Genetics of lagging strand DNA synthesis and maturation in fission yeast: suppression analysis links the Dna2–Cdc24 complex to DNA polymerase δ

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ABSTRACT

The Cdc24 protein is essential for the completion of chromosomal DNA replication in fission yeast. Although its precise role in this process is unclear, Cdc24 forms a complex with Dna2, a conserved endonuclease–helicase implicated in the removal of the RNA–DNA primer during Okazaki fragment processing. To gain further insights into Cdc24–Dna2 function, we screened for chromosomal suppressors of the temperature-sensitive *cdc24-M38* allele and mapped the suppressing mutations into six complementation groups. Two of these mutations defined genes encoding the Pol3 and Cdc27 subunits of DNA polymerase δ. Sequence analysis revealed that all the suppressing mutations in Cdc27 resulted in truncation of the protein and loss of sequences that included the conserved C-terminal PCNA binding motif, previously shown to play an important role in maximizing enzyme processivity *in vitro*. Deletion of this motif is shown to be sufficient for suppression of both *cdc24-M38* and *dna2-C2*, a temperature-sensitive allele of *dna2*[+], suggesting that disruption of the interaction between Cdc27 and PCNA renders the activity of the Cdc24–Dna2 complex dispensable.

INTRODUCTION

In all eukaryotic cells, chromosomal DNA replication is achieved by the coordinated action of a large number of protein factors. Replication is a semi-discontinuous process: the leading strand is synthesized continuously, while the lagging strand is synthesized as a series of discrete Okazaki fragments that are later processed to yield a continuous DNA strand. Genetic and biochemical studies in yeast and higher

eukaryotic systems, notably the SV40 *in vitro* replication system, have identified ∼20 polypeptides that are essential for Okazaki fragment synthesis and processing, namely DNA polymerase α-primase (Pol α-primase), replication protein A (RPA), DNA polymerase δ (Pol δ), replication factor C (RFC), the sliding clamp PCNA, the nucleases Fen1 and Dna2, and DNA ligase I (1). All these factors appear to be conserved in all eukaryotic cells.

Current models for Okazaki fragment synthesis and processing can be summarized as follows. First, Pol α-primase synthesizes a short RNA–DNA primer on the template DNA. Then polymerase switching takes place, resulting in displacement of the non-processive low-fidelity Pol α-primase enzyme and its replacement by Pol δ, which is both highly processive when complexed with its processivity factor PCNA and also possesses proofreading activity, ensuring high-fidelity DNA synthesis. Polymerase switching requires the clamp loader complex RFC to recognize the primer-template junction and load the sliding clamp PCNA onto the dsDNA. PCNA encircles the DNA and tethers Pol δ to it. The Pol δ–PCNA complex then continues to synthesize DNA until it encounters the 5′ end of the previously synthesized Okazaki fragment, at which point displacement synthesis results in the formation of an RNA–DNA flap structure. Studies with purified proteins suggest that the extent of displacement synthesis is governed by the binding of the single-stranded binding complex RPA to the displaced flap once the length of the flap has reached ∼35 nt. RPA binding, and its recruitment of the Dna2 helicase–endonuclease, ensures that further displacement synthesis is inhibited. Dna2 then cleaves the flap, most likely removing the entire RNA–DNA segment originally synthesized by the non-proofreading Pol α–primase complex, leaving a shortened flap structure that can be cleaved by the non-essential Fen1 nuclease. The nicked DNA produced is finally sealed by DNA ligase I.

In fission yeast *Schizosaccharomyces pombe*, Pol δ is composed of four subunits, three of which are essential for cell viability, Pol3, Cdc1 and Cdc27 (2). Orthologues of all four

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proteins, including the non-essential subunit Cdm1 (3), are found as subunits of mammalian Pol δ (4). Pol3 is the catalytic subunit of the complex. Pol3 binds directly to the B-subunit Cdc1 via a conserved C-terminal zinc finger module (5). The Cdc1 protein then binds to the C-subunit Cdc27 (6). Cdc27 comprises a globular ∼160 residue N-terminal domain (NTD), through which it binds Cdc1, and an extended C-terminal domain of ∼210 residues, at the extreme C-terminus of which is located a PCNA binding motif (PIP box) of the type first identified in the mammalian DNA replication inhibitor p21^{Cip1} (7). Also located in the C-terminal region of the protein is a conserved, non-essential binding site for Pol1, the catalytic subunit of Pol α–primase (8–10). The D-subunit of the complex, Cdm1, is not essential for cell viability and its role in Pol δ function is not known (3).

In addition to the conserved proteins mentioned above, the fission yeast Cdc24 protein has also been suggested to have a function in lagging strand DNA synthesis. Cdc24 is a 501 amino acid protein with no significant similarity to proteins in current databases (11,12). The involvement of Cdc24 in lagging strand DNA synthesis is suggested from genetic and/or physical interactions with RFC, PCNA, Pol δ and Dna2 (3,11–13). However the precise role of Cdc24 in lagging strand synthesis has not yet been established. In this report, Cdc24 is shown to form a complex with Dna2 in fission yeast protein extracts, extending previous results (13) obtained using the two-hybrid system. We also report the isolation and analysis of *cdc24* suppressor mutants. Together with complex formation between Cdc24 and Dna2, we propose that Cdc24 has a role in the processing of flap structures in Okazaki fragment maturation during lagging strand DNA synthesis.

MATERIALS AND METHODS

General fission yeast techniques and reagents

S.pombe genetic techniques and media were as described in reference (14). The *S.pombe* cDNA library used was described previously (12). The *S.pombe* genomic DNA libraries were constructed by inserting restriction enzyme digested genomic DNA into plasmid pALSK$^+$.

Epitope tagging

For epitope tagging of *cdc24*$^+$ and *dna2*$^+$, plasmid pKSU–XHA was first generated by subcloning the genomic *ura4*$^+$ HindIII fragment and 3xHA-nmt1 3′ UTR region NotI-SacI fragment from plasmid pREP2–XHA, a version of pREP2 carrying sequences encoding three HA epitopes for C-terminal tagging, into pBluescript II KS+ (Stratagene). Next, *cdc24*$^+$ genomic DNA was amplified by PCR, digested with EcoRI and NotI (the sites were present in the oligonucleotides used for PCR) and ligated into pKSU–XHA, such that the *cdc24*$^+$ open reading frame (ORF) was fused in frame at its 3′ end to sequences encoding three copies of the HA mAb epitope. Next, the nmt1 3′ UTR in this plasmid was replaced with the 3′ UTR of *cdc24*$^+$ by replacing the BamHI-SacI region with a BamHI-SacI *cdc24*$^+$ fragment generated by PCR, to make pKSU–cdc24HAUTR. For *dna2*$^+$, plasmid pKSU–dna2HAUTR was constructed in a similar fashion by subcloning *dna2*$^+$ genomic DNA and *dna2*$^+$ 3′ UTR sequence

into pKSU–XHA. For construction of pKSU–cdc24MycUTR and pKSU–dna2MycUTR, the NotI-BamHI 3xHA fragment in the plasmids described above was replaced with a PCR amplified 13xMyc fragment. All DNA sequences amplified by PCR were confirmed by DNA sequencing. The sequences of the primers used will be provided by the authors on request.

To construct the epitope tagged strains, the pop-in/pop-out homologous recombination method was used (15). The pKSU–cdc24HAUTR and pKSU–cdc24MycUTR plasmids were linearized by HpaI digestion, while pKSU–dna2HAUTR and pKSU–dna2MycUTR plasmids were linearized by BglII digestion. The linear plasmid DNAs were then transformed into *ura4-D18 leu1-32* h$^-$ cells. After selection of stable ura$^+$ transformants, PCR was used to confirm the correct integration (pop-in) of the plasmids at the desired loci. Ura$^-$ cells were then selected on 5-fluoroorotic acid (5-FOA) plates. Loss of the plasmid (pop-out) was confirmed by PCR and followed by immunoblotting analysis. The tagged strains were backcrossed three times and double *cdc24 dna2* mutants were obtained by crossing the single mutants.

