al.*, 2001) and consistent with the increased HU sensitivity of this double mutant relative to the *ctf18Δ* and *rad24Δ* single mutants (Figure 3). The *elg1Δ rad24Δ* and *elg1Δ ctf18Δ* double mutants displayed slight defects in Rad53 activation, as evidenced by the smaller fraction of Rad53 present in the most slowly migrating form when compared with wild-type (Figure 4A). As with *ctf18Δ rad24Δ*, this might account for the increased HU sensitivity of the double mutants compared with the single mutants (Figure 3). In the *elg1Δ rad24Δ ctf18Δ* triple mutant, phosphorylation of Rad53 was almost completely absent (Figure 4A). We conclude that *ELG1* contributes to the S–M checkpoint in the context of a *rad24* mutation. The near absence of Rad53 phosphorylation in the triple mutant indicates that all of the Rfc1 homologs can contribute to Rad53 activation in S phase.

We next treated cells with MMS to assess the role of Elg1 in the intra-S phase checkpoint (Figure 4B). Again, *elg1Δ* cells displayed no defect in Rad53 activation. However, when combined with *rad24Δ*, both *elg1Δ* and *ctf18Δ* have significant Rad53 activation defects, consistent with roles in intra-S checkpoint response. It is worth noting, however, that the *elg1Δ ctf18Δ* double mutant had little, if any, Rad53 activation defect, indicating that Rad24 plays the more important role in the intra-S checkpoint.

Since we had hypothesized a role for Elg1 in replication fork integrity, we assessed intra-S phase checkpoint function directly. Cells in asynchronous culture were treated with MMS, and their accumulation in S phase was monitored by flow cytometry (Figure 4C). Wild-type cells, with an intact intra-S phase checkpoint, accumulate in S phase by 2 h, and remain blocked with an intermediate DNA content for the 4 h duration of the experiment. This S phase accumulation is due to checkpoint-independent slowing of DNA replication fork progression combined with a checkpoint-dependent inhibition of dormant and late origin firing (Tercero and Diffley, 2001). In the *rad53* mutant, the checkpoint-dependent inhibition of origin firing is abrogated and cells appeared to move through S phase more rapidly, accumulating with a 2C DNA content by 3 h. In the *rad24Δ* mutant, which is partially defective in the intra-S checkpoint (Paulovich *et al.*, 1997), cells moved through S phase more rapidly than wild-type but not as rapidly as *rad53* mutant cells. The *elg1Δ* resembled wild-type, and deleting *elg1* did not enhance the intra-S checkpoint defect in *rad24Δ*. In contrast, the *ctf18Δ rad24Δ* mutant was more defective in the intra-S checkpoint, accumulating with 2C DNA content with kinetics similar to those observed with the *rad53* mutant.

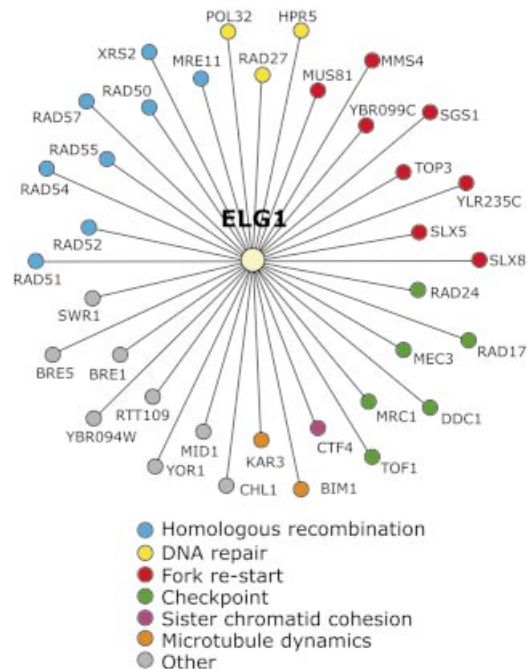


Fig. 5. Genome-wide synthetic genetic screens with *elg1Δ* identify homologous recombination, fork re-start and S phase checkpoint pathways. The results of synthetic genetic array analysis with *elg1Δ* presented as a genetic interaction map. Lines connecting genes represent synthetic lethality or synthetic slow growth. Colored circles designate the cellular role of the interacting genes.

