Functional Analysis of RFC and RFC-like Complexes in Fission yeast

by

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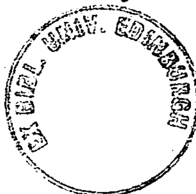

Declaration of Authenticity

I declare that this thesis was composed by myself and that the research presented is my own. Due acknowledgement is made within the text for the assistance of others.

Jiyoung Kim

January 2005

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Abstract

RFC plays an essential role in DNA replication in eukaryotic cells by loading the sliding clamp PCNA onto DNA in order to give processivity to DNA polymerase δ to DNA.. RFC consists of five subunits, one large subunit and four small subunits. The large subunit of RFC contains an extended C-terminal domain that is not present in the small subunits and whose function remains unknown. In addition to RFC, eukaryotic cells contain two more putative PCNA loaders known as RLCs. These other PCNA loaders have similar structures to RFC and contain the RFC small subunits. However the large subunit is replaced with a different protein, either Elg1 or Ctf18. The function of the three PCNA loaders is not clear.

In this work the function of the *S.pombe* Rfc1 C-terminal domain (CTD) was examined. The analysis of an Rfc1 CTD deletion mutant showed that the domain is essential for cell viability. *rfc1-44,* a temperature-sensitive mutant with a mutation in the C-terminal domain, displayed sensitivity to DNA damaging agents, abnormal chromosome structure and a synthetic lethal phenotype when combined with DNA replication mutants. *rfc5* mutants were isolated as suppressors of *rfc1-44* suggesting that the defect in *rfc1-44* may be in the Rfc1-Rfc5 interaction.

Ctf18, Dcc1 and Ctf8, components of Ctf18-RLC, were required for the viability of *rfc1-44* whilst Elg1 was not. Deletion of Elg1 restored the viability of *rfc1-44 ctf18Δ* double mutant cells, suggesting that Elg1 plays a negative role. The negative role of Elg1 was confirmed by over-expression of Elg1 in *rfc1-44* cells showing a lethal phenotype at permissive temperature. These results suggest that in *S.pombe,* RFC plays a key role in DNA replication and that Elg1-RLC and Ctf18-RLC can play negative and positive roles respectively, when RFC function is impaired.

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Abbreviations

°C	degree Celcius
μ	micro
5-FOA	5-fluoroorotic acid
A	adenine
ACS	ARS consensus sequence
ARS	autonomous replicating sequence
ATP	adenosine triphosphate
BSA	bovine serum albumin
bp	base pair
C	cytosine
Cdc	cell division cycle
CDK	cyclin-dependent protein kinase
Cm	centimetre
CPT	camptothecin
DW	distilled water
DMSO	dimethylsulphoxide
DNA	dieoxyribonucleic acid
dNTP	deoxynucleotide triphosphate
DSB	double strand break
dsDNA	double stranded DNA
EDTA	ethylene-diamine-tetraacetic acid
EMM	Edinburgh minimal media
G	guanine
g	gram
HU	hydroxyurea
IPTG	isopropylthio-β-D-galactoside
IR	ionising radiation
kb	kilo base
kDa	kilo Dalton
kV	kilo Volt
l	litre
LB	Luria broth
LiAc	lithium acetate
M	molar
m-_	milli
_m	metre
Mb	megabase
MCM	minichromosome maintenance
ME	malt extract
mM	millimolar
MMR	mismatch repair
MMS	methyl methanesulfonate
n	nano
NER	nucleotide excision repair
NHEJ	non-homologous end joining
nt	nucleotide
OB	oligonucleotide
OD	optical density
ORC	origin recognition complex
PCNA	proliferating cell nuclear antigen

PCR	polymerase chain reaction
PEG	polyethylene glycol
PFGE	pulse-field gel electrophoration
PMSF	phenylmethylsulphonylfluoride
Pol	polymerase
Pre-RC	pre-replicative complex
RFC	replication factor C
RNA	ribonucleic acid
RP-A	replication protein A
rpm	revolutions per minute
S.cerevisiae	*Saccharomyces cerevisiae*
ssDNA	single stranded DNA
S.pombe	*Schizosaccharomyces pombe*
SSB	single strand DNA binding protein
T	thymine
ts	temperature sensitive
UV	ultraviolet
W/v	weight per volume
X.laevis	*Xenopus laevis*
YE	yeast extract
α	alpha
β	beta
δ	delta
ε	epsilon
γ	gamma

Chapter 1: Introduction

1.1 Eukaryotic DNA replication

1.1.1 Overview

Chromosomal DNA replication is the complex process of duplicating DNA using both DNA strands as a template and producing newly synthesized daughter DNA strands (Lewin 2004). The duplication process occurs in the S phase of the eukaryotic cell cycle and the duplicated DNAs are separated in mitosis. The faithful duplication of the genome is critical for genome integrity. Thus DNA replication takes place in accordance with the cell cycle and with DNA repair.

DNA replication occurs with the cooperation of large numbers of proteins involved in the accurately regulated initiation, extension and completion steps. Replication starts by the ordered binding of initiation proteins onto a specific site on the chromosome, the replication origin. Upon binding to the replication origin, these proteins recruit additional proteins and eventually form two multiprotein replication fork structures. The two replication forks proceed in opposite directions so that DNA is synthesized bidirectionally from the replication origin.

Because DNA polymerases synthesise DNA only in the 5'-3' direction, the replication of antiparallel DNA strands at the same replication fork presents mechanistic difficulties. To overcome this problem, cells have evolved the mechanism of semidiscontinuous DNA replication. On one strand, DNA is synthesized continuously (leading strand) and on the other strand (lagging strand), Okazaki fragment DNA is synthesized. The nascent Okazaki fragment are later ligated by DNA ligase.

Eukaryotic genomes are large, ranging from 10^7 to 10^9 base pairs and are organized into multiple chromosomes. To efficiently duplicate these large, discontinuous genomes DNA replication in eukaryotic cells takes place at multiple sites along the chromosomal DNA. This raises the possibility that a single origin may fire more than once in a single cell cycle. Cells have evolved multiple mechanisms to prevent this problem such as the nuclear export of initiation proteins mediated by CDKs and chromatin structure change (Bell and Dutta 2002).

