

# Contrasting effects of Elg1–RFC and Ctf18–RFC inactivation in the absence of fully functional RFC in fission yeast

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Received May 4, 2005; Revised June 16, 2005; Accepted July 5, 2005

## ABSTRACT

**Proliferating cell nuclear antigen loading onto DNA by replication factor C (RFC) is a key step in eukaryotic DNA replication and repair processes. In this study, the C-terminal domain (CTD) of the large subunit of fission yeast RFC is shown to be essential for its function *in vivo*. Cells carrying a temperature-sensitive mutation in the CTD, *rfc1-44*, arrest with incompletely replicated chromosomes, are sensitive to DNA damaging agents, are synthetically lethal with other DNA replication mutants, and can be suppressed by mutations in *rfc5*. To assess the contribution of the RFC-like complexes Elg1–RFC and Ctf18–RFC to the viability of *rfc1-44*, genes encoding the large subunits of these complexes have been deleted and over-expressed. Inactivation of Ctf18–RFC by the deletion of *ctf18*<sup>+</sup>, *dcc1*<sup>+</sup> or *ctf8*<sup>+</sup> is lethal in an *rfc1-44* background showing that full Ctf18–RFC function is required in the absence of fully functional RFC. In contrast, *rfc1-44 elg1Δ* cells are viable and overproduction of Elg1 in *rfc1-44* is lethal, suggesting that Elg1–RFC plays a negative role when RFC function is inhibited. Consistent with this, the deletion of *elg1*<sup>+</sup> is shown to restore viability to *rfc1-44 ctf18Δ* cells.**

## INTRODUCTION

The ring-shaped homotrimer proliferating cell nuclear antigen (PCNA) plays key roles in many aspects of DNA metabolism in eukaryotic cells. PCNA encircles DNA to form a sliding clamp that can move freely along the duplex and acts to tether

a host of interacting proteins implicated in DNA replication, DNA repair and DNA modification, such as DNA ligase I, the nucleases Fen1 and XP-G, uracil-*N*-glycosylase and cytosine-5-methyltransferase. However, the central function of PCNA is to act as a processivity factor for the replicative DNA polymerases. In the absence of polymerase tethering by PCNA, chromosomal DNA replication cannot be completed efficiently, if at all [reviewed in (1,2)].

The loading of PCNA onto DNA requires that the PCNA ring be opened and then closed around the duplex. This reaction is catalysed in an ATP-dependent manner by a clamp loader complex. Clamp loaders have been identified in all three kingdoms of life. In eukaryotic cells, the first clamp loader to be identified was replication factor C (RFC), discovered in the studies of SV40 viral DNA replication *in vitro* (3,4). RFC is a pentameric complex comprising a large subunit (Rfc1) and four smaller subunits (Rfc2–Rfc5). All the five subunits are members of the AAA<sup>+</sup> protein superfamily of ATPase and ATPase-related proteins, and are essential for chromosomal DNA replication and cell survival in yeast. Recently, the structure of the yeast RFC complex was solved, revealing a complex with spiral geometry that closely matches to that of the DNA duplex (5).

In addition to RFC, three RFC-like complexes have been identified in recent years, two of which have been demonstrated to possess clamp loading activity, albeit with different sliding clamps [reviewed in (2,6)]. In this study, the fission yeast protein nomenclature is used when describing these complexes, although it should be noted that much of the information that has been gathered on their functions has come from the analysis of budding yeast (see Table 1 for nomenclature summary).

The first RFC-like complex to be identified (Rad17–RFC in fission yeast) performs its function in DNA structure checkpoints [reviewed in (7)]. The Rad17–RFC comprises the same

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**Table 1.** Yeast RFC/RFC-like complex and sliding clamp nomenclature

Nomenclature used	Large subunit		Small subunits Sc and Sp	Additional subunits Sc and Sp
	Sc	Sp		
RFC	Rfc1/Cdc44	Rfc1	Rfc2–Rfc5	None
Rad17–RFC	Rad24	Rad17	Rfc2–Rfc5	None
Ctf18–RFC	Ctf18	Ctf18 <sup>a</sup>	Rfc2–Rfc5	Dcc1 <sup>a</sup> and Ctf8 <sup>a</sup>
Elg1–RFC	Elg1	Elg1 <sup>a</sup>	Rfc2–Rfc5	None <sup>b</sup>
PCNA	Pol30	Pen1		
9-1-1	Ddc1	Rad9		
	Rad17	Rad1		
	Mec3	Hus1		

<sup>a</sup>Fission yeast proteins identified in this study.

<sup>b</sup>Based on purification shown in Figure 4.

four small subunits as RFC, Rfc2–Rfc5, but the large subunit Rfc1 is replaced by the related Rad17 protein (8–10). The effect of substituting Rad17 for Rfc1 is to alter the biochemical properties of the complex, notably its DNA substrate specificity (11–13). However, the Rad17–RFC also functions with a different sliding clamp. Instead of PCNA, Rad17–RFC loads the 9-1-1 complex onto DNA. This alternative sliding clamp comprises the Rad9, Rad1 and Hus1 DNA checkpoint proteins (Table 1) (11–13). It is thought that the Rad17–RFC and 9-1-1 complex act together as sensors of DNA structure perturbations.

The second RFC-like complex to be identified was Ctf18–RFC (14,15). In this complex, the large subunit of RFC is substituted with Ctf18. Two additional factors, Dcc1 and Ctf8, also associate with Ctf18, resulting in a seven subunit complex. The Ctf18–RFC is required for the efficient establishment of chromosome cohesion during S-phase, the process by which the newly replicated sister chromatids are held together until anaphase. Although all the three genes are non-essential, *Saccharomyces cerevisiae* cells carrying *ctf18*, *dcc1* or *ctf8* mutations display genome instability and chromosome loss phenotypes (14,16). Biochemical evidence indicates that Ctf18–RFC functions as an ATP-dependent PCNA loader, loading PCNA onto primed templates ~10-fold less efficiently than RFC (17,18).

The third RFC-like complex sees Rfc1 substituted by Elg1 [reviewed in (6,19)]. Mutants in the budding yeast *ELG1* gene were identified in a number of independent genetic screens for factors affecting genome stability in diverse ways (20–27): *elg1*Δ mutants display increased levels of mitotic recombination, chromosome loss, transposition and gross chromosomal rearrangement. The *elg1*Δ mutants also display telomere lengthening and are synthetically lethal or sick when combined with mutations in various DNA processing pathways, such as homologous recombination (synthetic interactions with *rad50*, *rad51*, *rad52*, *rad54*, *rad55*, *rad57*, *mre11* and *xrs2*), replication fork re-start (*mus81* and *mms4*) and DNA replication (*pol32*, *dna2* and *rad27*). The *elg1*Δ cells are also mildly sensitive to the alkylating agent methylmethanesulphonate (MMS) but not to hydroxyurea (HU) or UV treatment. Despite this analysis, the precise function of the Elg1–RFC is unknown, although it has been shown that the complex co-immunoprecipitates with PCNA (23). The complete subunit composition of the Elg1–RFC is also unknown.

Although the small and large subunits of the clamp loader complexes are related to one another, the large subunits have

extended N- and C-terminal regions. In the case of Rfc1, the function of the N-terminal extended region has been analysed previously (28–30). This region includes both a conserved PCNA-binding motif with a potential role in targeting RFC to replication factories (31) and a BRCT domain (32), and possesses DNA-binding activity (28,33) but is not required either for PCNA loading *in vitro* or for RFC function *in vivo*. The function of the C-terminal domain (CTD) has been less well characterized, although *in vitro* reconstitution experiments point to an important role for this domain in RFC complex assembly (28). Recent structural studies suggest a role for this domain in stabilizing the RFC complex by bridging the ends of the spiral assembly of RFC subunits but the functional consequences of this are not known (5).

In this study, the CTD of *Schizosaccharomyces pombe* Rfc1 is shown to be essential for RFC function *in vivo*, and the isolation and the analysis of novel mutant *rfc1* alleles generated by random and targeted mutagenesis of the CTD, including a temperature-sensitive mutant, *rfc1-44*, is described. Analysis of *rfc1-44* shows the cells to be incapable of completing DNA replication at their restrictive temperature, sensitive to DNA damaging agents, and synthetically lethal with a number of DNA replication mutants. To investigate this further, genes encoding *S.pombe* orthologues of Elg1, Ctf18, Dcc1 and Ctf8 are identified, *elg1*Δ, *ctf18*Δ, *dcc1*Δ and *ctf8*Δ strains constructed and analysed, and *rfc1-44 ctf18*Δ, *rfc1-44 dcc1*Δ and *rfc1-44 ctf8*Δ double mutants shown to be inviable, implying that Ctf18–RFC function is essential for cell viability in the absence of fully functional RFC. The purification of the Elg1–RFC from fission yeast is also described, demonstrating for the first time in any organism that this complex has a five subunit structure. In support of a model in which Elg1–RFC plays a negative role when RFC function is impaired, elevated levels of Elg1–RFC are shown to be lethal in *rfc1-44* and the deletion of *elg1*<sup>+</sup> shown to restore viability to *rfc1-44 ctf18*Δ cells. Finally, the identification of suppressors of *rfc1-44* mapping within the *rfc5* gene is reported, suggesting that the functional defect in RFC in *rfc1-44* cells may lie in the Rfc1–Rfc5 interaction revealed by the crystal structure.