Cells lacking *Elg1* require homologous recombination and replication fork re-start pathways for optimum growth

To gain further insight into Elg1 function *in vivo*, we performed a genome-wide synthetic lethal screen with *elg1Δ*. The synthetic genetic interactions confirmed by tetrad analysis are presented in Figure 5 and in the Supplementary data. Several functional clusters of genes are readily apparent. First, *elg1Δ* interacts with members of the *RAD52* epistasis group (*rad51Δ*, *rad52Δ*, *rad54Δ*, *rad55Δ* and *rad57Δ*), which are required for homologous recombination. The genes *rad50Δ*, *mre11Δ* and *xrs2Δ* were also identified. These interactions are likely to reflect the role of these genes in homologous recombination rather than in non-homologous end joining, as *elg1Δ* is not synthetic lethal or sick with *dnl4Δ* or *yku80* (data not shown). Of particular significance, genes thought to be in redundant pathways for re-starting stalled replication forks (Kaliraman *et al.*, 2001; Mullen *et al.*, 2001) have synthetic genetic interactions with *elg1Δ*. *mus81Δ*, *mms4Δ*, *sgs1Δ*, *top3Δ*, *slx5Δ* and *slx8Δ* all displayed a fitness defect in combination with *elg1Δ*. The last functional group of note comprises *rad24Δ*, *rad17Δ*, *mec3Δ*, *ddc1Δ*, *mrc1Δ* and *tof1Δ*. These genes are all linked to defects in S phase checkpoints (Alcasabas *et al.*, 2001; Foss, 2001; Tanaka and Russell, 2001; Osborn *et al.*, 2002), and their identification in the screen is consistent with lack of Elg1 causing DNA lesions or arrested replication forks during S phase. Our comprehensive genetic analysis indicates that cells lacking Elg1 and homologous recombination, replication fork re-start or S phase checkpoint pathways have a significant fitness defect, indicating that these pathways are required for

optimal growth when *ELG1* is deleted. Finally, the functional classes of genes identified in the *elg1* synthetic genetic screen bear a striking similarity to those identified in screens with *rad27Δ* (Tong *et al.*, 2001). Therefore, the genetic data suggest that Elg1 performs a function that is similar to that of Rad27, which plays an important role in Okazaki fragment maturation (Merrill and Holm, 1998; Parenteau and Wellinger, 1999).

Elg1 is required for replication fidelity

Mutants defective in Okazaki fragment maturation, such as *rfc1-1*, *pol30-52* and *rad27Δ*, all have increased forward mutation rates, but differ in the spectrum of mutants produced (Xie *et al.*, 1999, 2001). Whereas *rfc1-1* and *pol30-52* cause predominantly point mutations and small insertions or deletions, *rad27Δ* causes large rearrangements. We assessed the forward mutation rate to canavanine resistance of *elg1Δ*, and found that it was almost 8-fold higher than the wild-type strain (24.2×10^{-7} versus 3.24×10^{-7}), indicating that Elg1 is important for replication fidelity. To determine the spectrum of mutations caused by *elg1Δ*, we examined the *CAN1* gene from 20 independent *Can^r* mutants from both wild-type and *elg1Δ* for the presence of large insertion or deletion mutations. A difference in the size of a *CAN1*-derived fragment was observed in two out of 20 *Can^r* wild-type strains, and in only one out of 20 *Can^r* *elg1Δ* strains (data not shown). DNA sequencing revealed that the mutations in the *elg1Δ* strains were predominantly base substitution mutations, with some small (<5 bp) deletions and insertions. Therefore, the *elg1Δ* mutation spectrum resembles that caused by defects in Rfc1 or PCNA.