1.1.2 Initiation of DNA replication

1.1.2.1 Replication origin

The replication origin is the site at which DNA replication starts. Eukaryotic cells contain many replication origins while *E.coli* cells use only one replication origin to copy a whole genome (reviewed in Gilbert DM 2001, Biamonti *et al.*, 2003). Replication origins differ from organism to organism in their size and content; this makes it hard to identify higher eukaryotic replication origins. *S.cerevisiae* replication origins contain only one origin recognition complex (ORC) binding site while others contain more than one ORC binding site. With these multiple replication origins, eukaryotic cells can copy longer and more complex chromosomes at a comparable speed to that of *E.coli* DNA replication. *S.cerevisiae* replication origins have been studied extensively. In comparison, the study of metazoan replication origins seems to need the development of more sensitive and sophisticated techniques. Figure 1.1 summarizes what is known of eukaryotic replication origins.

In *S.cerevisiae*, replication origins are composed of short chromosomal sequences that enable plasmids to replicate; these are called autonomously replicating sequences (ARS). The best studied *S. cerevisiae* replication origin is *ars1*. *ars1* is composed of four important elements called A, B1, B2, and B3 (Celniker *et al.*, 1984). The A element contains an essential ARS consensus sequence (WAAAYRTAAAW) which, if mutated, will abolish origin activity. In addition to the A element, the *ars1* origin requires one or more of the B elements for origin activity. If more than two simultaneous mutations occur in the B elements origin activity is abolished (Marahrens and Stillman 1992). However, the composition and sequence of each ARS is different from origin to origin. For example, ARS307 does not contain the B3 element and ARS305 is composed of A, B1 and B4 elements.
At 500-1500bp, *S.pombe* replication origins are much larger than *S.cerevisiae* origins (reviewed in Bryant *et al.*, 2001). *ars2004*, an efficient replication origin, contains

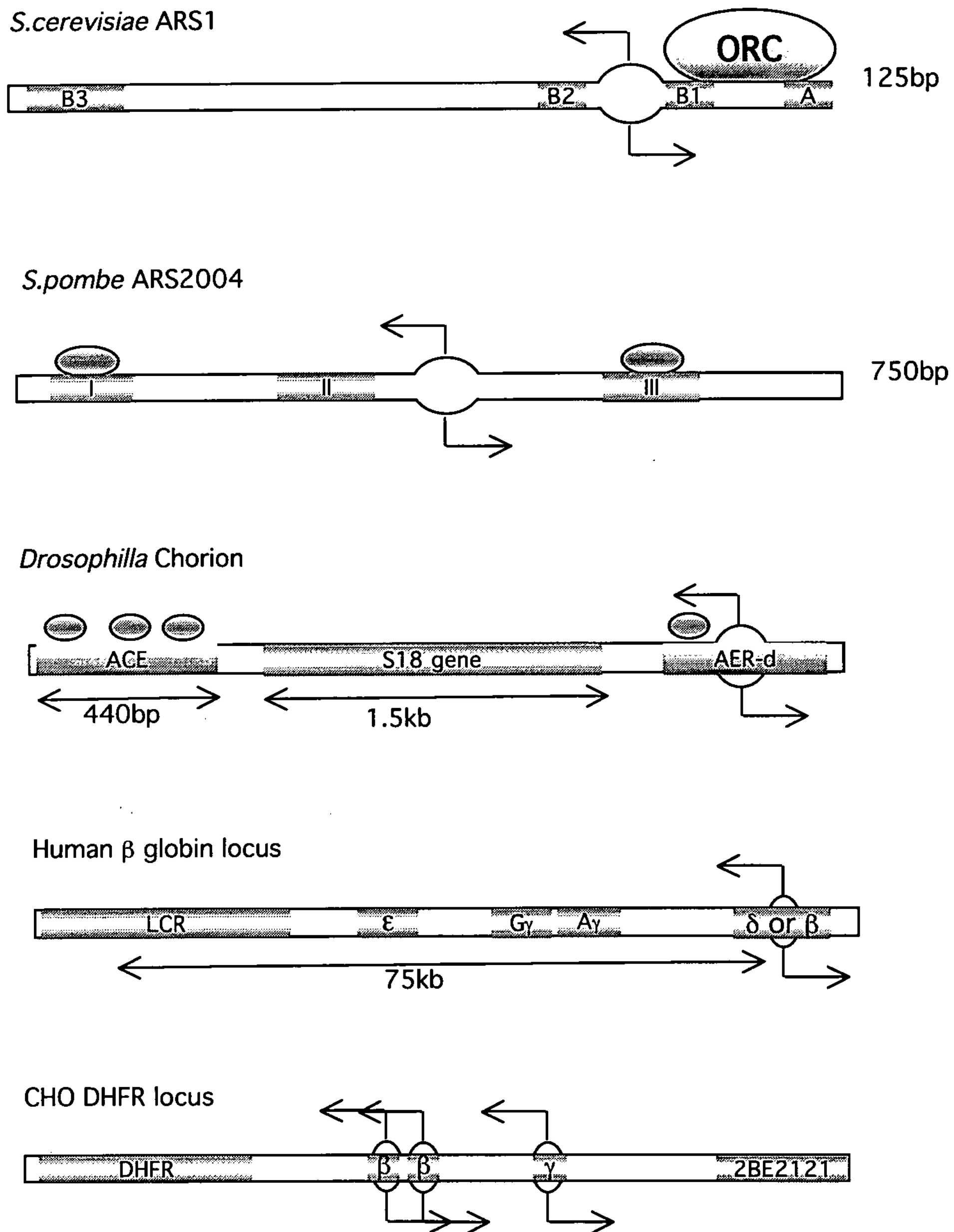


Figure 1.1 The DNA replication origins in eukaryotic cells. Known ORC binding sites are indicated with oval shape. Sites of initiation of replication are indicated with a bidirectional arrow and a bubble. The important regions for replication initiation are indicated with box. The genes around the replication origin are indicated.

three functional regions (region I, II and III) required for autonomous replication (Okuno *et al.* 1999). DNA replication starts in the area between regions II and III (Okuno *et al.* 1997) while ORC binds on region I and III. In contrast to *S. cerevisiae* ARS sequences, *S.pombe* replication origins do not contain consensus sequences. Multiple regions are important for origin activity (Kim and Huberman 1998) and the clustered A/T rich stretches within the origin are essential for its activity (Okuno *et al* 1999).