## MATERIALS AND METHODS

### Yeast strains, media and methods

Haploid *S.pombe* strains used for strain construction were as follows: Sp236 (*leu1-32 ura4-D18* h<sup>+</sup>), Sp292 (*leu1-32 ura4-D18 ade6-M210* h<sup>-</sup>), Sp293 (*leu1-32 ura4-D18 ade6-M216* h<sup>+</sup>), Sp322 (*leu1-32 ura4-D18* h<sup>-</sup>), Sp347 (972 h<sup>-</sup>) and Sp348 (h<sup>+</sup><sup>S</sup>). PCR-mediated gene targeting was used to construct the following strains: Sp323 (*elg1::natMX6 leu1-32 ura4-D18* h<sup>-</sup>), Sp325 (*elg1::kanMX6 leu1-32 ura4-D18* h<sup>-</sup>), Sp359 (*dcc1::natMX6* h<sup>-</sup>), Sp360 (*dcc1::natMX6* h<sup>+</sup><sup>S</sup>), Sp365 (*ctf18::natMX6* h<sup>-</sup>), Sp378 (*ctf8::natMX6* h<sup>-</sup>), Sp380 (*ctf8::natMX6* h<sup>+</sup><sup>S</sup>), Sp382 (*ctf18::natMX6* h<sup>+</sup><sup>S</sup>) and Sp402 (*elg1-TAP-natMX6* h<sup>-</sup>). A wild-type diploid strain (*leu1-32/leu1-32 ura4-D18/ura4-D18 ade6-M210/ade6-M216* h<sup>-</sup>/h<sup>+</sup>) was created by standard methods by mating haploids Sp292 and Sp293 and used to construct various *rfc1* mutants (see below). The following *rfc1* strains were generated by subsequent matings: HSp1 (*rfc1-44* h<sup>+</sup>), HSp9 (*rfc1-54* h<sup>+</sup>),

HSp17 (*rfc1-44 h<sup>-</sup>*), HSp23 (*rfc1-54 h<sup>-</sup>*), HSp25 (*rfc1-44 elg1::natMX6 h<sup>+</sup>*), HSp26 (*rfc1-44 elg1::natMX6 h<sup>-</sup>*), HSp27 (*rfc1-44 elg1::kanMX6 ctf18::natMX6 h<sup>+</sup>*) and KSp1 (*elg1::kanMX6 ctf18::natMX6 h<sup>-</sup>*). Yeast growth media (YE, EMM and ME) were used essentially as described (34). 5-Fluoroorotic acid (5-FOA) selection was performed using YE medium supplemented with 1 mg/ml 5-FOA. G418 (Geneticin; from Invitrogen/GibcoBRL) and nourseothricin (ClonNAT; from Werner BioAgents) were used at 100 mg/l in YE agar. Transformations were performed by electroporation (35) or lithium acetate methods (36) as appropriate. Tetrad dissection was accomplished using a micromanipulator (Singer Instruments). Flow cytometry on fixed propidium-iodide stained cells was performed by using a FACS Calibur instrument and CellQuest software (BD Biosciences) as described previously (37), cell number counts by using a Z2 particle counter (Beckman Coulter), and pulse-field gel electrophoresis (PFGE) as described by Kelly *et al.* (38) using a BioRad CHEF DR11 system.

### Construction and analysis of *rfc1<sup>+</sup>/rfc1-ΔC* strain

For plasmid integration into the genome, a series of constructs based on plasmid pJKE1 were used. pJKE1 corresponds to pUR19 (39) from which *arsI<sup>+</sup>* has been deleted by ClaI digestion and re-ligation. Initially, sequences encoding amino acids 590–935 of Rfc1 (corresponding to the CTD plus 130 amino acids N-terminal to the CTD) were amplified by PCR using pUR19-Rfc1 as template and cloned into plasmid pJKE1 to generate plasmid pJKE11. The PCR overlap extension method (39) was then used, with pJKE11 as template and complementary 50mer oligonucleotides, to introduce stop codons in all three frames into the *rfc1<sup>+</sup>* sequence, so that the encoded protein would terminate after Pro719. The product of the second round of PCR was cloned into pJKE32 (see below) to generate pJKE59. Linearized plasmid DNA was then transformed into an *S.pombe* diploid strain (*leu1-32/leu1-32 ura4-D18/ura4-D18 ade6-M210/ade6-M216 h<sup>-</sup>/h<sup>+</sup>*) and stable integrative transformants isolated and characterized by PCR using genomic DNA as template. Sequencing of PCR products generated in this manner allowed confirmation of the genotypes. The sequences of all the oligonucleotides used can be obtained from the authors on request. Diploids were then induced to sporulate on ME medium and asci dissected using a micromanipulator (Singer Instruments). Following re-growth, the genotypes of viable colonies were confirmed by replica plating.

### Pentapeptide insertion mutagenesis

To mutagenize *rfc1<sup>+</sup>* using the pentapeptide insertion method, sequences encoding amino acids 590–935 of Rfc1 (corresponding to the CTD plus 130 amino acids N-terminal to the CTD) were amplified by PCR and cloned into plasmid pJKE31, a derivative of pJKE1 with the polylinker KpnI site removed (see below), to generate pJKE32. Next, pJKE32 was mutagenized by the PCR overlap extension method (40) to remove the KpnI site present in the *rfc1<sup>+</sup>* sequence (the sequence G GTA CC was changed to G GTT CC, with no effect on encoded amino acid sequence), to make pJKE42. Pentapeptide insertion mutagenesis was performed using the Tn4430 system (41,42). pJKE42 was transformed

into *E.coli* FH1046 and individual transformant colonies were mated with *E.coli* DS941 after which the mating mixtures were plated on the medium to select for clones containing pJKE42 carrying the Tn4430 transposon. Isolates containing a Tn4430 insertion within the *rfc1* region were then identified by colony PCR and plasmid DNA prepared. This was restricted by KpnI and re-ligated to remove the bulk of the transposon, before being transformed into *E.coli* DH5α. Seventeen individual transposon-free clones were isolated and the position of the 15 bp insertion determined by KpnI restriction mapping and DNA sequencing. Six duplicated mutants were discarded at this point, leaving a collection of twelve different pentapeptide insertion alleles (*rfc1-43-rfc1-54*, carried by plasmids pJKE43–pJKE54). Site-specific mutations (*rfc1-33-rfc1-41*, carried by plasmids pJKE33–pJKE41) were generated by PCR overlap extension mutagenesis (40) using complementary 36mer oligonucleotides and plasmid pJKE11 as a template. The sequences of the oligonucleotides used can be obtained from the authors upon request. Following the second round of PCR the products were cloned into pJKE32. Subsequently, linearized plasmid DNA was transformed into diploid *S.pombe* and integrative transformants isolated and analysed as described above. For further analysis, 5-FOA was used to select for *rfc1-44* and *rfc1-54* strains in which the plasmid had recombined out of the genome leaving a mutated copy of the *rfc1* gene on the chromosome. These strains were then backcrossed to wild type to remove auxotrophic markers, resulting in strains HSp1 (*rfc1-44 h<sup>+</sup>*), HSp9 (*rfc1-54 h<sup>+</sup>*), HSp17 (*rfc1-44 h<sup>-</sup>*) and HSp23 (*rfc1-54 h<sup>-</sup>*).

### Suppressor screening

To screen for suppressors of *rfc1-44*, a 1.3 kb fragment was amplified by PCR from the *rfc5<sup>+</sup>* chromosomal region and subcloned into plasmid pJKE31 (above). This corresponded to ~200 bp 5' to the *rfc5<sup>+</sup>* open reading frame (ORF) and 1.2 of sequence encoding the first 289 (of 358) amino acids of Rfc5. The resulting plasmid pJKE55 was subjected to mutagenesis with Tn4430 as described above except that, following mating with *E.coli* DS941, a library of transposon-containing plasmids was prepared, digested with KpnI, re-ligated and transformed in *E.coli* DH5α. Plasmid DNA was prepared from the >3000 pooled colonies, linearized and used to transform *rfc1-44*.