elg1Δ mutants are defective in S phase progression

If *elg1Δ* cells are defective in fork re-start or have increased levels of fork stalling, we would expect to detect defects in S phase progression. We arrested cells in G₁ phase and released them synchronously into the cell cycle. Progression through S phase was measured by flow cytometry. As shown in Figure 6A, the wild-type culture completed S phase by 60 min after release, as evidenced by the accumulation of cells with a 2C DNA content. In the *elg1Δ* culture, however, a significant fraction of cells still had a 1C or intermediate (<2C) DNA content at 60 min. Even at 80 min post-release, some *elg1Δ* cells had not completed S phase. Consistent with a role for Elg1 in S phase progression, we found that *elg1Δ* has a probability of plasmid loss that is 7.6 times higher than wild-type in a minichromosome maintenance assay (Figure 6B). This elevated plasmid loss was not suppressed significantly by the presence of additional origins of replication, and 2D gel analysis did not reveal any initiation defects in *elg1Δ* (data not shown). Furthermore, we found that *elg1Δ* did not have any detectable defect in sister chromatid cohesion (data not shown), suggesting that plasmid loss in *elg1Δ* was not due to a segregation defect.

Since Elg1-RFC most probably functions as a clamp loader, we tested whether overexpression of PCNA could suppress the *elg1Δ* phenotype. As shown in Figure 6C, overexpression of PCNA rescued the MMS sensitivity of *elg1Δ*, as evidenced by improved growth on MMS plates when PCNA overexpression was induced by the presence

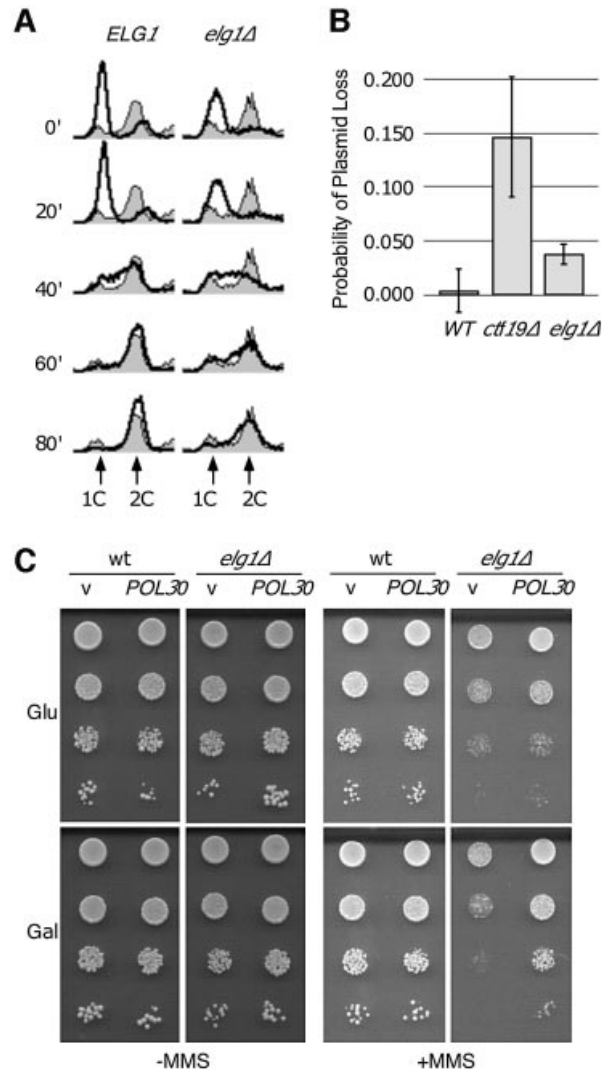


Fig. 6. *elg1Δ* mutants display DNA replication defects. (A) Progression through S phase. Wild-type or *elg1Δ* cells were arrested in G₁ ($t = 0$) and released synchronously into the cell cycle. Samples were removed at the indicated times and analyzed by flow cytometry. The shaded histograms represent the cell cycle distribution of the asynchronous cultures before the G₁ arrest. Overlaid histograms represent the cell cycle distribution at the indicated times after release from the G₁ arrest. The positions of cells with 1C and 2C DNA contents are indicated. (B) Plasmid loss in wild-type, *elg1Δ* and *ctf19Δ*. The probability of plasmid loss per generation is plotted, and error bars span 1 SD. (C) Suppression of *elg1Δ* MMS sensitivity by PCNA overexpression. Serial dilutions of wild-type or *elg1Δ* cells carrying empty vector (v) or *GAL1-POL30* plasmid (*POL30*) were plated on synthetic medium with 2% glucose (Glu; uninduced) or 2% galactose + 2% raffinose (Gal; induced), plus or minus 0.01% MMS.

of galactose. Taken together, these results suggest that Elg1 plays a direct role in DNA replication, most probably during the elongation phase of DNA synthesis.