In *Drosophila*, the chorion gene locus has been extensively studied (reviewed in Biamonti *et al.*, 2003). The chorion gene locus is replicated repeatedly in one cell cycle to produce large amount of egg shell proteins. Large amount of egg shell proteins are required for the formation of follicle cells that protect the oocyte. Although it replicates repeatedly, which is different from normal DNA replication, the replication initiation machinery is similar and the proteins involved in normal DNA replication play important roles in replicating the chorion gene locus. The genes encoding the major chorion proteins are arranged in two clusters in genome; one on the X chromosome and one on the chromosome 3. The replication origin of third chromosomal locus of chorion gene contains a 440 bp amplification control element 3 (ACE3). This is the site at which ORC binds (Orr-Weaver *et al.*, 1989). ACE alone is not sufficient for efficient origin activity and an amplification enhancing element (AER) is required (Delidakis and Kafatos 1989). ORC binds both ACE and AER but the replication starts in AERd, the most proximal AER 1.5kb away from ACE. Tandem ACEs, inserted in random positions along a chromosome can recruit ORC and facilitate replication, suggesting that ACE itself is an intrinsic replication origin (Austin *et al.,* 1999, Lu *et al.*, 2001).

Two more metazoan replication origins have been well studied. The human β-globin locus consists of five genes, ε, γA, γB, δ, and β that exhibit developmentally-regulated expression. Replication starts within a few kilobases located between δ and β-globin genes and the replication timing is regulated by a sequence called the β-globin locus control region (LCR). LCR regulates transcription, chromatin structure and replication timing (Kim *et al.*, 1992). However, the deletion of LCR abolishes replication initiation suggesting that the interaction between these two regions is

important for the initiation of replication (Aladjem *et al.,* 1995). The β-globin origin when transferred to a different chromosomal location, can start replication indicating that higher eukaryotic cells require specific DNA sequences for the initiation of replication (Aladjem *et al.*, 1998)

Another metazoan replication origin that has been extensively examined is the Chinese hamster ovary (CHO) dihydrofolate reductase (DHFR) locus. In the DHFR locus replication is initiated in the 55kb region between the DHFR gene and 2BE2121 gene. However, there is controversy over whether DNA replication starts from a defined position in the 55kb region (Pelizon *et al.* 1996) or from a large number of sites distributed through the 55kb region (Dijkwel and Hamlin 1995, Kalejta *et al.*1998). A recent study has suggested that transcription of the DHFR gene in S phase is important for the initiation of replication. DHFR promoter deletion mutant cells failed to show origin firing in 2D gel analysis (Saha *et al.* 2004). Like the β-globin origin, the DHFR origin when transferred into a new position in the chromosome is capable of replication initiation (Altman and Fanning 2001).

1.1.2.2 Initiation proteins

The assembly of an initiation complex on a replication origin is an ordered process that is regulated by protein kinases. The assembly starts from the recruitment of Cdc6 and Cdt1 by ORC in early G1 phase (reviewed in Bell and Dutta 2002). Cdc6 and Cdt1 then recruit the minichromosome maintenance (MCM) protein complex resulting in the formation of a pre-replicative complex (pre-RC). Once the MCM complex has been loaded onto the replication origin, the initiation of DNA synthesis is triggered by the activity of CDK and CDC7 family protein kinases (reviewed in Kelly and Brown 2000). At the same time, the CDK protein kinase phosphorylates Cdc6 resulting in the release of Cdc6 and Cdt1 from the replication origin. Release of Cdc6 and Cdt1 from the replication origin is one of the multiple redundant mechanisms that prevent the re-initiation of DNA replication in a single cell cycle. Activation of kinases also facilitates the recruitment of Cdc45 protein which in turn recruits DNA polymerase α/primase complex and RPA onto the replication origin (Mimura and Takisawa 1998). Recently, GINS was identified as an initiation factor

that is essential for replication in *S.cerevisiae.* It interacts with Cdc45 and Sld3 to recruit Cdc45 onto the replication origin (Takayama *et al.*, 2003, Kanemaki *et al.*, 2003). The initiation of DNA replication and formation of the pre-initiation complex is summarised in Figure 1.2.