### Gene deletions

The *elg1<sup>+</sup>*, *ctf18<sup>+</sup>*, *dcc1<sup>+</sup>* and *ctf8<sup>+</sup>* genes were deleted in wild-type 972 h<sup>-</sup> (SP347) and h<sup>+</sup>S (Sp348) strains using the PCR-mediated gene targeting method for fission yeast as described by Bähler *et al.* (36) using either pFA6a-kanMX6 or pFA6a-natMX6 as PCR templates. The latter (a gift from P. Hentges and T. Carr, University of Sussex) confers resistance to nourseothricin (also known as clonNAT, supplied by Werner BioAgents), which was added to YE plates at 100 μg/ml. Oligonucleotides (100mer with 80 nt of gene-specific sequence) were supplied by DNA Technology A/S. The deletions were confirmed by PCR using oligonucleotide primers flanking the cassette. The sequences of all the oligonucleotides used can be obtained from the authors upon request.

## Purification of the Elg1-RFC

To facilitate purification of the Elg1-RFC, the chromosomal *elg1*<sup>+</sup> gene was tagged at its 3' end with sequences encoding the TAP module marked with the *natMX6* cassette. For this, the *kanMX6* module was first excised from pFA6a-CTAP-kanMX6 (43) and replaced with the *natMX6* cassette from pFA6a-natMX6 (above). The resulting plasmid, pFA6a-CTAP-natMX6, was then used as the template for PCR using 100mer oligonucleotides (sequences from the authors upon request), the PCR product transformed into *S.pombe* strain Sp347 and nourseothricin-resistant colonies identified. Successful tagging was confirmed by PCR and direct sequencing of amplified chromosomal DNA fragments. For purification, a 12 litres culture of the *elg1-TAP-natMX6* h<sup>-</sup> strain (Sp402) was grown to an OD<sub>600 nm</sub> of 0.8 (1 × 10<sup>7</sup> cells/ml) at 32°C, before the cells were harvested and processed as described previously (43) except that the cells were broken by grinding in liquid nitrogen using an RM100 mortar grinder (Retsch) for 15 min. Following TCA precipitation, the samples were resolved by SDS-PAGE and silver stained. Trypsin digestion of excised bands and mass spectrometric analysis was carried out commercially at the Proteomics Facility at the University of Dundee, UK. (<http://www.dundee.ac.uk/biocentre/FingerPrints/>).

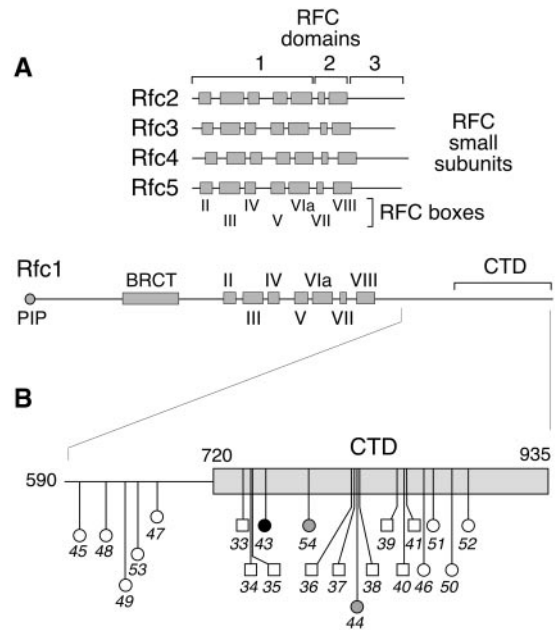
## RESULTS

### The conserved CTD of fission yeast Rfc1 is required for RFC function

In all eukaryotic species, the large subunit of RFC differs from the small subunits in having extended N- and C-terminal regions (Figure 1A). In order to probe the function of the CTD in greater detail, one copy of the chromosomal *rfc1*<sup>+</sup> gene in a diploid *S.pombe* strain was altered to encode a truncated Rfc1 protein lacking the entire 215 amino acid CTD (Materials and Methods). This *rfc1*<sup>+</sup>/*rfc1*-ΔC diploid was sporulated and the properties of haploid *rfc1*-ΔC cells analysed following meiotic tetrad dissection. Spores expressing the truncated Rfc1-ΔC protein were capable of germination and 1–5 cell divisions, but incapable of colony formation (data not shown). Similar to *S.pombe rfc1*Δ cells (44), the arrested *rfc1*-ΔC cells were often elongated, indicative of a cell cycle defect. Transformation of the *rfc1*<sup>+</sup>/*rfc1*-ΔC diploid prior to sporulation with a plasmid carrying wild-type *rfc1*<sup>+</sup> rescued the *rfc1*-ΔC haploid, allowing efficient colony formation (data not shown). The CTD is therefore required for RFC function *in vivo*, consistent with the earlier observation that the deletion of sequences from the C-terminus of the human Rfc1 orthologue hRFC140 abolished RFC complex formation *in vitro* (28).

### Mutational analysis of the CTD

To further probe its function, the CTD was subjected to mutational analysis. Both random and directed mutagenesis strategies were used to generate a total of 21 mutant *rfc1* alleles. Random mutagenesis was accomplished using the Tn4430-based pentapeptide insertion method (41,42). Twelve alleles were constructed by this method (Figure 1B, circles), seven of which contained insertions within the CTD. The remaining

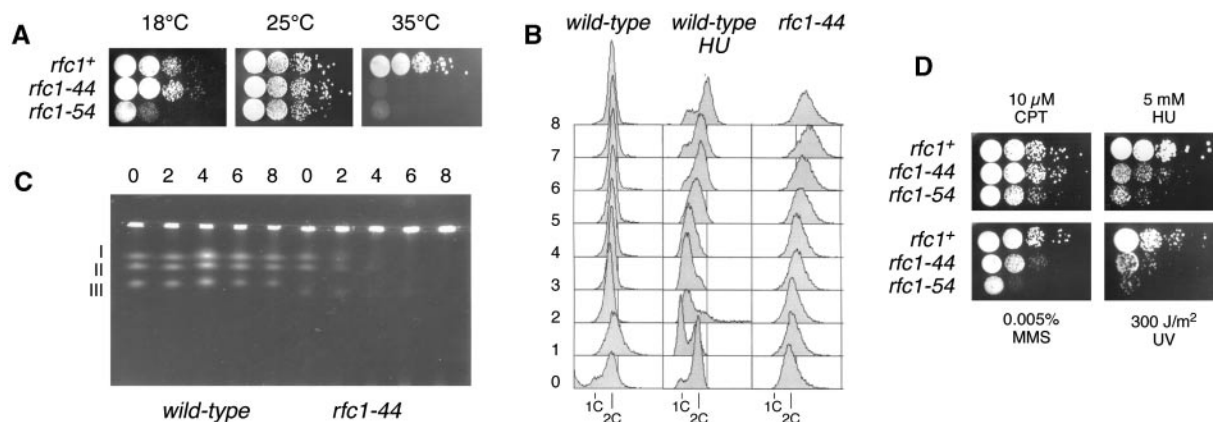


**Figure 1.** (A) Schematic structure of the RFC subunits. Each small subunit (Rfc2–Rfc5) is a member of the AAA<sup>+</sup> family of proteins and comprises three structural domains I–III (indicated above the Rfc2 protein). The large subunit Rfc1 is also an AAA<sup>+</sup> protein and possesses the same domains but has additional N- and C-terminal regions. The N-terminal region contains a PIP (PCNA-interacting protein) motif and a single BRCT motif of unknown function. The function of the CTD is not known. Each RFC subunit also contains a number of conserved sequence blocks, RFC boxes II–VIII (grey boxes, shown numbered on the Rfc1 protein). (B) Location of mutants within the Rfc1 CTD. An expanded view of part of domain III and the CTD is shown, spanning amino acids 590–935 (the CTD spans 720–935). The mutants generated in this study are indicated as follows: open circles (pentapeptide insertion mutants, no significant effect on protein function), grey circles (pentapeptide insertion mutants, temperature-sensitive), closed circles (pentapeptide insertion mutants, non-functional), open squares (amino acid substitutions, no significant effect on protein function). See Supplementary Information for more details.

five insertions mapped N-terminal to the CTD, within the RFC collar domain (5). Oligonucleotide-directed *in vitro* mutagenesis was used to generate a further nine *rfc1* alleles with mutations within the CTD (Figure 1B, squares). All nine mutations changed individual conserved charged amino acids to alanine (see Supplementary Information for details).