Immunoprecipitation and immunoblotting

Exponentially growing cells in EMM medium supplemented with uracil and leucine were collected and washed with ice-cold STOP buffer (150 mM NaCl, 50 mM NaF, 10 mM EDTA and 1 mM NaN$_3$). Cells were resuspended in Buffer B (10 mM sodium phosphate buffer pH 7.0, 1% Triton X-100, 1mM EDTA and 100mM NaCl) with protease inhibitors, transferred into a tube containing glass beads, and disrupted using a Ribolyser (Hybaid). Cell extracts (2.0 mg or 1.0 mg of proteins in buffer B in panels A and B, respectively) were mixed with anti-HA rat monoclonal antibody (clone 3F10) affinity matrix (Roche) that had been pre-treated with BSA. After incubation for 1 h at 4°C with rotation, the immunoprecipitates were washed four times with buffer B. Crude extracts (20 μg in panel A or 10 μg in panel B) and immunoprecipitates from 2.0 mg or 1.0 mg of extract (as above) were separated by SDS–PAGE, transferred to membrane and detected by anti-HA (clone 12CA5) or anti-Myc (clone 9E10) mouse monoclonal antibodies (Roche).

Classification of *cdc24-M38* suppressor mutants

Spontaneous genetic suppressors of *cdc24-M38* h$^-$ were isolated as described previously (16). Briefly, *cdc24-M38 leu1-32* h$^-$ cells were incubated at 36°C for 3 days on YE medium. Spontaneous revertant colonies were then replica-plated to 18°C for 3–5 days to test for cold-sensitivity. Following back-crossing, the cold-sensitive suppressor strains were crossed with *leu1* h^{+S} to determine whether the suppressing mutations were intra- or extragenic. Subsequently, to group the suppressors into complementation groups, the suppressed *cdc24-M38* h$^-$ strains were crossed to *cdc24-M38* h^{+S}, to allow the isolation of suppressed *cdc24-M38* strains of opposite mating type. The different suppressed strains (*cdc24-M38 cstX, cdc24-M38 ctsY*, etc.) were then crossed with one another and the progeny analysed by random spore analysis. Where all the progeny were cold-sensitive, the suppressing mutations were assigned to the same complementation group. Otherwise, the suppressors were assigned to different complementation

groups. In all, the suppressing mutations fell into six complementation groups, designated *cst1–cst6*.

Construction of *cdc27* mutants

Three types of *cdc27* deletion mutations were generated by PCR. DNA fragments were digested with EcoRI and NotI, and subcloned into pBluescript II-ura4+ plasmid. The resultant plasmids, pKSU–cdc27-D1, pKSU–cdc27-D2 and pKSU–cdc27-D3, contain 0.5 kb of 3′ UTR derived from the *cdc27+* gene and a varying length of coding sequence depending on the length of deletion (see Results). All DNA sequences generated by PCR were confirmed by DNA sequencing. After linearization by PstI digestion, the three plasmids were transformed to *ade6-M210/ade6-M216 ura4-D18/ ura4-D18 leu1-32/leu1-32* h⁻/h⁺ diploid cells. Successful homologous recombinants were selected following PCR analysis of genomic DNA. To test whether the *cdc27* mutants are viable or not, diploid cells were spread on MEA plates to induce meiosis and sporulation. Spores were then plated on YE medium to allow re-growth, and viable colonies analysed to determine their genotypes. All three *cdc27* mutants were viable. These were then plated on 5FOA plates to select for ura⁻ cells, after which PCR analysis was used to confirm the loss of the plasmid sequences. Finally, the *cdc27* mutant strains were backcrossed three times with h⁻ *leu1-32* or h⁺ *leu1-32* cells to obtain *cdc27-D1 leu1-32* h⁻, *cdc27-D2 leu1-32* h⁻ and *cdc27-D3 leu1-32* h⁻ mutants for further phenotypic analysis.

Forward mutation rate assay

Wild-type control (*leu1-32* h⁺S) and *cdc24-M38* strains (*cdc24-M38 leu1-32* h⁺S) were streaked to single colonies on EMMGL plates at 28°C and eight single colonies of each strain were inoculated into 10 ml of EMMGL medium (EMM with 5 g/l of glutamic acid replacing ammonium chloride, supplemented with 150 mg/l of leucine) and grown to saturation at 28°C for 48 h. From each saturated culture, 1×10^7 cells per plate were plated onto EMMGL agar containing either 40 μg/ml of filter-sterilized L-canavanine sulphate (Sigma) or thialysine (S-2-aminomethyl-L-cysteine hydrochloride, Sigma). The plates were then incubated at 28°C for 8 days and the number of L-canavanine- or thialysine-resistant clones counted. Plating efficiencies were determined by plating 1000 cells of each strain onto EMMGL plates.

RESULTS

Cdc24 and Dna2 form a complex

The fission yeast Dna2 and Cdc24 proteins are essential for the completion of DNA replication (11,12). In the course of previous analysis of fission yeast Dna2, an interaction between Cdc24 and Dna2 was observed using a two-hybrid assay, in which the two proteins were expressed to a high level in *Saccharomyces cerevisiae* as Gal4 activation domain and Gal4 DNA binding domain fusions (13). To determine whether Cdc24 and Dna2 also formed a complex *in vivo* in *S.pombe* when present at their normal cellular levels, fission yeast strains were constructed, in which the endogenous *cdc24+* and *dna2+* genes were precisely replaced with the alleles

encoding epitope tagged versions of the two proteins (see Materials and Methods), thereby ensuring that the tagged proteins were expressed to a normal level only.

To test whether Cdc24 and Dna2 were physically associated, co-immunoprecipitation assays were performed. Initially, protein extracts were prepared from *dna2-Myc cdc24-HA* and *dna2-Myc* strains, and subjected to immunoprecipitation using anti-HA antibodies. The immunoprecipitated material was then resolved on SDS–PAGE and immunoblotted using anti-HA and anti-Myc antibodies. Figure 1A shows the results of these experiments: Dna2-Myc could be immunoprecipitated by the anti-HA antibody but only in the presence of Cdc24-HA (compare lanes 3 and 4), that is, only from extracts prepared from the *dna2-Myc cdc24-HA* strain (Figure 1A).

This result was confirmed with another combination of tags. Native proteins extracts were prepared from *dna2-HA cdc24-Myc* and *cdc24-Myc* strains. After immunoprecipitation using anti-HA antibody and immunoblotting, we found that Cdc24-Myc was specifically immunoprecipitated by anti-HA antibody only in the presence of Dna2-HA, that is, only from extracts prepared from the *dna2-HA cdc24-Myc* strain (Figure 1B, compare lanes 3 and 4). These results indicate that
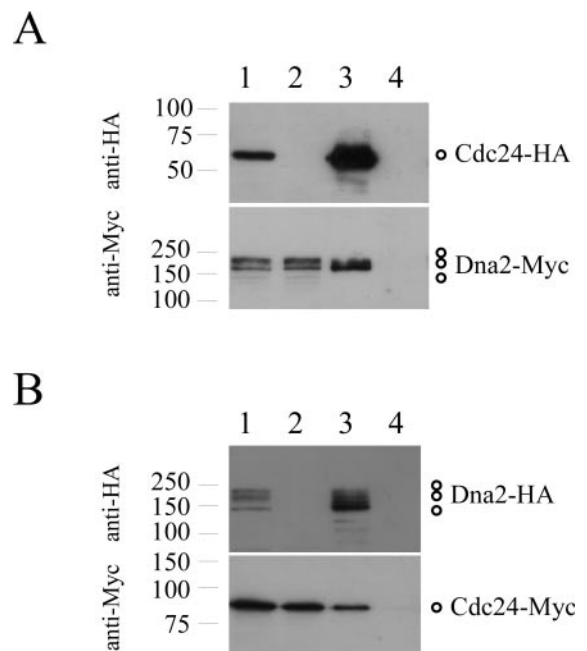