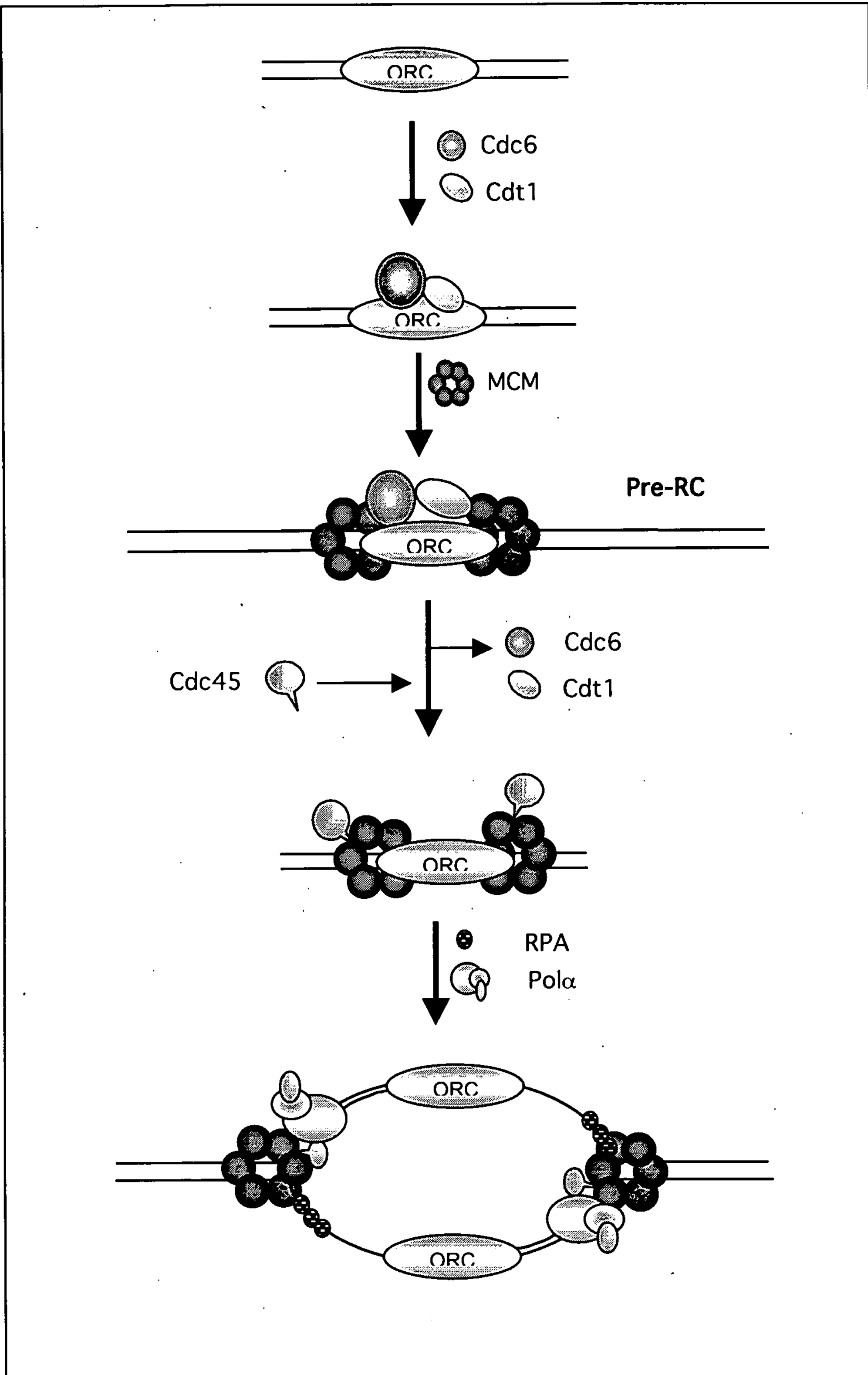


Figure 1.2 Formation of initiation complex. ORC recruits Cdc6 and Cdt1 that in turn recruit MCM complex. With the phsophorylation of Cdc6, the Cdc6 and Cdt1 proteins are dissociated from the replication origin. Cdc45 proteins are loaded onto the chromatin through the interaction with MCM complex. The binding of DNA polymerase α/ primase and RPA are mediated by Cdc45

ORC is a protein complex composed of six subunits (Orc1-Orc6) that initiates DNA replication by recruiting proteins at the replication origin. Like most replication proteins, ORC is well conserved in eukaryotic cells. In *S.cerevisiae* (Liang and Stillman 1997), *S.pombe* (Ogawa *et al.,* 1999) and *Drosophila* (Royzman *et al.*, 1999) ORC is constitutively bound to the replication origin, while in *Xenopus laevis* egg extracts the ORC is removed from the nucleus to prevent re-replication in one cell cycle (Romanowski *et al.* 1996). ORC can recognize and bind to the replication origin and for this process it requires the binding of ATP. However, the hydrolysis of ATP is not required for the binding (Klemm *et al.*, 1997). Accumulating data shows that Orc1 binds to heterochromatin and the heterochromatin protein 1 (HP1) and facilitates origin activity in late firing origin or inactive origins (Pak *et al.* 1997, Shareef *et al.* 2001).

Cdc6/Cdc18 is required for the loading of the MCM complex onto the replication origin (Tanaka *et al.*, 1997). In addition to the loading of MCM, Cdc6/Cdc18 plays an important role in preventing the re-initiation of DNA replication in one cell cycle (Liang and Stillman 1997). The level of Cdc6/Cdc18 within the nucleus is regulated throughout the cell cycle by CDK. In *S.cerevisiae* (Petersen *et al.* 1999) and *S.pombe* (Jallepalli *et al.* 1997) the Cdc6/Cdc18 protein is degraded by ubiquitination triggered by CDK phosphorylation. In mammalian cells the Cdc6/Cdc18 protein is exported from the nucleus after the phophorylation by CDK in S phase (Jiang *et al.* 1999). Cdc6/Cdc18 is a member of the AAA^+ ATPase family of proteins. The binding and hydrolysis of ATP is important for Cdc6/Cdc18 function. A mutation in either the ATP binding or ATP hydrolysis domain can abolish the loading of MCM onto chromatin and the maintenance of checkpoint control during S phase (Perkins and Diffley 1998, Liu *et al.*, 2000).

Cdt1 promotes the association of MCM with the chromosome when it interacts with Cdc6 (Nishitani *et al.* 2000). In mammalian cells, Cdt1 plays a role as an antagonist of geminin – a protein that inhibits the loading of MCM onto chromatin and thus prevents a second round of firing of the replication origin in one cell cycle (Wohlschlegel *et al.* 2000). However, a recent study with human Cdt1 and geminin

suggest that geminin promotes pre-RC formation by protecting Ctd1 from degradation by inhibiting its ubiquitination (Ballabeni *et al.* 2004). Consistent with the role as an MCM loading protein, the level of Cdt1 peaks in G1 phase and decreases through S phase (Nishitani *et al.* 2000).

MCM is a protein complex composed of six subunits (Mcm2-Mcm7). The crystal structure of the archaeal MCM complex shows that the protein forms a ring-shaped complex with a wide central cavity. The channel running through the complex is wide enough to accommodate double-stranded DNA (Pape *et al.* 2003). Like Cdc6 and many other replication proteins, MCM also binds and hydrolyzes ATP – this function is present in Mcm4, Mcm6 and Mcm7 subgroups (Schwacha and Bell 2001). The level of MCM in the nucleus is regulated according to the cell cycle. MCM proteins are present in the nucleus in G1 and S phase but are exported to the cytoplasm in G2 and M phase. In *S.cerevisiae*, this process is regulated by CDK phosphorylation (Labib *et al.* 1999) while in *S.pombe* MCM is exported by Crm1 nuclear export factor (Pasion and Forsburg 1999). Many data suggest that MCM is the replicative helicase that unwinds the chromosomal DNA at the replication fork. MCM is found both at the replication origin and at DNA sequences far from the replication origin, suggesting that MCM is involved in both replication initiation and elongation (Aparicio *et al.* 1997). Consistent with the chromosome binding assay, the depletion of MCM complex after initiation of DNA replication irreversibly blocks the progression of the replication fork, indicating the requirement of MCM at the replication fork (Labib *et al.* 2000). Recombinant archaeal MCM complex showed 3' to 5' DNA helicase activity that requires ATP hydrolysis, supporting the idea that MCM may be a replicative helicase (Chong *et al.* 2000).

Cdc45 is the protein loaded onto the chromosome that interacts with DNA polymerase α and helps the loading of this polymerase onto chromatin (Mimura and Takisawa 1998). In addition to the interaction with polymerase α, *S.cerevisiae* Cdc45 also associates with ORC (Zou *et al.* 1997), DNA polymerase ε (Aparicio *et al.* 1999), RPA (Zou and Stillman 2000), Sld3 (Kamimura *et al.* 2001) and MCM (Zou *et al.* 1997) proteins. From these interactions Cdc45 is thought to coordinate the

function of these proteins. Like MCM, Cdc45 is also found both at the replication origin and the replication fork, suggesting that Cdc45 plays a role in both initiation and elongation of DNA replication (Tercero *et al.* 2000). The localization of Cdc45 in the nucleus is regulated dependent on the cell cycle like Cdc6 and MCM proteins. Cdc45 is released from the chromatin as cells progress through S phase suggesting that the release of Cdc45 is a way to prevent the initiation of another round of replication in one cell cycle (Zou and Stillman 1998).