As above, the mutant alleles were targeted to one copy of the *rfc1*<sup>+</sup> gene in a diploid strain (Materials and Methods). Following meiosis and sporulation, the properties of the *rfc1* mutants were analysed by tetrad dissection and re-growth. The results are summarized in the Supplementary Information. Briefly, 13 of the 21 alleles were viable in haploid form and essentially indistinguishable from wild type (Figure 1B, open circles or squares) whereas only one, *rfc1*-43, was inviable (Figure 1B, closed circle). The latter encodes an Rfc1 protein with a pentapeptide insertion within the CTD. Two of the mutant alleles, *rfc1*-44 and *rfc1*-54, displayed a temperature-sensitive phenotype (Figure 1B, grey circles and Figure 2A) that could be rescued by wild-type *rfc1*<sup>+</sup> (data not shown).

One of these alleles, *rfc1*-44, was characterized further. When shifted to the restrictive temperature of 35°C, *rfc1*-44 cells arrest with a 2C DNA content (determined by flow cytometry of propidium iodide stained cells, Figure 2B) indicating



**Figure 2.** Characterization of temperature-sensitive *rfc1* alleles. **(A)** Serial dilutions of wild-type (*rfc1*<sup>+</sup>), *rfc1-44* and *rfc1-54* cells incubated at 18, 25 and 35°C, respectively, for 3–4 days. **(B)** Flow cytometry of propidium iodide stained wild-type and *rfc1-44* cells following shift from 25 to 35°C with or without the addition of HU. Samples for analysis were taken every hour from 0 to 8 h. **(C)** PFGE analysis of chromosome structure in wild type or *rfc1-44* following shift from 25 to 35°C. Samples for the analysis were taken every 2 h from 0 to 8 h. Note that chromosome III in *rfc1-44* cells appears somewhat shorter than in the wild-type control, indicative of the loss of rDNA repeats. **(D)** Sensitivity of wild-type, *rfc1-44* and *rfc1-54* cells to camptothecin (CPT, 10 μM), hydroxyurea (HU, 5 mM), methylmethane sulphonate (MMS, 0.005%) and UV light (300 J/m<sup>2</sup>). Serial dilutions were spotted and incubated at 25°C for 4 days.

that bulk DNA replication is completed prior to arrest. Note that the apparent increase in DNA content seen following shift to the restrictive temperature (seen as a gradual rightwards drift in the position of the 2C peak over the timecourse of the experiment shown in Figure 2B) is the result of ongoing mitochondrial DNA replication in elongating (i.e. cell cycle arrested) *S.pombe* cells, as described previously (37). Similar peak movements are shown in Figure 6 for *rfc1-44* single-, double- and triple-mutant combinations (see below), but not in cells of normal length.

To analyse DNA structure in the arrested cells, PFGE of chromosomal DNA from wild-type and mutant cells was performed. Many previously isolated yeast DNA replication mutants display a 2C DNA content by flow cytometry but their chromosomes cannot enter a PFGE gel (45). Similar results are obtained when chromosomes are prepared from the cells arrested in S-phase with HU, for example, and are taken as an indication that the chromosomes remain at least partly unreplicated (45). Significantly, by 4 h after temperature shift, the chromosomes from *rfc1-44* cells failed to enter the PFGE gel, indicating that the replication is not complete under these circumstances (Figure 2C). At the permissive temperature of 25°C, *rfc1-44* cells display increased sensitivity compared with wild type to the DNA replication inhibitor HU and to the DNA damaging agents, MMS and UV, consistent with the previous data implicating RFC and PCNA in various DNA repair mechanisms (Figure 2D). In addition, *rfc1-44* is synthetically lethal at 25°C with a number of temperature-sensitive DNA replication mutants (data not shown), including *pol3-ts3*, *cdc1-P13*, *cdc27-P11*, *cdc27-D1* and *dna2-C2* (46–49). The *pol3*<sup>+</sup>, *cdc1*<sup>+</sup> and *cdc27*<sup>+</sup> genes encode the catalytic, B and C subunits, respectively, of DNA polymerase δ in *S.pombe*, while *dna2*<sup>+</sup> encodes an endonuclease-helicase implicated in Okazaki fragment processing.

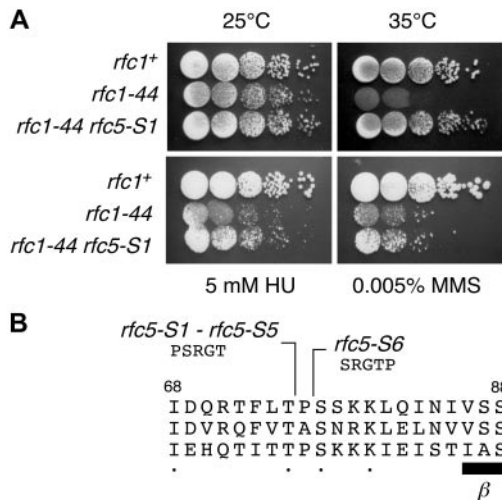
#### The *rfc1-44* mutant can be rescued by mutations in *rfc5*

The structure of the budding yeast RFC complex has been solved recently (5). The structure shows the ATPase domains

of all five subunits forming a right-handed spiral whose pitch matches that of duplex DNA and the CTD forming a physical link between the two ends of the spiral, bridging Rfc1 and Rfc5. This suggested to us that the RFC complex in *rfc1-44* cells might be defective in this linking function and that it might be possible to identify suppressors of *rfc1-44* by randomly mutagenizing *rfc5*<sup>+</sup>. The cloned *rfc5*<sup>+</sup> gene was therefore subjected to pentapeptide insertion mutagenesis and a library of mutagenized plasmids constructed (Materials and Methods). The library DNA was then linearized by restriction enzyme digestion and transformed into *rfc1-44* cells. Approximately 2400 integrative transformants obtained at 25°C were tested for their ability to grow at 35°C and six clones capable of growth selected for further analysis, including sequencing of the chromosomal *rfc5* gene (Figure 3). Remarkably, all six contained pentapeptide insertions within a very short stretch of the Rfc5 protein, despite the random distribution of insertions in individual library clones as determined by restriction mapping (data not shown). Indeed, five of the six insertions (named *rfc5-S1* through *rfc5-S5*, see Figure 3B) were identical, containing an insertion of the pentapeptide sequence PSRGT between amino acids 75 and 76. The sixth allele (*rfc5-S6*) contained an insertion of the sequence SRGTP between amino acids 76 and 77. Unfortunately, amino acids 68–85 of budding yeast Rfc5 are absent from the published three-dimensional structure, making it impossible to locate these in relation to the CTD (5). However, these genetic observations suggest that there may be a close functional relationship between those regions of the Rfc1 and Rfc5 proteins defined by *rfc1-44* and *rfc5-S* and support a model in which the biochemical defect in the RFC (Rfc1–44) complex lies in bridging the ends of the RFC spiral.

#### Fission yeast Elg1–RFC

During the course of this work, several groups reported the isolation and genetic analysis of the budding yeast *ELG1* gene encoding the large subunit of a novel RFC-like complex (20–27). To investigate whether a similar complex



**Figure 3.** Suppression of *rfc1-44* by *rfc5-S1*. (A) Upper parts: serially diluted cultures of wild-type, *rfc1-44* and *rfc1-44 rfc5-S1* double mutant cells plated at 25°C (left panel, 4 days) or 35°C (right panel, 3 days). Lower parts: the same serial dilutions plated on either 5 mM HU (left panel) or 0.005% MMS (right panel) and incubated at 25°C for 4 days. Although *rfc5-S1* can suppress *rfc1-44* at 35°C, it does not fully restore *rfc1-44* activity as the double mutant cells are still sensitive to HU and MMS. See the text for details. (B) Sequence alignment of *S.pombe*, *S.cerevisiae* and human Rfc5 proteins showing location of *rfc1-44* suppressing insertion alleles. The structure of this region is unknown, except for a short  $\beta$ -strand (1SXJ  $\beta$ 22) encompassing amino acids 86–88. Conserved residues are indicated by dots beneath the aligned sequences.