Figure 1. Cdc24 and Dna2 form a complex *in vivo*. (**A**) Dna2-Myc co-purifies with Cdc24-HA. Native protein extracts were prepared from *cdc24-HA dna2-Myc ura4-D18 leu1-32* h⁻ (lanes 1 and 3) and *dna2-Myc ura4-D18 leu1-32* h⁺ (lanes 2 and 4) cells growing exponentially in EMM medium supplemented with leucine and uracil, and Cdc24-HA immunoprecipitated with anti-HA antibody. Cell extracts (lanes 1 and 2) and immunoprecipitates (lanes 3 and 4) were analysed by immunoblotting with anti-HA (upper panel) and anti-Myc (lower panel) antibodies. (**B**) Cdc24-Myc co-purifies with Dna2-HA. The same procedures were performed with *dna2-HA cdc24-Myc ura4-D18 leu1-32* h⁻ (lanes 1 and 3) and *cdc24-Myc ura4-D18 leu1-32* h⁺ (lanes 2 and 4) cells, but in this case, Dna2-HA was immunoprecipitated with anti-HA antibody. Cell extracts (lanes 1 and 2) and immunoprecipitates (lanes 3 and 4) were then analysed by immunoblotting with anti-HA (upper panel) and anti-Myc (lower panel) antibodies. Molecular weight markers (kD) are shown to the left of each panel. Note that multiple forms of Dna2 can be detected by immunoblotting. Whether these represent post-translationally modified isoforms of the protein, or merely degradation products, is not known.

Cdc24 forms a complex with Dna2 in yeast protein extracts, and therefore, most likely *in vivo* also, and suggests that, like Dna2, Cdc24 functions in Okazaki fragment maturation.

Complementation groups of *cdc24-M38* suppressing mutations

In order to investigate the function of *cdc24*+ further, and in particular, to question its potential roles during Okazaki fragment synthesis and maturation, extragenic suppressors of the temperature-sensitive *cdc24-M38* allele were isolated as has been previously described (16). Briefly, exponentially growing temperature-sensitive *cdc24-M38* cells were spread onto YE plates and incubated at the restrictive temperature of 36°C for 3 days, in order to allow the growth of spontaneous revertant colonies. In order to facilitate subsequent analyses, cold-sensitive revertants were then selected following replica plating and incubation at 18°C for 3–5 days. The suppressing mutants were designated *cst* mutants, for cold-sensitive suppressors of twenty-four. Note that the *cst* mutations confer two properties on cells: cold-sensitivity *and* suppression of the temperature-sensitivity of *cdc24-M38*.

In total, 37 such mutants were isolated from $\sim 3 \times 10^9$ plated cells. Subsequent genetic analyses showed that all the *cst* mutant phenotypes were caused by single gene mutations and not by reversion of the *cdc24-M38* allele to wild type. To classify the *cst* mutants into complementation groups, the mutants were crossed to one another and the resulting spores were plated and incubated at 18 and 36°C. Where all the progeny were cold-sensitive, the suppressing mutations were assigned to the same complementation group. Otherwise, the suppressors were assigned to different groups. In all, the 37 mutants were classified into six complementation groups, *cst1–cst6*, containing between 1 and 17 alleles (Table 1). One of these complementation groups, *cst6*+ has been described

Table 1. Complementation groups of *cdc24* suppressing mutants

	No. of alleles	Morphology[a]	Gene	Encoded protein
cst1+	17	Elongated	*cdc27*+	Pol δ subunit
cst2+	4	Normal	*sup45*+	eRF1
cst3+	8	Normal	*sup35*+	eRF3
cst4+	2	Elongated	*pol3*+	Pol δ subunit
cst5+	1	Elongated	–	–
cst6+	5	Elongated	*pfh1*+	DNA helicase

[a]Cell morphology of double mutant with *cdc24-M38* at 18°C.

(16,17). The *cst6*+ gene, which encodes an essential DNA helicase enzyme, was renamed *pfh1*+.

Figure 2 shows representative alleles from the *cst1–cst5* complementation groups. The mutant cells show cold-sensitivity, in the presence and absence of *cdc24-M38*, as well as the suppression of the temperature-sensitivity of *cdc24-M38*. Microscopic observation of *cst* mutants incubated at 18°C revealed that they could be divided into two groups. Mutants in the first group displayed an elongated morphology at 18°C. This group consisted of mutants of *cst1*+, *cst4*+, *cst5*+ and *cst6*+/*pfh1*+. In contrast, the second group of mutants, comprising alleles of *cst2*+ and *cst3*+, did not show the elongated cell phenotype. The cell elongation phenotype suggested that the *cst1*+, *cst4*+, *cst5*+ and *cst6*+/*pfh1* genes could be involved in cell cycle events (Table 1).

Identification of *cst* genes

To identify the *cst* genes, two approaches were taken. The first was to examine whether the cold-sensitive phenotype of the *cst* mutants could be rescued by the introduction of known genes. Previously, a variety of genetic interactions between *cdc24* and genes encoding components of the Pol δ-RFC-PCNA holoenzyme have been reported. For example, overproduction of PCNA will partially rescue *cdc24-M38* (3,12), as will overproduction of Rfc1, the large subunit of the RFC complex (12). Six candidate genes were therefore tested, for their ability to complement the cold-sensitivity of individual *cst* or *cdc24 cst* double mutants, when expressed from multi-copy plasmids. The genes examined were *pol3*+/*cdc6*+, *cdc1*+ and *cdc27*+ (encoding the catalytic, B and C subunits of Pol δ, respectively), *cdc17*+ (DNA ligase I), *pcn1*+ (PCNA) and *rfc1*+ (encoding the large subunit of RF-C). Among these genes, two were observed to efficiently rescue particular *cst* mutants: *pol3*+ rescued *cst4* and *cdc27*+ rescued *cst1* (data not shown). To test whether *pol3*+ was isogenic with *cst4*+, genetic linkage between these loci was analysed by crossing *pol3/cdc6* and *cst4* mutants. The results of these experiments showed that *pol3*+ and *cst4*+ are tightly linked, implying that *cst4*+ is *pol3*+. By similar analysis, it was also concluded that *cst1*+ is isogenic with *cdc27*+. Thus, mutations in genes encoding two different subunits of Pol δ were identified as *cst* mutations. This strongly suggests a physiological interaction between Cdc24-Dna2 and Pol δ.

In contrast, none of the six candidate genes suppressed the cold-sensitivity of *cst2*, *cst3* and *cst5* mutants. Therefore,
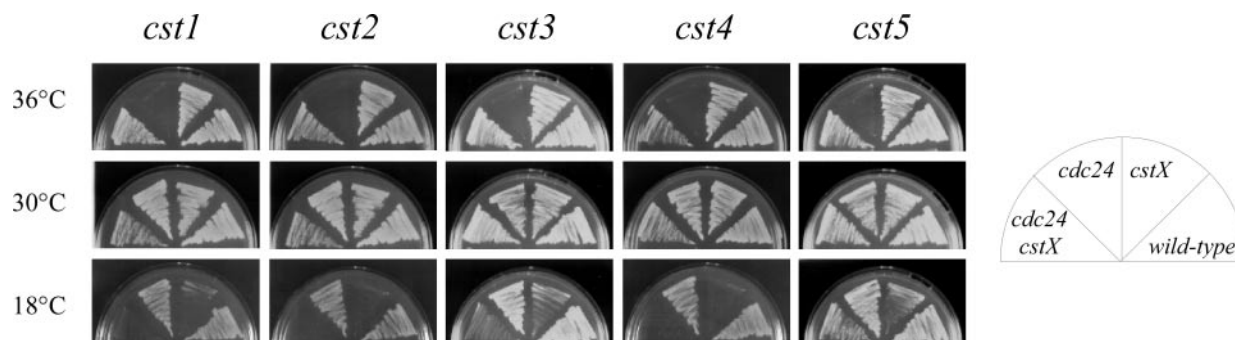