1.1.3 Replication fork proteins

After the unwinding of the double-strand DNA by the MCM complex, RPA binds to the single-strand DNA that is produced. In accordance with Cdc45 and RPA, the DNA polymerase α/primase complex is recruited onto the DNA and synthesises the RNA/DNA primer. With the cooperation of RPA, RFC and PCNA, the DNA polymerase α/primase is dissociated from the DNA and DNA polymerase δ or ε is recruited. The synthesis of DNA at the replication fork by DNA polymerase δ or ε requires two accessory proteins (PCNA and RFC) to overcome the distributive activity of DNA polymerase δ and complete the DNA synthesis. On the lagging strand, short Okazaki fragments are synthesised by polymerase δ and further processed by Fen-1 and Dna2 to remove the RNA/DNA primer that was synthesised by polymerase α/primase. The newly synthesized Okazaki fragments are ligated to the lagging strand DNA by DNA ligase I.

Many cellular replication factors have been identified using the SV40 *in vitro* replication system. Simian virus 40 infects in monkey cells and duplicates its genome by using the cellular replication system. For the SV40 genome to be replicated it requires its own replication origin and the large T antigen. Using this property, Li and Kelly established an *in vitro* replication system. The replication assay involves adding a plasmid containing the SV40 replication origin and purified large T antigen to virus uninfected monkey cell extracts. Duplication of the plasmid occured in a replication origin- and T-antigen-dependent manner (Li and Kelly

1984). In a subsequent study, it was shown that the replication of the plasmid takes place not only in monkey cell extracts but also in human cell extracts. This indicates that *in vitro* replication assay can be performed easily with any mammalian cell extracts if large T antigen and SV40 replication origin are provided (Li and Kelly 1985). Figure 1.3 summerizes the replication fork proteins involved in the DNA replication.

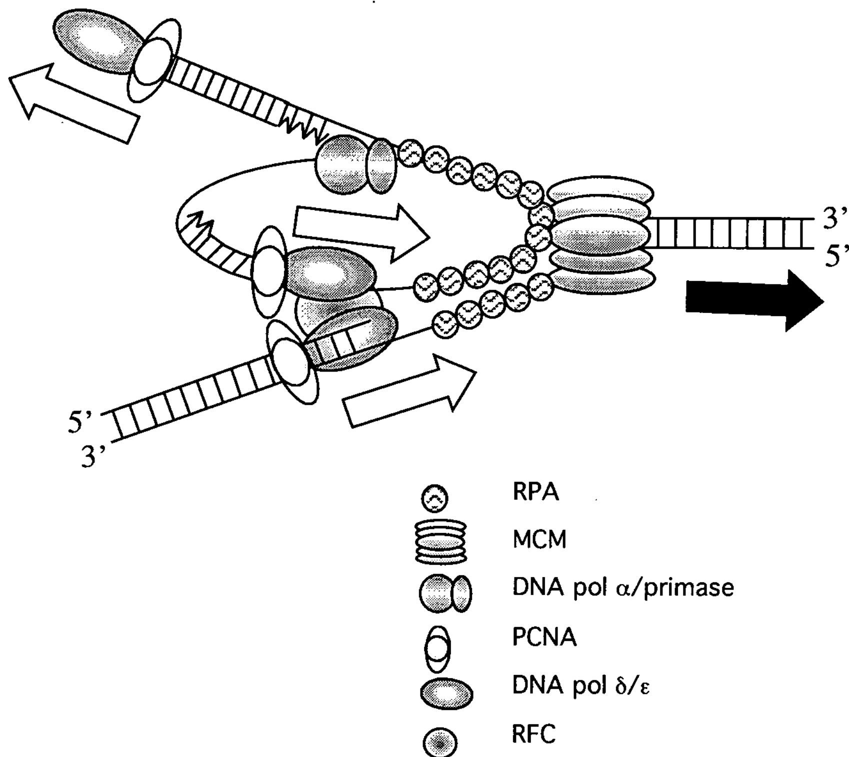


Figure1.3 Replication fork protein. The proteins playin a role in DNA replication fork are indicated. Single-stranded DNA that is unwound by MCM complex is stabilized by the binding of RPA. RNA-DNA primer is synthesized by DNA polymerase α/primase that is further extended by DNA polymerase δ/ε. RFC load PCNA that tethers DNA polymerase δ/ε to DNA. The direction for replication fork movement is indicated by gray arrow.

1.1.3.1 DNA polymerase α/primase

DNA polymerase α/primase is composed of four proteins called p180, p70, p58 and p48; each of these subunits is essential for cell viability. DNA polymerase α/primase can synthesize around 12 nucleotides of RNA primer by *de novo* primase activity. These primers are then extended by DNA polymerase α for a further 30-40 nucleotides of DNA (reviewed in Foiani *et al.*, 1997) After the unwinding of the double-strand DNA, the polymerase α/primase complex is loaded onto chromatin by a direct interaction between Cdc45 and DNA polymerae α/primase that is controlled by p21, a CDK inhibitor (Mimura and Takisawa 1998). In addition to Cdc45, polymerase α/primase interacts with RP-A, T-antigen (Dornreiter *et al.,* 1992), and Mcm10 (Fien *et al.*, 2004). Human DNA polymerase α is phosphorylated in a cell cycle dependent manner suggesting that the activity of DNA polymerase α is regulated by phosphorylation. The p180 and p58 subunits are phosphorylated throughout the cell cycle while the phosphorylation of p70 occurs only in cells in M phase (Nasheuer *et al.*, 1991). p180 is the catalytic subunit of DNA polymerase α that extends the RNA primer synthesized by primase. p48 is the catalytic subunit of the primase molecule and is sufficient for the synthesis of the RNA primer (Santocanale *et al.*, 1993). In *S.cerevisiae*, the cells containing mutant p48 did not arrest in S phase in the presence of a DNA damaging agent (UV or MMS), suggesting an important role for p48 in the DNA damage checkpoint (Marini *et al.*, 1997). The function of p58 is not well understood. A mutation in p58 results in the absence of the RNA primer suggesting that this subunit is involved in primer synthesis (Zerbe and Kuchta 2002). Early studies indicated that p70 binds p180 and SV40 T antigen (Collins *et al.*, 1993). However, more recent studies have suggested another possible function of p70. *In vitro* SV40 DNA replication analysis using the DNA polymerase α/primase complex with or without the p70 subunit has shown that the trimeric complex without p70 could not initiate DNA replication although RPA and SV40 T antigen were present in the reaction mixture. This result suggests that the p70 is essential for the priming activity of polymerase α/primase complex (Ott *et al.*, 2002). Recently, another important function of the p70 subunit has been suggested. In this study a mutation in Pol12, the *S.cerevisiae* p70 subunit, did not affect the normal function of polymerase α nor increase the length of telomeric