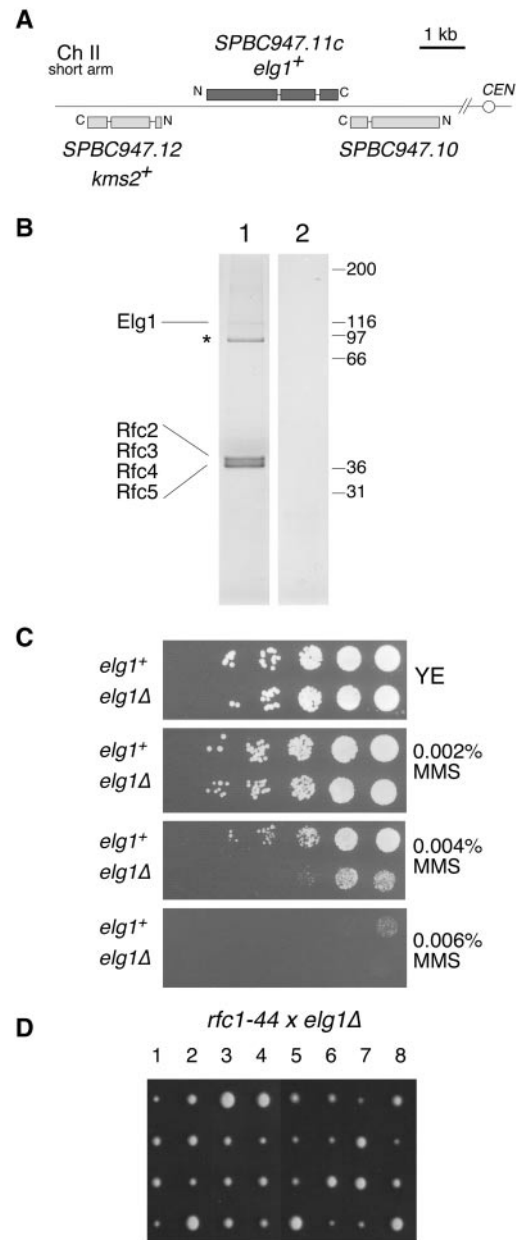
**Table 2.** RFC and RFC-like complex components in fission yeast

Protein	Systematic name	Size	E-score <sup>a</sup>	Essential function?
Elg1	SPBC947.11c	920	$6.7 \times 10^{-16}$	No
Ctf18	SPBC902.02c	960	$3.7 \times 10^{-70}$	No
Dcc1	SPAC31A2.15c	349	$5.1 \times 10^{-05}$	No
Ctf8	SPAC19D5.11c	109	0.26	No

<sup>a</sup>BLAST E-score versus *S.cerevisiae* orthologue.

existed in *S.pombe*, the fission yeast *elg1*<sup>+</sup> gene was identified by BLAST searching (E-value  $6.7 \times 10^{-16}$ ). *elg1*<sup>+</sup> (systematic name SPBC947.11c, see Table 2 and Figure 4A) lies on *S.pombe* chromosome II and encodes a 2760 bp ORF interrupted by two introns of 44 and 47 nt, resulting in a predicted protein of 920 amino acids and Mr of 103.9 kDa.

To determine the composition of the Elg1–RFC complex, the chromosomal *elg1*<sup>+</sup> gene was tagged at its 3' end with sequences encoding the tandem affinity purification (TAP) tag [reviewed in (50)] and the Elg1–RFC purified by the TAP technique (43). Silver staining and subsequent mass spectrometric analysis of the purified material identified Elg1 in both full-length and N-terminally degraded forms, as well as all four small RFC subunits (see Figure 4B and Supplementary Material for the coverage of individual proteins). No additional proteins were identified in these purified preparations, strongly suggesting that Elg1–RFC comprises the Elg1, Rfc2, Rfc3, Rfc4 and Rfc5 proteins only. If the Elg1–RFC complex contains additional proteins, perhaps similar to Dcc1 and Ctf8 in Ctf18–RFC, these must be present in sub-stoichiometric amounts relative to full-length Elg1, be refractive to silver



**Figure 4.** Fission yeast Elg1. (A) Chromosome context and schematic structure of the *elg1*<sup>+</sup> gene on chromosome II. (B) TAP of Elg1–RFC. Protein extracts were prepared from *elg1*-TAP cells and subject to TAP. Following TCA precipitation, the purified proteins were subjected to SDS–PAGE and silver staining (lane 1). The bands indicated were excised and identified by mass spectrometry (see Supplementary Information). Wild-type cells (lane 2) were processed in parallel. The species marked with an asterisk corresponds to N-terminally degraded Elg1. (C) The *elg1* $\Delta$  cells display increased sensitivity to MMS. Serial dilutions were plated on YE medium containing 0, 0.002, 0.004 or 0.006% MMS and incubated for 4 days at 32°C. (D) The *rfc1-44 elg1* $\Delta$  cells are viable at 28°C. Eight tetrad type tetrads (labelled 1–8) are shown. The genotypes of colonies were confirmed by replica plating and/or PCR with genomic DNA templates as appropriate.

staining, or lost during the TAP purification. Further analysis will be required to address these issues.

To study its function, *elg1*<sup>+</sup> was deleted from the chromosome and replaced with the *natMX6* cassette by the PCR-mediated gene targeting method (36). Cells carrying the

*elg1Δ* allele (*elg1::natMX6*) were viable and displayed mild sensitivity to MMS (Figure 4C) but were insensitive to UV, HU and the topoisomerase inhibitor camptothecin (data not shown) as is the case for budding yeast *elg1Δ* cells (21–23).

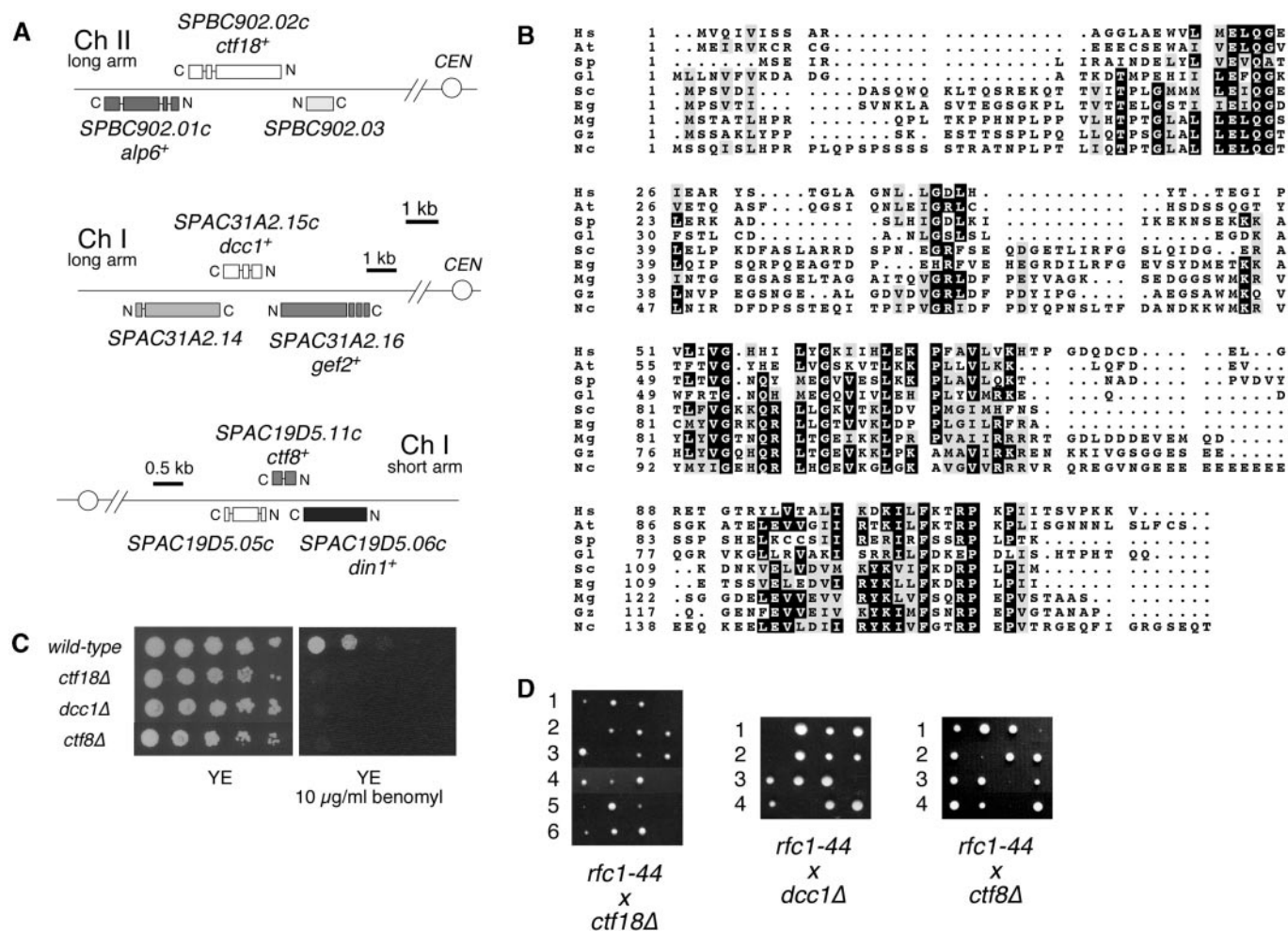
Next, *rfc1-44* was crossed to *elg1Δ*. The double mutant *rfc1-44 elg1Δ* was viable at 28°C (Figure 4D) and essentially indistinguishable from *rfc1-44*. However, closer inspection suggested that at 36.5°C the *rfc1-44 elg1Δ* cells grew marginally better than the *rfc1-44* mutant alone, suggesting that *elg1+* might exert a negative effect on *rfc1-44* cells (data not shown). This is explored further below.