Figure 2. Suppression of the temperature-sensitivity of *cdc24-M38* by the *cst1-R3*, *cst2-R11*, *cst3-R13*, *cst4-R18* and *cst5-R19* mutations. Cells from the indicated strains were streaked on YE plates and incubated for 3 days at 36°C or 30°C, or 5 days at 18°C.

genomic and cDNA libraries were screened for genes that suppressed the cold-sensitivity of these mutants. No suppressors of *cst5* were isolated, and the identity of this gene remains unknown. A single gene, *sup45*⁺, was identified as a suppressor of *cst2-R11*. This gene (systematic name SPAC1834.01, see http://www.sanger.ac.uk/Projects/S_pombe for details) was isolated from both cDNA and HindIII digested genomic DNA libraries and encodes the translation termination factor eRF3. *In vivo*, eRF3 forms a complex with eRF1, which is encoded by *sup35*⁺ (18). Strikingly, *sup35*⁺ (SPCC584.04) was identified as the only suppressor of *cst3-R13*. A single *Hin*dIII DNA fragment was isolated from genomic DNA libraries as a *cst3-R13* suppressor and subsequent deletion analysis revealed that the complementation activity in this plasmid was derived from the *sup35*⁺ gene (data not shown). These results suggest that *cst2*⁺ and *cst3*⁺ correspond to the fission yeast *sup45*⁺ and *sup35*⁺ genes, respectively. To examine this, the *sup45*⁺ and *sup35*⁺ genes in the genomes of *cst2-R11*, *cst3-R13* and wild-type cells were sequenced. A single point mutation in *sup45* was found in *cst2-R11* but not in *cst3-R13* or wild-type strains. The *cst2-R11* allele contains a single C to A (CAA to AAA) point mutation, resulting in a single amino acid change, from proline to lysine, at residue 400 in the predicted Cst2/Sup45-R11 protein. Similarly, a point mutation in *sup35* gene was only found in *cst3-R13* strain. The *cst3-R13* allele contains a single C to A (GAC to GAA) point mutation, resulting in a single amino acid change, from aspartic acid to glutamic acid, at residue 292 in the predicted Cst3/Sup35-R13 protein. Therefore, *cst2*⁺ and *cst3*⁺ are indeed *sup45*⁺ and *sup35*⁺, respectively. Since the molecular defect in the *cdc24-M38* allele is a nonsense mutation at codon 370, it is probable that this mutant can be suppressed by mutations that suppress premature translational termination at this codon. This interpretation is consistent with our observation that neither *cst2* nor *cst3* mutants displayed an elongated cell phenotype (Table 1), suggesting that the mutants do not have a cell cycle phenotype.

Sequencing of *pol3* and *cdc27* mutant alleles

To gain insights into the type of mutations obtained and the effect of these mutations on the mutant proteins, all the *cdc27* and *pol3* mutant alleles were amplified by PCR and the PCR products were directly sequenced. Among the seventeen *cdc27* alleles, we found six different mutations (Figure 3, Table 2). Eight of the mutant alleles (*cdc27-R22*, etc.) contained a single nucleotide insertion of a cytosine residue in a stretch of six

consecutive cytosines (Figure 3A, indicated by yellow bar). This frame-shift mutation causes truncation of the native Cdc27 protein after amino acid 164, although the mutant protein gains some extra 12 amino acids derived from another reading frame before terminating. All the other *cdc27* mutant alleles contained sequence duplications ranging in size from 16 to 173 bp. Each duplicated sequence is flanked by a short direct repeat, 4–7 nt in length, with no apparent sequence specificity. The *cdc27-R3* allele has 16 bp duplication between two GACA sequences (Figure 3A, green bars), while *cdc27-R39* allele has 22 bp duplication between GACA sequences (Figure 3A, pink bars). One of the GACA sequence surrounding the duplicated sequence in *cdc27-R39* is identical to that of *cdc27-R3* (Figure 3A). Of the remaining alleles, *cdc27-R40* has 58 bp duplication between AGAAG sequences (Figure 3A, blue bars), *cdc27-R25* has 121 bp duplication between ACAATCG sequences (red bars) and *cdc27-R5* has a 173 bp duplication between AAAT repeats (orange bars). All these sequence duplications result in truncation of the C-terminal region of the Cdc27 protein due to frame-shift. Table 2 summarizes these results and the predicted lengths of the mutant proteins.

The two cold-sensitive *pol3* alleles were also sequenced. The *pol3-R18* allele contains a single C to T point mutation, resulting in a single amino acid change, from histidine to tyrosine, at residue 969 in the Pol3-R18 protein. In *pol3-R37* allele, there is a 27 bp sequence duplication (Figure 3B, orange bars). This duplication also occurred between short repeat sequences (TGGTGA) and is located only a few bases from the position of the *pol3-R18* mutation (Figure 4). The duplication causes an in-frame nine amino acid insertion (sequence EKASSLLHG) into the Pol3 protein sequence between amino acid 967 and 968. Both mutations are located in a region of the Pol3 protein of unknown function that lies between the catalytic (polymerase) domain and the tandem C-terminal zinc finger modules responsible for the interaction between Pol3 and the B-subunit of the Pol δ complex, Cdc1 (5).

Phenotype of Cdc27 C-terminal deletion mutants

The Cdc27 protein is essential for complete DNA replication in fission yeast (19). Biochemical studies have shown that the 372 amino acid protein has a globular N-terminal domain of ~160 amino acids that binds to Cdc1, and a highly extended C-terminal region (6,20,21). There is a binding site for PCNA at the extreme C-terminus of the protein (amino acids 363–372) (20). Also within the C-terminal region is a short conserved sequence motif that binds to the catalytic subunit of the Pol α-primase complex (10). Previous studies have suggested that the C-terminal region of the Cdc27 protein is essential for its *in vivo* function (20,21). Deletion of the last 20 amino acids of Cdc27 has been shown to render the protein unable to complement a *cdc27* allele when the truncated mutant protein is expressed from a heterologous promoter on a multi-copy plasmid (20,21). *In vitro*, loss of the PCNA binding site greatly reduces the processivity of the *S.pombe* Pol δ enzyme (21).

Despite the apparent importance of PCNA binding for Cdc27 function, the identification of truncated Cdc27 proteins that act as suppressors of *cdc24-M38* indicates that, when the

Table 2. Summary of *cdc27* mutant alleles

Mutation type	Repeated sequence	Duplication size	Occurrence	Mutant protein length[a]
Insertion[b]	–	–	8/17	164 + 12
Duplication	GACA	16	4/17	179 + 2
Duplication	GACA	22	1/17	179 + 4
Duplication	AGAAG	58	1/17	245 + 5
Duplication	ACAATCG	121	2/17	175 + 2
Duplication	AAAT	173	1/17	271 + 24