single-stranded DNA. Instead, a double mutation between *pol12* mutant and *stn1* mutant, encoding a protein involved in telomere capping resulted in a synthetic lethal phenotype. In addition to this Pol12 and Stn1 have shown genetic and biochemical interactions suggesting that the p70 subunit of polymerase α/primase plays an important role in telomere capping (Grossi *et al.*, 2004).

1.1.3.2 RP-A (Replication protein A)

RPA is a single-strand DNA binding protein that binds to the chromosome after the double-strand DNA has been unwound by MCM DNA helicase (reviewed in Wold 1997). RPA was identified as an essential component in the SV40 *in vitro* replication system. RPA is composed of three subunits all of which are essential for cell viability (Brill and Stillman 1991). The subunits have been named according to the molecular weight of human RPA - Rpa70, Rpa32 and Rpa14. RPA binds both single- and double-strand DNA but the affinity for double-strand DNA is half of that for single-strand DNA (Brill and Stillman 1989). The association of RPA with the chromosomal replication origin requires the MCM complex, which is regulated by CDK kinase (Tanaka and Nasmyth 1998). The level of human RPA does not vary during the cell cycle (Din *et al.*, 1990) although in *S.cerevisiae* mRNA for each subunit is expressed only in G1 and S phase (Brill and Stillman 1991). The activity of RPA is regulated by the phosphorylation of Rpa32. Rpa32 is phosphorylated in a cell-cycle dependent manner (Din *et al.*, 1990) or by DNA damage (Fried *et al.*, 1996). In *S.cerevisiae*, the phosphorylation of Rpa32 in response to DNA damage is dependent on Mec1, a key kinase in DNA damage response. These results suggest that RPA is also involved in the DNA damage repair pathway in addition to DNA replication. Recent evidence supports the idea of RPA as a key component in the DNA damage checkpoint pathway (Zou and Elledge 2003). RPA senses and binds to the single-strand DNA that is produced as a result of DNA damage. This results in the recruitment of ATR-ATRIP to the site of DNA damage. ATR is a protein kinase that involved in the checkpoint pathway and is regulated by ATRIP. ATRIP also plays a role in the activation of Chk1, a key component of the DNA damage checkpoint (Zou and Elledge 2003). The function of RPA appears not to be restricted

to DNA replication and DNA damage response. Recent data suggest that Rpa32 is phosphorylated in a Mec1-independent, but p53-dependent manner resulting in the transcriptional activation of repair genes. RPA can activate the repair pathway by increasing the level of repair proteins (Schramke *et al.*, 2001). RPA is also involved in telomere length regulation through its interaction with one of the telomerase subunits (Schramke *et al.* 2004).

1.1.3.3 DNA polymerase δ

The possibility that DNA polymerase δ was a replicative polymerase first arose from the identification of PCNA. When PCNA was purified from human 293 cell extract and characterized, Prelich *et al.* found that PCNA and calf thymus DNA polymerase δ auxiliary protein showed identical chromatographical and biochemical behaviour in regards to gel mobility, SV40 DNA replication stimulation and immunoprecipitation inhibition. These results indicated the involvement of DNA polymerase δ in the DNA replication process (Prelich *et al.*, 1987b). Later, DNA polymerase δ was purified from the human 293 cell extract and the protein was shown to stimulate the SV40 DNA replication 10-20 fold. The polymerase activity was dependent on PCNA, RFC and RPA (Melendy and Stillman 1991).

DNA polymerase δ contains both DNA polymerase and 3'-5' exonuclease activity that can proofread the mismatched template primer (Bauer *et al.*, 1988). Mutational analysis of Pol3, the large subunit of *S.cerevisiae* DNA polymerase δ, has shown that it bears both DNA polymerase activity and 3'-5' exonuclease activity. In the same analysis, the mutant cells exhibiting exonuclease dysfunction showed drastically increased spontaneous mutation rates indicating that the 3'-5' exonuclease activity of DNA polymerase δ is required for accurate replication (Simon *et al.*, 1991).

When mammalian DNA polymerase δ was identified as a replicative polymerase, it was thought to be a two protein complex comprising 125kDa and 50kDa proteins. Later two more subunits were identified by PCNA-affinity chromatography (Hughes *et al.*, 1999) and sequence homology search (Liu *et al.*, 2000). In *S.cerevisiae,* DNA polymerase δ is composed of three subunits comprising Pol3, Pol31 and Pol32

(Gerik *et al.*, 1998). In *S. pombe* DNA polymerase δ is thought to consist of four subunits - a large catalytic subunit (Pol3) and three small subunits, Cdc1, Cdc27 and Cdm1 (Zuo *et al.*, 1997, MacNeill *et al.*, 1996).

The large subunit of DNA polymerase δ is essential for cell viability and DNA replication. Mutant cells harbouring a temperature sensitive mutation in the catalytic domain of DNA polymerase δ arrest in the S phase of the cell cycle when these cells are shifted to restrictive temperature (Francesconi *et al.* 1993).

cdc1 deleted *S.pombe* cells could not survive, indicating that Cdc1 is essential for cell viability. In the same study, Cdc1 displayed a physical interaction with Pol3 and physical and genetic interaction with Cdc27 suggesting that Cdc1 plays a role as a linker between Pol3 and Cdc27 (MacNeill *et al.*, 1996).