### Fission yeast Ctf18–RFC

To complement our studies on the *S.pombe* Elg1–RFC, the role of the fission yeast Ctf18–RFC in *rfc1-44* cells was examined. In *S.cerevisiae*, the Ctf18–RFC comprises seven subunits: Ctf18, Rfc2, Rfc3, Rfc4, Rfc5, Dcc1 and Ctf8 (2). The same appears to be true of the human Ctf18–RFC

also (17). To investigate whether *S.pombe* possessed Ctf18, Dcc1 and Ctf8 proteins and to test their function in relation to RFC, the fission yeast genome sequence was probed for genes encoding putative fission yeast Ctf18, Dcc1 and Ctf8 orthologues. Searches using the budding yeast proteins as the query sequences readily identified putative Ctf18 and Dcc1 orthologues (BLAST *E*-scores of  $3.7 \times 10^{-70}$  and  $5.1 \times 10^{-05}$ , respectively; Table 2). *ctf18+* (systematic name SPBC902.02c) lies on chromosome II and is predicted to encode a 960 amino acid protein with an Mr of 108.6 kDa (Figure 5A). This is somewhat larger than the *S.cerevisiae* protein (741 amino acids, Mr 84.3 kDa). The two proteins are 28% identical over their C-terminal 720 amino acids. The *dcc1+* gene on chromosome I (SPAC31A2.15c) is predicted to encode a 349 amino acid protein with predicted Mr 40.9 kDa, that is, ~22% identical to *S.cerevisiae* Dcc1 (Figure 5A).

A gene encoding a Ctf8 homologue was also identified. This gene, designated *ctf8+*, contains a single 50 bp intron



**Figure 5.** Fission yeast Ctf18, Dcc1 and Ctf8. (A) Chromosome context and schematic structure of the *ctf18+*, *dcc1+* and *ctf8+* genes. (B) Multiple sequence alignment of Ctf8 homologues from various species. Key: Hs (human, A1301014), At (*Arabidopsis thaliana*, BAA97465.1), Sp (*S.pombe*, this study), Sc (*S.cerevisiae*, accession number NP\_012061), Eg (*Eremothecium gossypii*, NP\_985024), G1 (*Giardia lamblia*, EAA38993), Nc (*Neurospora crassa*, XP\_328185), Gz (*Gibberella zeae*, EAA67941) and Mg (*Magnaporthe grisea*, EAA51651) (C) Sensitivity of wild-type *ctf18Δ*, *dcc1Δ* and *ctf8Δ* strains to the microtubule inhibitor benomyl. Serial dilutions were plated on YE plates with/without 10 μg/ml benomyl and incubated at 32°C for 4 days. (D) All three double mutants (*ctf18Δ rfc1-44*, *dcc1Δ rfc1-44* and *ctf8Δ rfc1-44*) are synthetically lethal. Four to six tetraplate tetrads are shown for each cross. The genotypes of colonies were confirmed by replica plating and/or PCR with genomic DNA templates as appropriate.

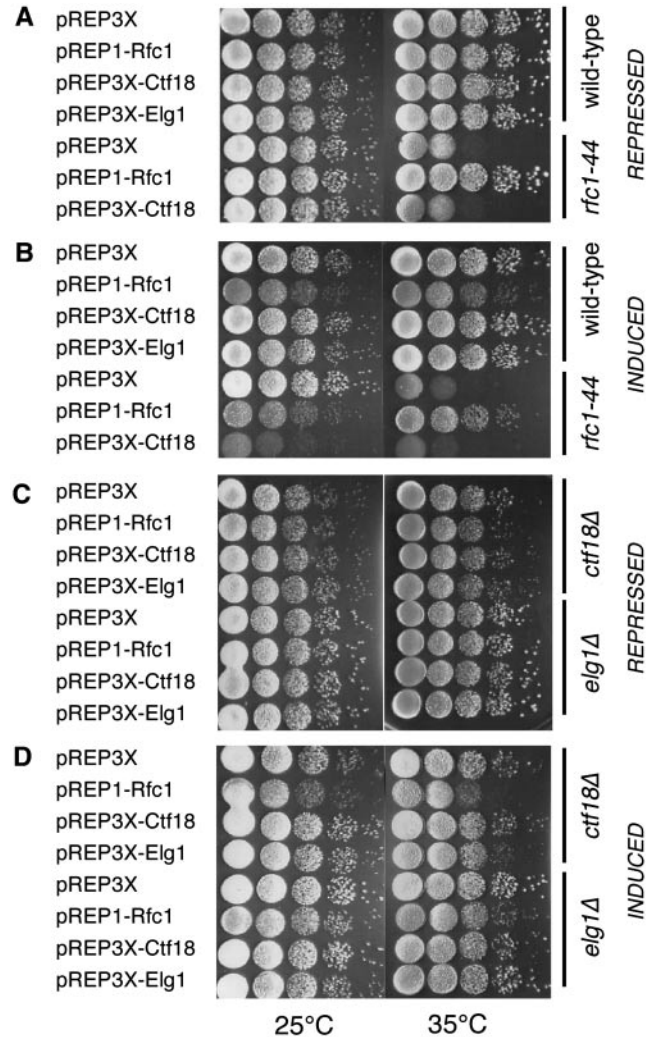
(confirmed by cDNA sequencing; data not shown) and encodes a 109 amino acid protein. The *ctf8<sup>+</sup>* is located on chromosome I between the SPAC19D5.06c and SPAC19D5.05c genes. Owing to its small size, the presence of the intron, and the low sequence similarity between Ctf8 family members (*S.pombe* Ctf8 is only ~20% identical to its *S.cerevisiae* counterpart, see Figure 5B), this region of the chromosome was not previously predicted to encode a protein. The gene has now been assigned the systematic name SPAC19D5.11c (Table 2).

To investigate the role of these three proteins in *S.pombe*, all three genes were deleted from the chromosome and replaced by the *natMX6* cassette. Cells carrying one of the *ctf18::natMX6* (*ctf18Δ*), *dcc1::natMX6* (*dcc1Δ*) or *ctf8::natMX6* (*ctf8Δ*) alleles were viable and indistinguishable from wild type under normal growth conditions. Deletion of *CTF18* in *S.cerevisiae* renders cells sensitive to the microtubule inhibitor benomyl (16). This was also true of *S.pombe* *ctf18Δ*, *dcc1Δ* and *ctf8Δ* cells (Figure 5C), suggesting that the Ctf18–RFC complexes may perform similar, if not identical, functions in the two yeasts. Consistent with this, while this work was in progress, Watanabe and co-workers (51) reported the isolation of *ctf18* and *dcc1* mutant alleles in a screen for meiotic chromosome non-disjunction mutants. Inactivation of either gene resulted for the most part in sister chromatid non-disjunction during the second meiotic division. Taken together, these data suggest that the identified genes above encode components of the *S.pombe* Ctf18–RFC.

Next, it was intended to examine the properties of double mutants of *rfc1-44* and *ctf18Δ*. However, when *rfc1-44* was crossed to *ctf18Δ*, no *rfc1-44 ctf18Δ* double mutant progeny were obtained at 25°C, the permissive temperature for *rfc1-44* (Figure 5D). Thus in contrast to *elg1<sup>+</sup>*, *ctf18<sup>+</sup>* is required for viability of *rfc1-44*, indicating that Ctf18–RFC plays an essential role when RFC function is impaired. Whether *ctf8<sup>+</sup>* and *dcc1<sup>+</sup>* were required for *rfc1-44* viability was also tested, by crossing *dcc1Δ* and *ctf8Δ* strains to *rfc1-44*, but in each case the double mutant, *rfc1-44 dcc1Δ* or *rfc1-44 ctf8Δ*, was inviable (Figure 5B), as was the case for *rfc1-44 ctf18Δ*. We conclude that the full Ctf18–RFC activity is required for the rescue of *rfc1-44*.