[a]The length of the mutant protein is shown as number of amino acids from Cdc27 + number of additional amino acids resulting from frame-shift.
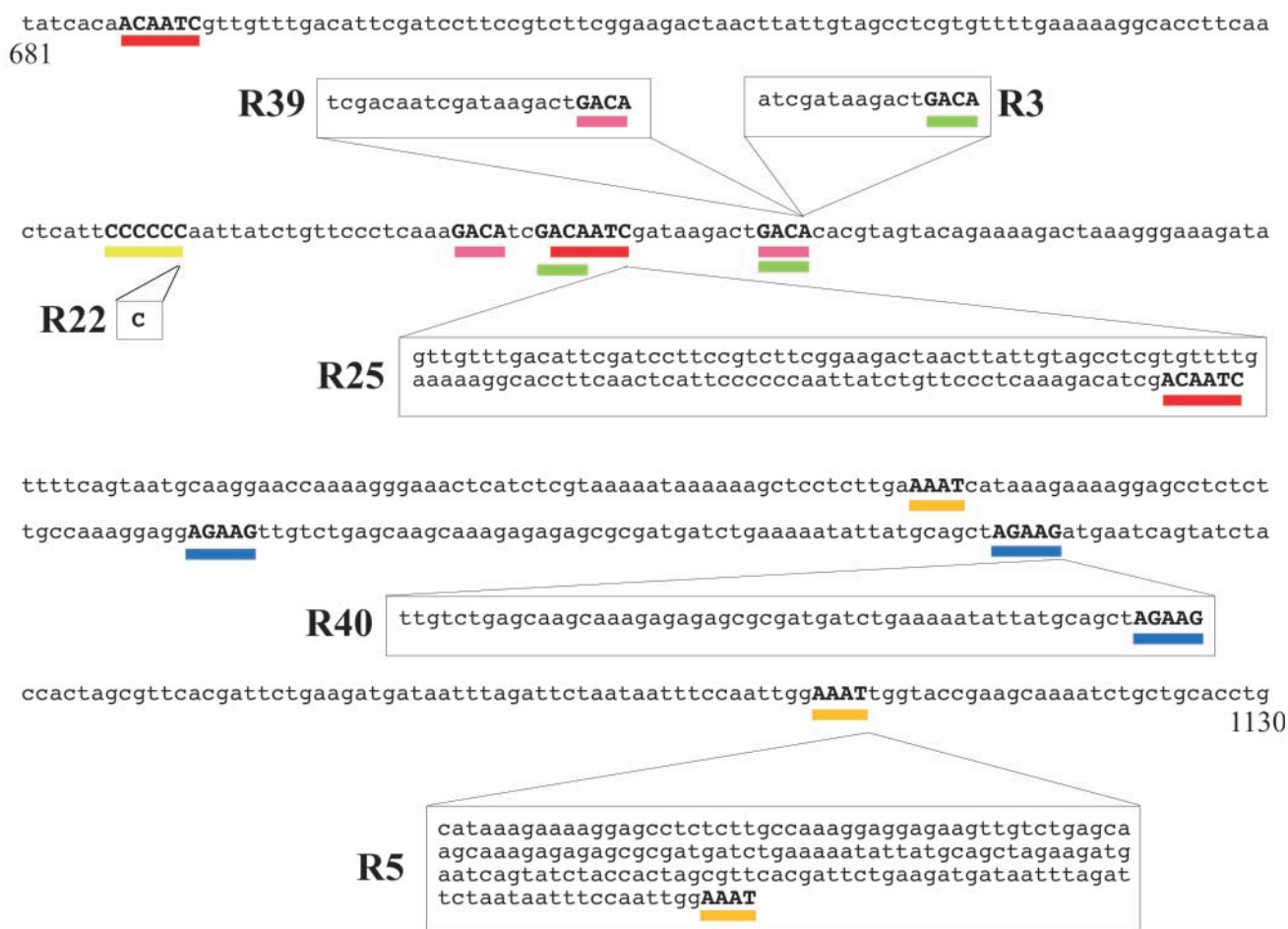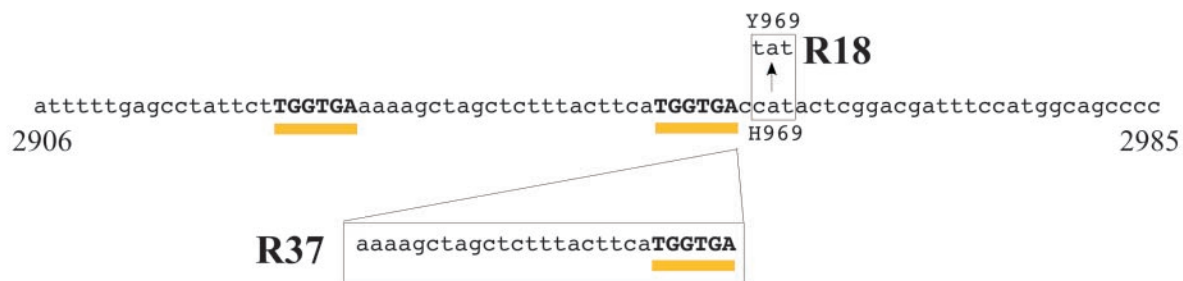[b]The insertion alleles contained one additional C residue inserted into a run of six C residues.

Figure 3. Mutation spectra for *cst* alleles of *cdc27* and *pol3*. (**A**) Nucleotide sequence of a 450 bp region of *cdc27⁺* genomic DNA (from nucleotide 681–1130, where the ATG is at 1) showing the molecular structure of the *cdc27 cst* alleles. Six different mutation types are indicated, as follows: found in R39 allele (22 bp duplication of region bounded by GACA repeats underlined in pink), R3 and three isoalleles (16 bp duplication, GACA repeats, green), R25 and one isoallele (121bp, ACAATCG, red), R40 allele (58 bp, AGAAG, blue), R5 allele (173 bp, AAAT, orange) and R6 and seven isoalleles (insertion of single C in CCCCCC sequence, indicated by yellow bar). (**B**) Nucleotide sequence of an 80 bp region of *pol3⁺* genomic DNA (from nucleotide 2906–2985, where the ATG is at 1) showing the molecular structure of the *pol3 cst* alleles. Two different mutants were obtained: R37 (27 bp duplication of region bounded by TGGTGA repeats underlined in orange), and R18 (C to T mutation, causing H969 to be mutated to Y969).

mutant proteins are expressed from the endogenous *cdc27⁺* promoter, the PCNA binding motif is non-essential. To confirm this (and to rule out the possibility that sequences encoding the PCNA binding motif might be restored to the 3′ end of the mutated *cdc27* ORF by some aberrant splicing event), three new mutant alleles were constructed by replacing the endogenous *cdc27⁺* gene with truncated gene sequences (Figure 4A). Of the encoded proteins, Cdc27-D1 possesses

only the N-terminal Cdc1 binding domain (160–372), Cdc27-D2 lacks most of the of C-terminal region but retains the PCNA binding motif (160–352), while Cdc27-D3 lacks only the PCNA binding motif (353–372). By using a two-step homologous recombination method in diploid cells, heterozygous mutant strains were obtained that contained, on one chromosome, the mutant genes under the control of the native *cdc27* promoter. The diploids were then induced to sporulate, the haploid spores were allowed to germinate on rich medium, and the genotypes of the resulting clones analysed by PCR.

All three haploid *cdc27-D* mutants were viable at 32°C. The mutant strains were then examined for temperature, cold or drug sensitivities (Figure 4B). All three mutants grew well at 32°C. However, both *cdc27-D1* and *cdc27-D3* showed cold-sensitivity; *cdc27-D3* cells could grow only slowly at 18°C, and *cdc27-D1*, not at all. Cells of both mutants were highly elongated at 18°C, indicating cell cycle delay. In addition to this, *cdc27-D1* and *cdc27-D3* cells were highly sensitive to the ribonucleotide reductase inhibitor hydroxyurea (HU), the DNA damaging agent methyl methanesulphonate (MMS) and the topoisomerase I inhibitor camptothecin (CPT). These

results indicated that, while the PCNA binding motif at the C-terminus of Cdc27 is not absolutely required for cell growth, it is required for the efficient growth at low temperature and for cell viability after DNA damage.