Abnormal recovery from replication fork stalling in *elg1Δ*

We measured S phase progression in medium containing 0.035% MMS, a concentration of MMS known to cause extensive fork stalling in wild-type cells (Tercero and Diffley, 2001) (Figure 7A). In the presence of MMS, S phase was prolonged in the wild-type cells, which take

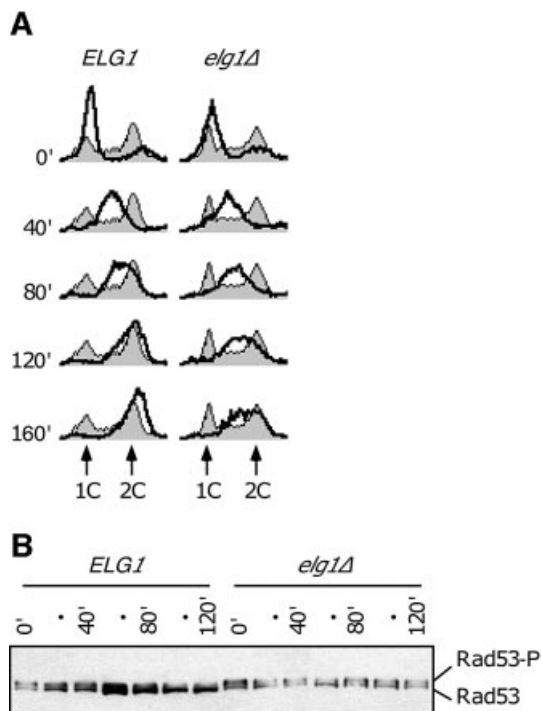


Fig. 7. *elg1Δ* mutants are defective in recovery from MMS-induced replication fork stalling. (A) S phase progression in the presence of MMS. Wild-type or *elg1Δ* cells were arrested in G₁ ($t = 0$) and released synchronously into medium containing 0.035% (v/v) MMS. Samples were removed at the indicated times and analyzed by flow cytometry. The shaded histograms represent the cell cycle distribution of the asynchronous cultures before the G₁ arrest. Overlaid histograms represent the cell cycle distribution at the indicated times after release from the G₁ arrest. (B) Checkpoint activation of Rad53 during recovery from MMS damage. Cells were arrested in G₁, released into MMS for 1 h, and then transferred to medium lacking MMS ($t = 0$). At the indicated times, samples were withdrawn and Rad53 activation was analyzed by immunoblotting.

120 min to accumulate with 2C DNA content. The *elg1Δ* mutant was clearly defective in S phase progression in the presence of MMS, with a significant fraction of cells containing <2C DNA at 160 min post-release. Thus the introduction of fork stalls causes further defects in DNA synthesis in *elg1* mutants, consistent with Elg1 functioning in preventing fork stalling or in re-starting stalled replication forks.

If Elg1 is required for replication fork integrity, we expected to see prolonged activation of Rad53 following MMS treatment during S phase. Wild-type and *elg1Δ* cells were arrested in G₁ and released into medium containing MMS for 1 h to activate Rad53. The MMS was then washed out and cells were allowed to recover. Rad53 activation during recovery from MMS damage was assayed by immunoblot analysis (Figure 7B). In the wild-type strain, Rad53 is dephosphorylated by 80 min following removal of MMS. In contrast, activated Rad53 persisted in the *elg1Δ* strain for at least 120 min, indicating that Elg1 is required for downregulation of the intra-S checkpoint response. Since the *elg1Δ* strain is at least 10-fold less sensitive to MMS than mutants in MMS repair pathways (data not shown), these results suggest that Elg1 is required for efficient fork re-start rather than direct repair of DNA lesions.