### Overproduction of RFC and RFC-like complex large subunits in *rfc1-44*

In order to test the effects of overproducing the large subunits, each gene was cloned into a fission yeast expression plasmid, 3' to the thiamine-repressible *nmt1* promoter (52). The resulting pREP plasmids were then transformed into wild-type, *rfc1-44*, *elg1Δ* and *ctf18Δ* strains. With one exception, the transformed cells were viable and suitable for further analysis (Figure 6). The exception was *rfc1-44* transformed with pREP3X-Elg1. Only microcolonies could be obtained following transformation and plating onto minimal medium with or without 5 μg/ml thiamine (i.e. with the *nmt1* promoter repressed or fully derepressed) at the permissive temperature of 25°C. Microscopic examination revealed that the transformed cells were highly elongated and only marginally viable, as even after prolonged incubation the formation of larger colonies was not possible. Strikingly, transformation of wild-type, *elg1Δ* or *ctf18Δ*, strains with the same pREP3X-Elg1 plasmid did not affect their growth, either in the presence or in



**Figure 6.** Regulated overproduction of RFC/RFC-like complex large subunits in wild-type *rfc1-44*, *ctf18Δ* and *elg1Δ* cells. (A) Expression in wild-type and *rfc1-44* cells growing on medium containing thiamine (promoter repressed). (B) Expression in wild-type and *rfc1-44* cells growing on medium lacking thiamine (promoter derepressed). (C) Expression in *ctf18Δ* and *elg1Δ* cells growing on medium containing thiamine. (D) Expression in *ctf18Δ* and *elg1Δ* cells growing on medium lacking thiamine. Cells were transformed with the indicated plasmids (pREP3X, etc.) and serial dilutions plated on EMM plates either with or without 5 μg/ml thiamine, to either repress or induce large subunit expression from the *nmt1* promoter. Plates were incubated at 25 or 35°C as indicated for 3–5 days.

the absence of thiamine. Thus, cells in which RFC activity is impaired by mutation within the CTD are highly sensitive to increased Elg1 protein levels. The extent of Elg1 overproduction under these circumstances is not known, as our recent attempts to generate anti-Elg1 antibodies have not been successful (data not shown). Nevertheless, it is tempting to speculate that in these cells excess Elg1 out-competes Rfc1–44 for binding to the small RFC subunits, resulting in an increase in the levels of Elg1–RFC. Additional dominant-negative phenotypes, albeit non-lethal ones, were seen when *rfc1<sup>+</sup>* was overexpressed in wild-type, *rfc1-44*, *elg1Δ* and *ctf18Δ*, cells grown in the absence of thiamine (promoter fully derepressed), and when *ctf18<sup>+</sup>* was overexpressed in *rfc1-44* cells under the same conditions at 25°C (Figure 6).



### Inactivation of the Elg1–RFC restores viability to *rfc1-44 ctf18Δ* cells

The results above suggested that the Elg1–RFC complex might play a negative role in *rfc1-44* cells, since elevated levels of *elg1*<sup>+</sup> expression prevented *rfc1-44* colony formation. To test this further, construction of an *rfc1-44 elg1Δ ctf18Δ* triple mutant was attempted by crossing *rfc1-44 elg1Δ* to *ctf18Δ elg1Δ*, for the reason that viability of *rfc1-44 ctf18Δ* might be restored by inactivation of the negatively acting *elg1*<sup>+</sup>. As predicted, the *rfc1-44 elg1Δ ctf18Δ* triple mutant was viable (Figure 7A). Fifteen tetrads, each of which gave rise to four viable colonies, were obtained following tetrad dissection of meiotic asci from this cross. Further analysis of the 24 meiotic products from six of these asci (using PCR and replica plating to nourseothricin or G418 to identify *ctf18Δ* and *elg1Δ*, respectively, and replica plating to 35°C to identify *rfc1-44*; Materials and Methods) identified six triple mutants, as predicted, among four tetratypic asci, one parental ditype and one non-parental ditype. Viable *rfc1-44 elg1Δ ctf18Δ* triple mutants, together with inviable *rfc1-44 ctf18Δ* double mutants, were also obtained by crossing *rfc1-44* with *elg1Δ ctf18Δ* (data not shown).

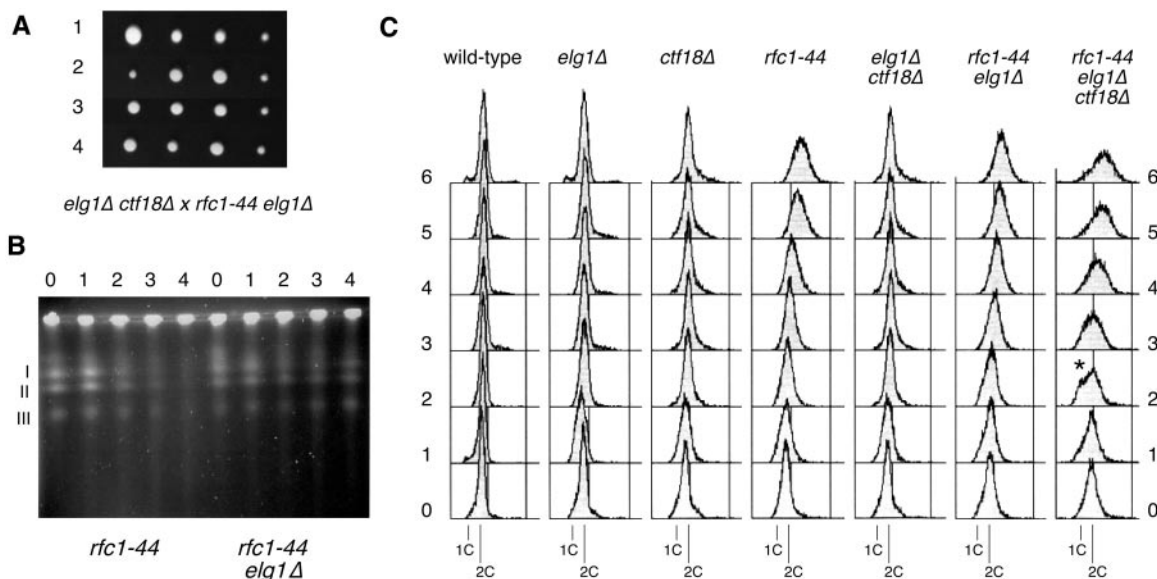
Further evidence in support of the notion that the Elg1–RFC plays a negative role in *rfc1-44* cells came from PFGE analysis of chromosome structure following shift of *rfc1-44* and *rfc1-44 elg1Δ* cells to the restrictive temperature (Figure 7B). As shown previously, the ability of the chromosomes from *rfc1-44* cells to enter a PFGE gel is significantly reduced by 2 h after shift to the restrictive temperature. In *rfc1-44 elg1Δ* cells, however, the chromosomes can still enter the gel at later time points (Figure 7B, compare 3 and 4 h timepoints), suggesting that their structure is stabilized by the loss of Elg1–RFC activity. However, note that *rfc1-44 elg1Δ* cells were still incapable of colony formation at the restrictive temperature, i.e. the

deletion of *elg1*<sup>+</sup> does not fully rescue *rfc1-44* (data not shown).

In the *rfc1-44 elg1Δ ctf18Δ* triple mutant cells, all three PCNA-interacting RFC and RFC-like complexes are functionally impaired or absent. The DNA content of these cells following shift to the *rfc1-44* restrictive temperature was examined and compared to the two viable double mutants *rfc1-44 elg1Δ* and *elg1Δ ctf18Δ*, the single mutants *rfc1-44*, *elg1Δ* and *ctf18Δ*, and wild type (Figure 7C). No significant differences in DNA content profile were apparent between wild type, *elg1Δ* and *ctf18Δ*, and the double mutant *elg1Δ ctf18Δ*. As shown in Figure 2C, shifting *rfc1-44* to 36.5°C results in rightward movement and broadening of the DNA content peak as the cells arrest in elongated form (37). However, no cells with 1C DNA content are apparent. Similar behaviour is seen with *rfc1-44 elg1Δ* cells. However, when the *rfc1-44 elg1Δ ctf18Δ* triple mutant cells are shifted to 36.5°C, there is a transient accumulation of cells with <2C DNA content, suggesting that simultaneous impairment or loss of RFC and Ctf18–RFC results in a severe failure in the replication process, consistent with the inviability of the *rfc1-44 ctf18Δ* double mutant.