S. pombe cells carrying a *cdc27* deletion construct do not form colonies. The cells were germinated but showed a highly elongated phenotype when examined under the microscope indicating that Cdc27 is essential for the viability of *S.pombe* (MacNeill *et al.*, 1996). In contrast, the third subunit of *S.cerevisiae* DNA polymerase δ (Pol32) is not essential for cell viability although the deletion of POL32 cause a cold-sensitive phenotype, damage sensitivity and synthetic lethality when combined with mutations in other polymerase δ genes (Gerik *et al.*, 1998). The third subunit of human DNA polymerase δ is dispensable for polymerase activity *in vitro* (Podust *et al.*, 2002). The function of Cdc27 was elucidated from biochemical and genetic analysis of the interaction between Cdc27 and PCNA. When Reynolds *et al.* used PCNA as a bait in yeast two hybrid system, they found that *S.pombe* Cdc27 interacts with PCNA. Subsequent biochemical and genetic analysis have shown that PCNA and Cdc27 interact via the C-terminal of Cdc27. The fact that Cdc27 can bind to Cdc1 and PCNA simultaneously suggests that Cdc27 acts as a linker between PCNA and DNA polymerase δ (Reynolds *et al.*, 2000). It has subsequently been shown that *S.cerevisiae* Pol32 interacts with PCNA (Johansson *et al.*, 2004).

In *S.pombe* a *cdm1* deletion strain is viable and exhibits no growth defects in the presence of various DNA damaging agents. This shows that Cdm1 is not essential for growth and division. However, in *in vitro* reconstitution experiments, human DNA polymerase δ showed remarkably reduced activity when the smallest subunit p12, a

human homologue of Cdm1, was omitted (Podust *et al.*, 2002), suggesting that p12 plays an important role in polymerase activity.

In addition to a role as a replicative polymerase, DNA polymerase δ also plays important roles in mismatch repair (Longley *et al.*, 1997), base excision repair (Matsumoto *et al.*, 1999), double-strand break repair (Holmes and Haber 1999) and trans-lesion DNA synthesis (Haracska *et al.*, 2001).

1.1.3.4 DNA polymerase ε

DNA polymerase ε has been purified as a pentameric complex from the yeast *S.cerevisiae* and has been well characterised. Yeast DNA polymerase ε contains 3'-5' exonuclease activity; however, in contrast to DNA polymerase δ, the activity of DNA polymerase ε is not stimulated by the addition of PCNA (Hamatake *et al.*, 1990). Cloning and characterization of Pol2, the large subunit of polymerase ε, has revealed that it is essential for cell viability and that the catalytic activity of DNA polymerase ε resides in the N-terminal portion of the subunit whilst the C-terminal portion is necessary for complex formation with other subunits (Morrison *et al.*, 1990).

Temperature-sensitive *pol2* mutant cells were arrested in S phase when shifted into restrictive temperature. Purified DNA polymerase ε from the mutant cells showed replication defect indicating that DNA polymerase ε is a replicative polymerase (Araki *et al.*, 1992). The deletion of the gene encoding the second largest subunit, Dpb2 resulted in a lethal phenotype and the temperature-sensitive *dpb2* mutant showed a partial DNA synthesis defect (Araki *et al.*, 1991). These results suggest that DNA polymerase ε is required for chromosomal replication.

However, the pol2 N-terminal portion deletion mutant that both the 3'-5' exonuclease and catalytic domains were deleted suppressed the temperature-sensitive phenotype of other *pol2* mutants suggesting that the catalytic domain of DNA polymerase ε is not required for DNA replication. It has also been shown that catalytic domain deficient cells can survive in the absence of the DNA damage checkpoint (Kesti *et al.*, 1999). However, the DNA replication in polymerase ε-depleted *Xenopus* egg extracts suggests that the DNA polymerase ε is required for

efficient chromosomal DNA replication. When the DNA polymerase ε catalytic subunit and p60 subunit were depleted from *Xenopus* egg extracts by using p60 antibody, the level of DNA replication was dramatically decreased although the level of chromatin bound DNA polymerase α was increased (Waga *et al.*, 2001). This result emphasizes the important role of DNA polymerase ε in DNA replication. Extensive studies are required to address the function of DNA polymerase ε at the DNA replication fork.

1.1.3.5 PCNA

PCNA was first identified as an antigen reactive with sera from patients with systemic lupus erythematosus (SLE). Also originally known as cyclin, it showed elevated expression levels in transformed and tumor cell lines. The link between the antigen and the accessory factor for polymerase δ was revealed in an early effort to identify cellular proteins involved in the SV40 replication system. In an experiment to identify cellular replication factors, human 293 cell extract was fractionated into three fractions and the replication activity of each fraction or combinations of each fraction, in the presence of T-antigen and a plasmid containing the SV40 origin, was measured. When a fraction was added to the reaction showing basic level of replication, the replication level increased by around 7 fold. The protein in the replication-stimulating fraction was further purified by conventional column fractionation and it was revealed as a 36 kDa protein. The 36 kDa protein comigrated with the replication stimulating activity through the column purification step. Circumstantial evidence suggested that PCNA was involved in DNA replication and/or cell cycle progression through S phase. In order to confirm this, PCNA was purified from human HeLa cells and the replication stimulation activity was measured. The replication activity coincided precisely with the presence of PCNA while depletion of PCNA from the column fraction with antibody from an SLE patient abolished the replication activity. Protein sequence analysis of both PCNA and the 36 kDa replication-stimulating factor proved conclusively that they were the same protein (Prelich *et al.*, 1987a).

In another study, it was found that PCNA and calf thymus DNA polymerase δ auxiliary protein are the same replication-stimulating protein. This was shown by an *in vitro* SV40 replication assay, where the addition of human PCNA dramatically increased the length of DNA polymerized by calf thymus DNA polymerase δ. This suggests that PCNA affects the processivity of polymerase δ (Prelich *et al.*, 1987b). A subsequent study identified that PCNA functions during the elongation stage of replication on both the leading and lagging strands (Prelich and Stillman 1988). Tetrad analysis of a diploid strain that one of the PCNA gene is deleted from the genome revealed that PCNA is essential for cell viability in *S.pombe* (Waseem *et al.*, 1992).

PCNA is a homotrimer (Figure 1.4). The three proteins form a doughnut-like structure with six-fold symmetry. Each individual subunit is divided into two symmetrical domains. Interestingly, the overall structure of PCNA is similar to the β subunit of *E. coli* polymerase III holocomplex. The β complex functions as a clamp for *E.coli* polymerase III, the replicative DNA polymerase. Each β subunit is divided into three symmetrical domains so that the overall structure of the homodimer has six-fold symmetry (Kong *et al.*, 1992). The central cavity that is formed by the three PCNA monomers is wide enough to encircle double-strand DNA and this encircling is thought to prevent non-specific interaction between PCNA and DNA (Krishna *et al.*, 1994).