Discussion

A novel RFC-like complex

Using a functional genomics approach, we have identified Elg1 as a novel DNA replication protein. Elg1 associates with Rfc2, Rfc3, Rfc4 and Rfc5, forming a fourth eukaryotic RFC-like complex that is functionally distinct from the canonical RFC, and from Rad24-RFC and Ctf18-RFC. Whether Elg1-RFC functions as a pentameric complex, like RFC and Rad24-RFC (Mossi and Hubscher, 1998; Green *et al.*, 2000), or requires accessory factors as found in Ctf18-RFC (Mayer *et al.*, 2001), awaits purification of Elg1-RFC. Eukaryotic cells have at least two sliding clamps that are loaded by RFC-like enzymes, PCNA and the 9-1-1 complex. RFC loads PCNA during leading and lagging strand DNA synthesis (reviewed in Mossi and Hubscher, 1998), whereas Rad24-RFC loads the 9-1-1 clamp *in vitro* but cannot load PCNA (Bermudez *et al.*, 2003; Majka and Burgers, 2003). Ctf18-RFC binds to PCNA *in vitro* and *in vivo* (Ohta *et al.*, 2002), and so may function as a PCNA clamp loader under specialized circumstances. Our data suggest a role for Elg1-RFC in DNA replication, and we therefore propose that it functions to load or unload PCNA. In support of this, we find that overexpression of PCNA suppresses the MMS sensitivity of *elg1Δ*. Furthermore, *elg1Δ* is synthetic sick when combined with *rad17Δ*, *mec3Δ* and *ddc1Δ*, which encode the 9-1-1 clamp, but has no genetic interaction with *pol30-1*, a mutant in the gene encoding PCNA (data not shown), suggesting that Elg1 functions in the same pathway as PCNA, but in a pathway that is parallel to that in which the 9-1-1 clamp functions.

Elg1-RFC and checkpoint activation

A number of RFC family proteins have been implicated in checkpoint activation. Rad24 is required for the G₁ and G₂ DNA damage checkpoints (Siede *et al.*, 1994; Weinert *et al.*, 1994), and is important (although not essential) for the intra-S damage checkpoint (Paulovich *et al.*, 1997; Pellicoli *et al.*, 1999). A role for Rad24 in the S–M checkpoint is revealed in the context of mutations in other RFC family genes (Shimomura *et al.*, 1998; Naiki *et al.*, 2001). Similarly, deletion of *ctf18* has little effect on checkpoint activation unless *rad24* is also deleted (Naiki *et al.*, 2001). Consistent with formation of Rad24–Rfc2–5 complexes (Green *et al.*, 2000), mutants in *rfc2*, *rfc4* and *rfc5* with checkpoint defects have also been described (Sugimoto *et al.*, 1996, 1997; Noskov *et al.*, 1998; Kim and Brill, 2001). Our data indicate that Elg1 does not play a primary role in checkpoint activation, as *elg1Δ* mutants show normal Rad53 activation in response to DNA damage in S phase and in response to replication fork arrest by HU. Double mutants in *rad24* and either *elg1* or *ctf18* were more defective in Rad53 activation in S phase, and the triple mutant lacked detectable Rad53 activation. Thus it appears that Ctf18, and to a lesser extent Elg1, can partially substitute for the primary role of Rad24 in Rad53 activation in S phase. Alternatively, Elg1 could contribute to Rad53 activation in an indirect manner, for example through its effects on DNA replication.

When assessed directly, *elg1Δ* mutants display an intact intra-S phase checkpoint response as these cells exhibit the slow progression through S phase in the presence of MMS