### DISCUSSION

RFC and the RFC-like complexes, Ctf18–RFC and Elg1–RFC, play vital roles in maintaining genome integrity in eukaryotic cells (2,6). RFC loading of PCNA is essential for chromosomal replication and optimal DNA repair, Ctf18–RFC is required for the establishment of sister chromatid cohesion in S-phase, and Elg1–RFC has been implicated in DNA replication, replication fork re-start, recombination and repair (2,6). This describes the results of experiments aimed at investigating the role of the CTD of the large subunit of



**Figure 7.** (A) The triple mutant *rfc1-44 elg1Δ ctf18Δ* is viable. Four tetratype tetrads derived from a cross of *rfc1-44 elg1Δ* × *ctf18Δ elg1Δ* are shown. In each case, all four meiotic products were capable of colony formation. The genotypes of colonies were confirmed by replica plating and/or PCR with genomic DNA templates as appropriate. (B) PFGE analysis of chromosome structure following shift of *rfc1-44* and *rfc1-44 elg1Δ* cells to 35°C for 4 h, with samples being taken for PFGE analysis every hour. (C) Flow cytometric analysis of propidium-iodide stained cells following shift of the indicated strains to 35°C for 6 h. The asterisk in the triple mutant indicates the position of a sub-population of cells with 1C DNA content.

RFC in fission yeast, Rfc1. Deletion of the entire CTD was shown to abolish RFC function *in vivo* and a collection of CTD mutant *rfc1* alleles generated by random or directed mutagenesis techniques were examined. Twelve alleles were constructed by random pentapeptide insertion. Perhaps surprisingly, only one of the twelve insertions disrupted RFC function, while nine of the twelve had no significant effect. The non-functional mutant protein Rfc143 carries an insertion of the sequence QGVPL between amino acids 753 and 754 (see Supplementary Data for details). Comparison with the *S.cerevisiae* Rfc1 structure (5) suggests that this region of the Rfc1 protein (amino acids 737–757) is likely to form a lengthy  $\alpha$ -helix that would be disrupted by the pentapeptide insertion. The remaining two alleles (*rfc1-44* and *rfc1-54*) caused a temperature-sensitive phenotype (Figure 2A). In this case, the encoded mutant proteins carried insertions of the sequences IRGTP and PVGVP between amino acids 811 and 812, and 780 and 781, respectively. Neither insertion can be mapped onto the *S.cerevisiae* Rfc1 structure as both fall within a region whose structure could not be determined. None of the nine site-directed mutants created in this study, all of which replaced well-conserved charged amino acids within the CTD with alanine (see Supplementary Information), disrupted RFC function.

Further analysis of *rfc1-44* showed that these cells were capable of bulk DNA replication after being shifted to the restrictive temperature (Figure 2B) but that the DNA was incapable of entering an agarose gel in PFGE experiments (Figure 2C). These properties are shared by a number of other DNA replication mutants in *S.pombe*, including the essential DNA helicases encoded by *pfh1* and *dna2* (49,53) and the Dna2 interacting factor *cdc24* (54,55). The *rfc1-44* is also more sensitive than wild-type to the DNA damaging agents MMS, HU and UV (Figure 2D) and displays synthetic lethality with conditional lethal *pol3*, *cdc1* and *cdc27* alleles, indicative of a close involvement in lagging strand DNA synthesis (46). In an unpublished work, the ability of a range of DNA replication proteins, including the four small RFC subunits, when overproduced, to suppress *rfc1-44* at restrictive or semi-restrictive temperatures was tested. However, none of the proteins tested was capable of rescuing *rfc1-44*. In a subsequent screening for multi-copy suppressors of *rfc1-44* using genomic DNA libraries, the same suppressing gene *sk2*<sup>+</sup> (SPBC1709.05) was identified from two of the three libraries screened (J. Kim and S.A. MacNeill, unpublished data). The *sk2*<sup>+</sup> (formerly known as *hsc1*<sup>+</sup>) encodes an HSP70 family member (56,57) that may act as a chaperone promoting the folding or assembly of the RFC complex in *rfc1-44* cells. Whether Sk2 plays a role in RFC function in wild-type cells awaits further analysis.

That bulk DNA replication was possible in *rfc1-44* cells at the restrictive temperature (Figure 2B) suggested that either the RFC complex was not completely inactive under these conditions or that other clamp loaders could partially compensate for the loss of RFC function. To investigate this, analysis of the RFC-like complexes Elg1–RFC and Ctf18–RFC was initiated. The Elg1–RFC complex was purified (Figure 4B) and genes encoding the unique Elg1–RFC and Ctf18–RFC subunits Elg1, Ctf18, Dcc1 and Ctf8 were identified and deleted from the genome (Figures 4 and 5). All three double mutants *rfc1-44 ctf18 $\Delta$* , *rfc1-44 dcc1 $\Delta$*  and *rfc1-44*

*ctf8 $\Delta$*  are non-viable at the permissive temperature for *rfc1-44* (Figure 5D). Biochemical evidence indicates that although Ctf18–RFC lacking Dcc1 and Ctf8 can load PCNA onto DNA *in vitro*, its activity is ~10-fold less than that of the seven subunit complex including Dcc1 and Ctf8 (17). Our genetic results suggest that maximal Ctf18–RFC activity is required when RFC function is impaired, as the both *dcc1*<sup>+</sup> and *ctf8*<sup>+</sup> are required for *rfc1-44* viability.

In sharp contrast, the double mutant *rfc1-44 elg1 $\Delta$*  was viable at the permissive temperature (Figure 4D) and PFGE results suggests that inactivation of the Elg1–RFC has a positive effect on *rfc1-44* at the restrictive temperature, as the chromosomes were able to enter the gel at later timepoints compared to the single mutant (Figure 7B). This negative effect of Elg1–RFC was most clearly seen when *ctf18 $\Delta$  elg1 $\Delta$*  was crossed with *rfc1-44 elg1 $\Delta$*  to generate a viable *rfc1-44 elg1 $\Delta$  ctf18 $\Delta$*  triple mutant. This result shows that inactivation of the Elg1–RFC restores viability to the *rfc1-44 ctf18 $\Delta$*  double mutant, reinforcing the view that the Elg1–RFC plays an opposing role to RFC and Ctf18–RFC in *S.pombe* cells.

Previously, various functions have been proposed for Elg1–RFC (6). For example, Elg1–RFC might function as a specialized PCNA clamp loader for DNA substrates associated with, for example, replication fork stalling or collapse. It is possible that in *rfc1-44 ctf18 $\Delta$*  cells, recognition of such structures and subsequent PCNA loading by Elg1–RFC has a harmful effect, whereas loss of Elg1–RFC function in the triple mutant *rfc1-44 ctf18 $\Delta$  elg1 $\Delta$*  allows replication fork recovery to be achieved by an alternative mechanism such as homologous recombination. Intriguingly, in *S.cerevisiae* *elg1 $\Delta$*  is synthetically lethal with genes involved in homologous recombination [reviewed in (19)]. Alternatively, it has been suggested that the Elg1–RFC could function as a PCNA clamp unloader (6). Recycling of PCNA is likely to be vital for efficient replication but exactly how this occurs is unclear. If Elg1–RFC acts as a dedicated clamp unloader, its inactivation might counteract, at least in part, the consequences of impairing RFC activity by the *rfc1-44* mutation. Finally, Elg1–RFC might have a role in loading or unloading a modified form of PCNA onto DNA. Ubiquitination and sumoylation of PCNA has been demonstrated to play an important role in replication and repair of damaged DNA [reviewed in (58)] but whether PCNA modification impinges on clamp loading and unloading is not known. Further detailed genetic and biochemical studies will be required to resolve these issues.

## SUPPLEMENTARY MATERIAL

Supplementary Material is available at NAR Online.

## ACKNOWLEDGEMENTS

We would like to thank our friends and colleagues in Edinburgh and elsewhere for their help during the course of this work, in particular Dr Hiroyuki Tanaka for many helpful discussions, Pierre Hentges and Professor Tony Carr (University of Sussex) for the pFA6A-natMX6 plasmid and Dr Finbarr Hayes (UMIST) for supplying the Tn4430 system components. S.M. and F.G. were supported by a Wellcome Trust Senior

Research Fellowship in Basic Biomedical Science, J.K. by a Wellcome Trust Prize Studentship, K.M. by the Wellcome Trust 4 year PhD Programme *Genetics and Disease*, I.C. by a Darwin Trust of Edinburgh vacation scholarship and K.R. by the European Union (contract QLK3-CT-2002-02071). Funding to pay the Open Access publication charges for this article was provided by the University of Copenhagen.

*Conflict of interest statement.* None declared.

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