Loss of PCNA binding domain suppresses *cdc24-M38*

Six different *cdc27* alleles were identified as suppressors of *cdc24-M38*, all of which appear to encode truncated proteins (Table 2). The longest protein encoded by the mutant alleles, Cdc27-R5, comprises 271 amino acids of Cdc27 sequence, plus an additional 24 amino acids that result from the frame-shift caused by the sequence duplication. Therefore, loss of 101 amino acids from the extended C-terminal part of Cdc27 appears to be sufficient for *cdc24-M38* suppression activity. This region of the protein contains binding sites for Pol1 (amino acids 293–332) and for PCNA (amino acids 353–372) (10,20).
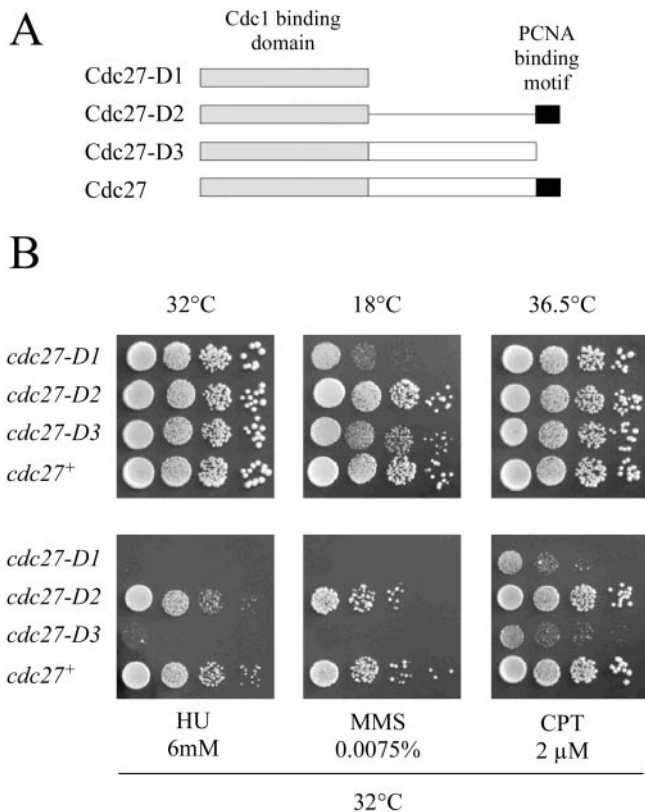

Figure 4. Properties of truncated Cdc27 proteins. (**A**) Schematic representation of wild-type and C-terminally truncated Cdc27 mutant proteins, showing the location and extent of the globular Cdc1 binding domain (speckled box) and the PCNA binding motif (black box). Cdc27-D1 lacks amino acids 160–372; Cdc27-D2 lacks 160–352; Cdc27 lacks 353–372. (**B**) Growth properties and drug sensitivities of *cdc27-D1*, *cdc27-D2* and *cdc27-D3* mutant strains at 32°C. Serial dilutions of exponentially growing *cdc27-D1*, *cdc27-D2*, *cdc27-D3* and wild-type cells in YE were spotted (10 000, 1000, 100, 10 cells per spot) onto YE plates or YE plates containing 6mM HU, 0.0075% MMS and 2 μM CPT. Plates at 32°C and 36.5°C were incubated for 3 days. The 18°C plate was incubated for 7 days.
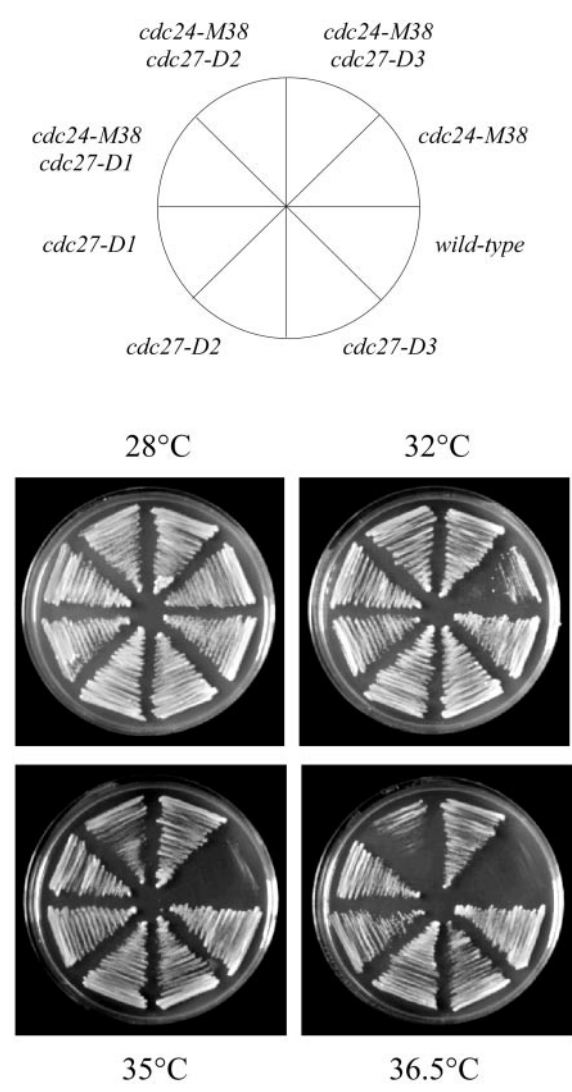

Figure 5. The *cdc27-D1* and *cdc27-D3* mutations suppress the temperature-sensitivity of *cdc24-M38* cells. *cdc24-M38 cdc27-D1*, *cdc24-M38 cdc27-D2* and *cdc24-M38 cdc27-D3* double mutants, plus single mutant and wild-type controls were streaked on YE plates and incubated at the indicated temperatures for 2 days.

To test whether loss of the PCNA binding motif alone was sufficient for suppression of *cdc24-M38*, the double mutants *cdc24-M38 cdc27-D1*, *cdc24-M38 cdc27-D2* and *cdc24-M38 cdc27-D3* were constructed by tetrad dissection and examined for temperature-sensitivity at 36.5°C (Figure 5). Both *cdc24-M38 cdc27-D1* and *cdc24-M38 cdc27-D3* cells were able to grow efficiently even at 36.5°C. The Cdc27-D1 protein lacks the entire C-terminal region of the protein, while Cdc27-D3 protein lacks the C-terminal 20 amino acids, containing PCNA binding domain only. Thus, loss of the PCNA binding domain alone is sufficient to restore growth to *cdc24-M38* cells at the restrictive temperature. Interestingly, although *cdc24-M38 cdc27-D2* cells were able to grow at 35°C, their growth was poor compared with either *cdc24-M38 cdc27-D1* or *cdc24-M38 cdc27-D3* cells (Figure 5), indicating that the presence of the PCNA binding motif prevents efficient rescue of *cdc24-M38*. This is discussed further below.

The *pol3* and *cdc27* mutations also suppress *dna2-C2*

The temperature-sensitive *cdc24-M38* and *dna2-C2* mutants show similar phenotypes. Cells carrying either mutant allele arrest the cell cycle in S phase with incompletely replicated chromosomes when shifted to the restrictive temperature (12,13,19). As shown above (Figure 1), the Dna2 and Cdc24 proteins form a complex in the cell. These data raised the possibility that the molecular defects of both mutants are the same. If this was the case, the *dna2-C2* mutant might be expected to be suppressed by the *pol3* and *cdc27* alleles that suppress the *cdc24-M38*. To assess this possibility, *dna2-C2 pol3-R18* and *dna2-C2 cdc27-R22* mutants were constructed and examined for temperature-sensitivity at 36.5°C (Figure 6). Both *pol3-R18* and *cdc27-R22* suppressed the temperature-sensitivity of *dna2-C2* mutant. This genetic result suggests that Cdc24 and Dna2 have a common function that can be bypassed by impairing the PCNA binding capability of Pol δ.

Forward mutation assay

Yeast strains carrying mutations in genes encoding important replicative enzymes often display elevated mutation rates when grown under permissive or semi-permissive conditions and the nature of the mutations that arise can be a useful indicator of the function of the protein in wild-type cells. In fission yeast, for example, inactivation of the Fen1 (*S.pombe* Rad2) nuclease results in a 16.5-fold increase in mutation rate over wild type, with many of the mutations being sequence duplications flanked by short direct repeat sequences (22), similar in nature to those seen in the *pol3* and *cdc27* alleles isolated as suppressors of *cdc24-M38* (Figure 3, Table 2). To test whether partial impairment of Cdc24 function also resulted in an elevated mutation rate, wild-type and *cdc24-M38* cells were plated at 1×10^7 cells per plate onto plates containing 40 μg/ml of either the toxic arginine analogues L-canavanine sulphate (23) or thialysine (S-2-aminoethyl-L-cysteine hydrochloride) (24) and incubated at 28°C for 8 days (see Materials and Methods). Resistance to canavanine or thialysine in fission yeast results from mutational inactivation of *can1⁺*, a gene that most likely encodes a regulator of arginine uptake (S. A. MacNeill, unpublished observations) rather than an arginine permease as originally proposed (23). No significant differences were observed between wild-type and *cdc24-M38* strains in this system, however, suggesting that *cdc24-M38* does not have a mutator phenotype at 28°C. Note that the choice of 28°C for these assays was dictated by the fact that, at higher temperatures, the plating efficiency of *cdc24-M38* (in the absence of either arginine analogue) dropped markedly in comparison to wild type.