that is seen in wild-type cells. We did note, however, that *elg1Δ rad24Δ* double mutants do not display an intra-S phase checkpoint defect that is greater than that seen with *rad24Δ* alone. This was unexpected, as *elg1Δ rad24Δ* was more defective in Rad53 activation than *rad24Δ*. In contrast, in *rad24Δ ctf18Δ*, S phase progression in the presence of MMS was more rapid than wild-type or *rad24Δ*, and resembled the progression seen in the completely checkpoint-defective *mec2-1* strain. Thus deletion of *ctf18* in a *rad24Δ* strain exacerbates the *rad24Δ* checkpoint defect, whereas deletion of *elg1Δ* does not. The implications of these data are 2-fold. First, the slow progression through S phase in *elg1Δ* is at least partially checkpoint independent, which is consistent with the *elg1Δ* mutant having a defect in DNA replication fork integrity or re-start. Secondly, there is not a strict correlation between Rad53 activation and S phase progression in the presence of MMS in these mutants. This probably reflects that the slow progression through S phase that is the hallmark of the intra-S phase checkpoint (Paulovich and Hartwell, 1995; Paulovich *et al.*, 1997) has both a checkpoint-dependent and a checkpoint-independent component (Tercero and Diffley, 2001). Our data suggest that Elg1 functions in the checkpoint-independent component of the slow S phase progression, again consistent with a role for Elg1 in preventing replication fork stalling or in re-starting stalled forks.

Elg1-RFC and genome integrity

The phenotypes of *elg1Δ* indicate that Elg1 has an important role in maintaining genome integrity. Elg1 suppresses recombination, protects against mutation and is important for recovery from DNA damage. Several aspects of the *elg1Δ* phenotype point to the presence of double-strand DNA breaks (DSBs), including the importance of homologous recombination for growth of the *elg1Δ* mutant and the hyper-recombination phenotype. Other aspects of the phenotype, while consistent with the presence of DSBs, point to a more fundamental role for Elg1 in DNA replication. These include the genetic requirement for the *SGS1/TOP3* and *MUS81/MMS4* replication fork re-start pathways (Kaliraman *et al.*, 2001; Doe *et al.*, 2002; Fabre *et al.*, 2002) in the *elg1Δ* mutant cells, the slow S phase progression observed in both the presence and absence of DNA damage, the increase in plasmid loss, the increase in mutation rate and the failure to recover efficiently from increased replication stalling induced by MMS. We propose that deletion of *ELG1* causes a decrease in replication fork processivity (an increase in fork stalling or a defect in re-start of stalled forks) perhaps due to defective Okazaki fragment maturation. Precedent for such a model comes from studies in both bacteria and yeast which have indicated that mutants in Okazaki fragment processing cause DSBs, stimulate recombination, confer DNA damage sensitivity, cause an increase in mutation frequency and render homologous recombination essential for viability (Rothstein *et al.*, 2000; Michel *et al.*, 2001), phenotypes similar to those observed in *elg1Δ*. Furthermore, defects in Okazaki fragment synthesis can cause replication fork stalling (Flores *et al.*, 2001), and there is a strong correlation between replication stalling and genome instability

(Aguilera *et al.*, 2000; Michel, 2000; Rothstein *et al.*, 2000; Michel *et al.*, 2001).

Given the similarity of Elg1 to Rfc1 and its presence in RFC-like complexes, it is likely that Elg1-RFC functions as a clamp loader or unloader. Biochemical experiments *in vitro* indicate that a complex interplay between RFC, PCNA and DNA polymerase δ governs appropriate polymerase switching and Okazaki fragment sealing during lagging strand DNA synthesis. Although it is clear that RFC is responsible for limiting the length of the nascent strand synthesized by pol α , and for loading of PCNA to initiate the switch to synthesis by pol δ (Maga *et al.*, 2001; Ayyagari *et al.*, 2003), it is not clear how PCNA is recycled on the lagging strand. Eukaryotic cells in S phase have a great excess of Okazaki fragments over molecules of PCNA (Mossi and Hubscher, 1998), and the stability of PCNA–DNA complexes suggests that an unloading activity is required to recycle PCNA (Yao *et al.*, 1996). A role for Elg1 in unloading and recycling PCNA on the lagging strand fits well with the available data. Additionally, this model predicts that PCNA should be limiting in *elg1Δ* mutants, and indeed we found that PCNA overexpression suppresses the MMS sensitivity of *elg1Δ*. Since Elg1 is not essential, its role in this process must be redundant, perhaps with RFC. Alternatively, Elg1 could be important for loading of PCNA during re-start of stalled replication forks, although in this model there must be a significant amount of fork stalling in an unperturbed S phase to account for the increased plasmid loss and the slow S phase progression observed in *elg1Δ*. It will be of great interest to determine if the human homolog of Elg1 plays a similar role in maintaining genome integrity.

Materials and methods

Yeast strains and media

Yeast strains used in this study are listed in table S1 in the Supplementary data. Non-essential haploid deletion strains were made by the Saccharomyces Gene Deletion Project (Winzler *et al.*, 1999) and can be obtained from Research Genetics (Huntsville, AL) or EUROSCARF (Frankfurt, Germany). Standard yeast media and growth conditions were used (Sherman, 1991).

Synthetic genetic array (SGA) analysis

SGA analysis was carried out as described (Tong *et al.*, 2001). The *MAT α* SGA starting strains containing *mus81Δ::natR* (Y3597), *mms4Δ::natR* (Y3561) and *elg1Δ::natR* (Y4521) were used to identify viable gene deletions that show synthetic genetic interactions with deletions in *mus81Δ*, *mms4Δ* and *elg1Δ*, respectively. Genetic interactions were confirmed by tetrad analysis on YPD (for *mms4Δ* and *mus81Δ*) or on synthetic medium supplemented with sodium glutamate as a nitrogen source (for *elg1Δ*). Confirmed interactions and extent of fitness defect are listed in table S2 in the Supplementary data.

Epitope tagging, immunoprecipitation and immunoblotting

The construction of strains carrying 3HA- or 13MYC-tagged Elg1 was performed as described (Longtine *et al.*, 1998). Immunoprecipitation was performed essentially as described (Naiki *et al.*, 2001). Proteins were resolved on 12% polyacrylamide–SDS gels, transferred to nitrocellulose membranes and subjected to immunoblot analysis with anti-HA (16B12; Covance), anti-myc (9E10; Santa Cruz) or anti-flag (M2; Sigma) antibodies. Immunoblots were developed using Supersignal ECL (Pierce). For detection of Rad53 phosphorylation, cells were fixed and extracts prepared essentially as described (Pelliccioli *et al.*, 1999). Proteins were separated on 8 or 4–12% polyacrylamide gels (Invitrogen), and immunoblots were probed with anti-RAD53 (γ C-19; Santa Cruz).

MMS, HU and UV sensitivity measurements

Cells were grown in YPD, serially diluted, spotted onto plates and incubated at 30°C. MMS (Aldrich) plates contained 0.01 or 0.035% (v/v) MMS in YPD and were used within 24 h of preparation. HU plates contained 50 mM HU in YPD. For the UV radiation sensitivity assay, cells were serially diluted, spotted onto YPD plates, exposed to UV light at 100 J/m² and incubated at 30°C. To determine viability after transient MMS treatment, mid-log phase cultures were incubated with 0.035% MMS in YPD liquid at 30°C. Samples were collected at the indicated time points, diluted, plated on YPD, and colonies were counted after incubation at 30°C for 3 days.

Synchronization and flow cytometry

Cells were arrested in G₁ by culturing in the presence of 2 µg/ml α-factor for 2 h at 30°C in YPD pH 3.9. Cells were released into the cell cycle by harvesting, washing and resuspending in YPD. Flow cytometry was performed as described (Chang *et al.*, 2002).

Plasmid loss, forward mutation rate and Can^r mutation spectra

Plasmid loss rate was measured using the plasmid YCp1 (Tye, 1999). Transformants were streaked on YPD, and single colonies were inoculated into YPD and grown to saturation. Probabilities of plasmid loss represent the averages of 10 independent experiments for wild-type and *elg1Δ*, and seven independent experiments for *ctf19Δ*, and were calculated as described (Boe and Rasmussen, 1996). Mutation rates were determined by measuring the rate of forward mutation to canavanine resistance, as described previously (Huang *et al.*, 2002). Fluctuation tests were performed with 10 parallel cultures, and the median value from each was used to calculate the spontaneous mutation rate by the method of the median (Lea and Coulson, 1949). Values represent the average of three experiments. To examine the spectrum of Can^r mutations, the complete open reading frame of the *CAN1* gene was amplified by PCR from independent Can^r colonies. Amplified DNA was analyzed by *HphI* restriction digestion and by sequencing.

Supplementary data

Supplementary data are available at *The EMBO Journal* Online.

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