DISCUSSION

Cdc24 is essential for DNA replication in fission yeast. Cells carrying temperature-sensitive mutations in the *cdc24⁺* gene undergo cell cycle arrest when shifted to the restrictive temperature, becoming arrested with incompletely replicated chromosomes (11,12,19). In this paper, it is shown that Cdc24 interacts with Dna2 in yeast protein extracts, confirming previous results obtained using the two-hybrid system (13). Taken together, these results strongly support a model in which Dna2 and Cdc24 form a functional complex *in vivo*. Dna2 is a helicase–nuclease that has been implicated in Okazaki fragment processing through a series of genetic and biochemical
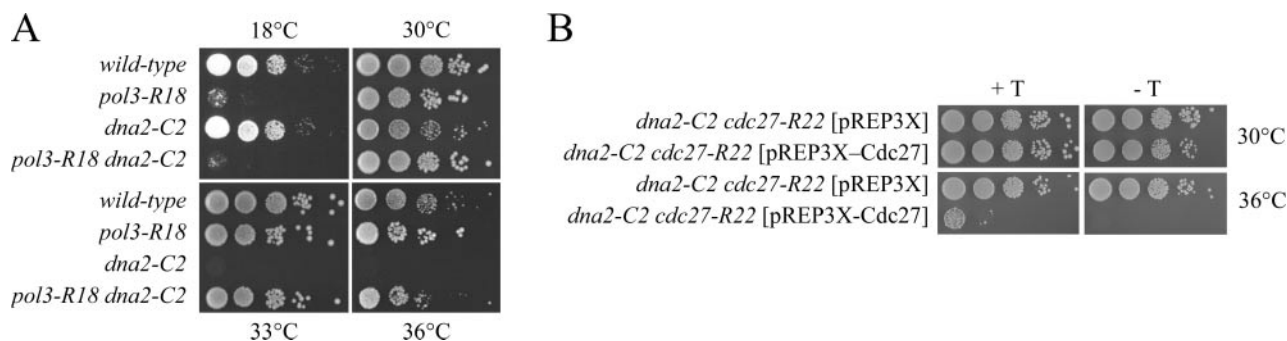

Figure 6. The *pol3* and *cdc27* mutations suppress *dna2-C2*. (**A**) The *pol3-R18* mutation suppresses the temperature-sensitivity of *dna2-C2*. Cells from the indicated strains were spotted in 10-fold dilutions (10^5, 10^4, 10^3, 10^2 and 10^1 cells) onto YE plates and incubated for 3 days at 36°C, 33°C or 30°C, or at 18°C for 6 days. (**B**) The *cdc27-R22* mutation suppresses the temperature-sensitivity of *dna2-C2* and the expression of wild-type *cdc27⁺* abolishes the suppression. The *dna2-C2 cdc27-R22* double mutant cells was transformed with pREP3X alone or pREP3X-cdc27 expressing wild-type *cdc27⁺*. Each transformant was grown in EMM containing thiamine (5 μg/ml) and the cells obtained were spotted in 10-fold dilutions (10^5, 10^4, 10^3, 10^2 and 10^1 cells) onto EMM plates or EMM plate containing thiamine (5 μg/ml). The plates were incubated for 3 days at 36°C or 30°C.

studies in budding and fission yeast (13,25–32). Dna2 is an essential protein that is conserved throughout eukaryotes. In contrast, homologues of Cdc24 have not been identified in other species, raising the possibility that the protein is unique to *S.pombe*. In budding yeast, the NTD of the Dna2 protein has been shown to have a regulatory function that is mediated via its interaction with the nuclease and helicase domains for the protein (33). Whether the N-terminus of the fission yeast Dna2 protein has a similar function or not is not clear. However, it is this region of Dna2 that is responsible for binding to Cdc24, as a C-terminally truncated form of Dna2 comprising the N-terminal 425 amino acids only, Dna2-C425, is able to bind Cdc24 in the two-hybrid system (J. Crossland and S. M., unpublished). Therefore, Cdc24 may have a role in regulating Dna2 activity via the Dna2 N-terminal domain. However, confirmation of this will require detailed biochemical analysis of Cdc24–Dna2 interactions. Intriguingly, Dna2 also appears to exist in several forms of differing mobility on SDS–PAGE (see Figure 1). Budding yeast Dna2 and probably the fission yeast Dna2 is phosphorylated by both the Rad53 and Cdc7-Dbf4 kinases (Y. -S. S, unpublished data). In the co-precipitation experiments shown in Figure 1, Cdc24 appears to bind preferentially to the faster migrating forms of Dna2, perhaps hinting at a role for phosphorylation in regulating the Dna2–Cdc24 interaction. Further analysis of this issue is underway.

Through suppressor mutant screening, genetic interactions have been uncovered between *cdc24* and genes encoding two of the four subunits of Pol δ, the catalytic subunit Pol3 and the C-subunit Cdc27 (Table 1). Previously, it was shown that overproduction of the D-subunit of Pol δ, Cdm1, was also able to suppress the *cdc24-M38* and *cdc24-C26* alleles (3). These results suggest that Cdc24 is intimately involved with Pol δ in lagging strand DNA synthesis, consistent with the observation that Cdc24 interacts biochemically with both RFC and PCNA (12).

Sequencing of the *cdc27* and *pol3* alleles that suppressed *cdc24-M38* revealed that many of the mutations (10 out of a total of 19) were DNA sequence duplications (Figure 3, Table 2). All the *cdc27* alleles, both sequence duplications and insertions, encoded C-terminally truncated proteins lacking the PCNA binding motif. Indeed, loss of the PCNA binding domain was sufficient to suppress the temperature-sensitivity of *cdc24-M38* (Figure 5). Although loss of the PCNA binding motif could result from either sequence insertions or deletion, it was striking that no deletions were found. Furthermore, all the sequence duplications were flanked by short direct repeats. This pattern of mutation has been observed previously in Fen1 mutants in both budding (34) and fission yeast (22). The nuclease Fen1 also plays an important, albeit non-essential, role in Okazaki fragment processing (35) and Fen1 mutant cells (*rad27* in *S.cerevisiae*, *rad2* in *S.pombe*) are thought to accumulate uncleaved flap structures generated during Okazaki fragment processing. Repeat slippages occurring in such a situation are thought to cause duplication mutations. Thus, the propensity for this type of mutation to arise in *cdc24-M38* cells suggests that these cells too have a defect in Okazaki fragment processing. Given the close functional and physical relationship between Cdc24 and Dna2, and the phenotypic similarity between mutants in the two genes, it is to be expected that sequence duplications will also be found with increased frequency in *S.pombe dna2*

mutants also, but this has not been tested. In addition, it is unclear whether sequence duplications will be the predominant type of mutation found in *cdc24-M38* cells or whether the selective pressure imposed in the search for suppressors of *cdc24-M38* that are able to grow at 36°C resulted in a bias towards this type of mutation.

In the course of this study, it was shown that the PCNA binding domain of Cdc27 is not essential for cell viability, contrary to previous reports (20,36). The reason for this discrepancy is unclear but could relate to the level of expression of the mutant proteins, as in both previous sets of experiments, the mutant proteins were expressed from cloned cDNAs under the control of various forms of the heterologous nmt promoter on multi-copy plasmids (20,36). To analyse the function of the C-terminal part of Cdc27 that includes the PCNA binding domain, we generated three new *cdc27* mutants, *cdc27-D1*, *cdc27-D2* and *cdc27-D3* (Figure 4A). Both *cdc27-D1* and *cdc27-D3* cells showed cold-sensitivity and sensitivities to the DNA damaging agents HU, MMS and CPT (Figure 4B). The Cdc27-D1 and Cdc27-D3 proteins both lack the PCNA binding motif. PCNA binding by Cdc27 is therefore essential for growth at low temperature and viability after exposure to certain DNA damaging agents. In contrast to *cdc27-D1* and *cdc27-D3*, *cdc27-D2* encodes a protein that lacks most of the C-terminal region (amino acids 160–352) but retains the PCNA binding motif. Cells expressing the Cdc27-D2 mutant protein, unlike those expressing either Cdc27-D1 or Cdc27-D3, were neither cold-sensitive nor sensitive to DNA damaging agents (Figure 4B). Similarly Cdc27-D2 failed to suppress *cdc24-M38* efficiently at 36.5°C, although some suppression was apparent at 35°C, suggesting that simply fusing the PCNA binding motif to the globular N-terminal domain of Cdc27 may not completely restore Cdc27 function to wild-type levels. Whether this is due to the Cdc27–PCNA interaction being less than optimal in *cdc27-D2* cells or whether the loss of amino acids 160–352 disrupts another important protein–protein interaction (10) awaits biochemical investigation. In addition, note that although interaction with PCNA is essential for the processivity of the Pol δ enzyme, Pol δ interacts with PCNA in at least two ways—via Cdc27 and via the Pol3 and/or Cdc1. Thus, disruption of the Cdc27–PCNA interaction does not abolish Pol δ–PCNA interactions altogether, but is likely to have more subtle effects on enzyme function that are manifest in the cold-sensitivity, drug sensitivity and *cdc24-M38* suppression phenotypes displayed by *cdc27-D1* and *cdc27-D3*.

The left side of Figure 7 shows a model for Okazaki fragment processing in fission yeast. Under normal circumstances, maximal processivity of Pol δ is ensured through interactions between Cdc27 and PCNA, and between Pol3 and/or Cdc1 and PCNA. As the polymerase approaches the 5′ end of the previously synthesized Okazaki fragment (Figure 7A), synthesis continues until a flap structure of at least 35 nt is displaced (Figure 7B and C). At this point, the single-stranded DNA binding factor RPA binds the displaced flap and recruits the Dna2–Cdc24 complex. (For clarity, RPA is omitted from the diagram.) Cdc24 may serve to regulate Dna2 or perhaps to link Dna2 to other components of the replication apparatus. Dna2 then cleaves the bulk of the flap, leaving behind a shorter flap that acts as a substrate for the non-essential nuclease Fen1 (Figure 7D). Finally, the single-stranded nick is ligated by
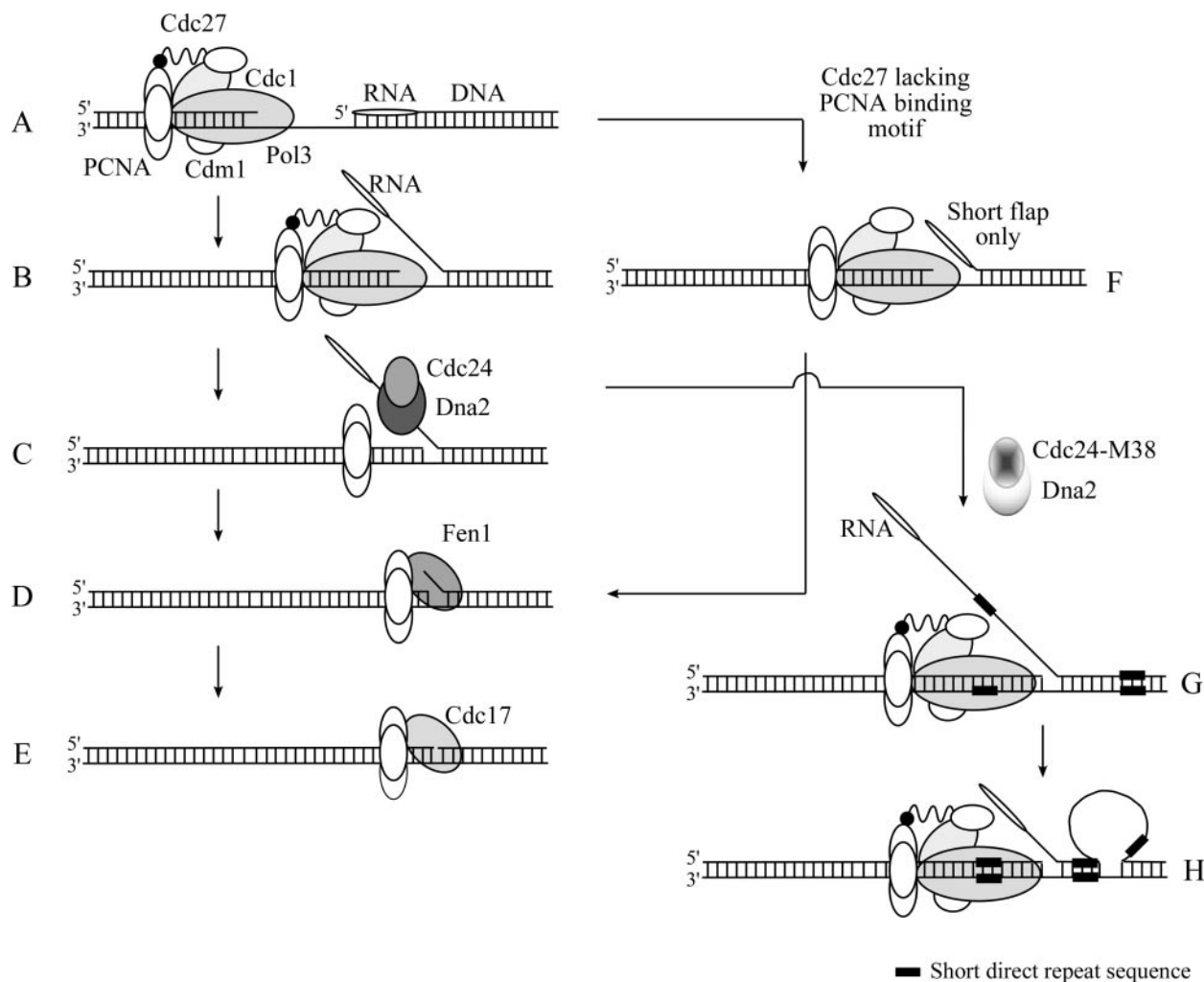
Figure 7. Model for Okazaki fragment processing in fission yeast. Left, schematic showing Cdc24-Dna2 processing of 5′ flap structures, from the first approach of the Pol δ-PCNA complex (**A**), through displacement synthesis (**B**) and Cdc24-Dna2 binding (**C**; for clarity, RPA is omitted), to Fen1 cleavage (**D**) and Cdc17 catalysed ligation (**E**). Right, upper part: when Pol δ processivity is reduced as a result of impairment of the Cdc27-PCNA interaction, a short flap only is generated (**F**) that can be processed by Fen1(D). Right, lower part: when Cdc24-Dna2 function is impaired, transient stabilisation of long flaps (**G**) can result in repeat slippage. See text for further discussion.

DNA ligase I, Cdc17 (Figure 7E). Both Cdc24 and Dna2 are essential proteins, implying that the efficient removal of long flap structures is necessary for successful replication. When the processivity of Pol δ is reduced by disruption of the Cdc27–PCNA interaction (notice the loss of the C-terminal region of Cdc27 in Figure 7F), it is possible that the formation of long flaps is reduced. Under these circumstances, Cdc24–Dna2 activity becomes dispensable and the step illustrated in Figure 7C is bypassed: in this way, *cdc27-D1* and *cdc27-D3* are able to suppress *cdc24-M38* and *dna2-C2* at their restrictive temperatures. The shorter flaps generated under these conditions (e.g. in *cdc27-D1 cdc24-M38* cells) are then presumably processed by Fen1. When Cdc24 function is partially impaired, as in *cdc24-M38* cells grown at their permissive temperature, lengthy uncleaved flap structures may transiently accumulate (Figure 7G). This appears to lead to the formation of slippage structures between short direct repeats (Figure 7H) and the generation of sequence duplications. Confirmation of this model will require further biochemical investigation.

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