The interaction between PCNA and various proteins involved in DNA metabolism and the cell cycle has been intensively studied. A well-studied protein that interacts with PCNA is the cell cycle-dependent kinase inhibitor $p21^{WAF1}$. The interaction between PCNA and $p21^{WAF1}$ was revealed by a yeast two hybrid screening using human PCNA as bait. The C-terminal 89 amino acids of $p21^{WAF1}$ was identified as a PCNA interacting protein. A motif important for the interaction of $p21^{WAF}$ with PCNA was identified by an intensive study of the interaction between PCNA and peptides containing overlapping $p21^{WAF1}$ C-terminal sequences. The motif known as $p21^{WAF1}$ PCNA binding peptide ($p21^{WAF1}$ PBP) is QTS**M**TD**F**Y (bold amino acid s are critical and underlined amino acids are important for the PCNA binding) (Warbrick *et al.*, 1995). Many other PCNA interacting proteins have been identified from PCNA binding motifs within the protein sequences and also by an *in vitro*

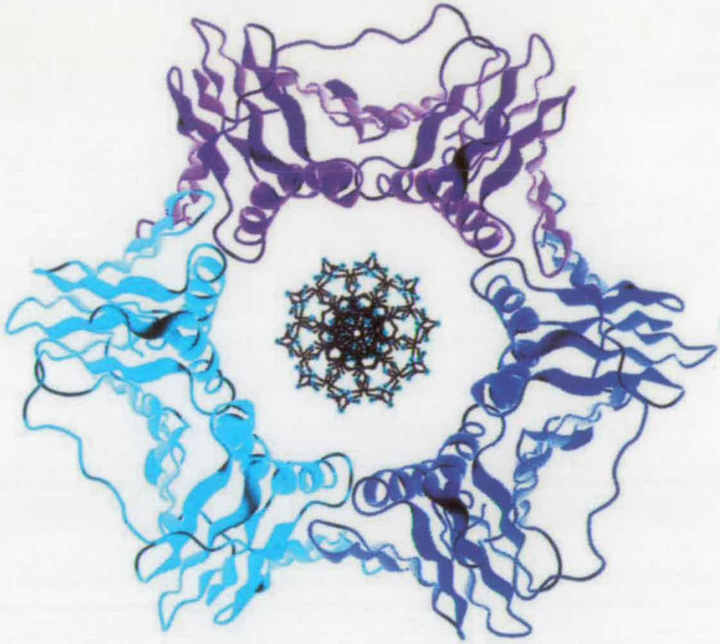


Figure 1.4 Structure of *S.cerevisiae* PCNA trimer. Individual monomers within the ring are distinguished by different colors. A hypothetical model of duplex B-form DNA is placed in the geometrical center of the structure. Image taken from Krishina *et al*., 1994.

p21^{WAF1} PBP binding inhibition assay. The Okazaki fragment maturation protein Fen-1 (Gary *et al.*, 1997, Gomes and Burgers 2000), DNA repair endonuclease XPG (Gary *et al.*, 1997), PDIP, a tumor necrosis factor α induced protein (He *et al.*, 2001), Cdc27, *S.pombe* DNA polymerase δ subunit (Reynolds *et al.*, 2000) and transcription cofactor p300 contain all PCNA binding motifs in their C-terminal ends. Similarly, DNA-(cytosine-5) methyltransferase (MCMT) (Chuang *et al.*, 1997), DNA ligase I (Levin *et al.*, 1997), base excision repair protein UNG (Otterlei *et al.*, 1999) and DNA mismatch recognition protein MSH3 and MSH6 (Kleczkowska *et al.*, 2001) all contain PCNA binding motifs in their N-terminal domains. As a result of the identification of these interactions with various proteins involved in DNA metabolism and cell cycle, PCNA is thought to play a role as a molecular mediator that coordinates molecular events such as DNA replication, DNA repair and cell cycle.

The replication factor C (RFC) complex is required for the opening of the circular structure of PCNA and to load it onto DNA. The interaction and loading mechanism is discussed below. Consistent with its function as a molecular clamp at the replication fork, mutations in PCNA result in a reduced processivity of DNA polymerase δ *in vitro*. Cells carrying a mutation in PCNA showed growth defects, sensitivity to DNA damaging agents, and an increase in spontaneous mutation rates (Ayyagari *et al.*, 1995, Arroyo *et al.*, 1996). Accumulating data suggests that PCNA is involved in the DNA repair pathways. Interactions between PCNA and mismatch binding factors via MSH3 and MSH6 have been identified (Umar *et al.*, 1996, Johnson *et al.*, 1996, Kleczkowska *et al.*, 2001). Both MSH3 and MSH6 contain highly conserved PCNA binding motif and the interaction between PCNA and MSH3 or MSH6 is inhibited by the addition of p21 peptide, a strong PCNA binding peptide (Kleczkowska *et al.*, 2001). PCNA also interacts with base excision repair protein UNG (Otterlei *et al.*, 1999), nucleotide excision repair protein XPG (Gary *et al.*, 1997) and transcription coactivator p300 (Hasan *et al.*, 2001) to modulate DNA repair. PCNA is thought to play a role in the DNA repair pathway by either recruiting repair proteins onto the damaged DNA or by recruiting DNA polymerase for DNA synthesis after the removal of damaged DNA (Kleczkowska *et al.*, 2001, Otterlei *et al.*, 1999). Recent research into PCNA modification has shed light on the

function of PCNA in the repair process. Hoege *et al.* found that PCNA is modified by ubiquitination or SUMO modification in response to DNA damage or the cell cycle respectively. The ubiquitination of PCNA is dependent on proteins in the Rad6 group that play a role in post-replication repair. Consistent with the idea of Rad6-dependent ubiquitination, a PCNA mutant that can not be ubiquitinated (*pol30-K164R*) was highly sensitive to UV and MMS (Hoege *et al.*, 2002). Subsequent research has shown that monoubiqutinated PCNA activates translesion DNA synthesis by DNA polymerase η and ζ (Stelter and Ulrich 2003). Colocalization of ubiquitinated PCNA and DNA polymerase η at the replication foci after the UV irradiation in human cells suggests that the ubiquitinated form of PCNA may mediate polymerase switching at the replication fork after DNA damage has occurred (Kannouche *et al.*, 2004).

1.2 Replication Factor C

1.2.1 Identification of RFC as a clamp loader

Replication Factor C (RFC) was first identified as a protein factor that is essential for the *in vitro* SV40 replication system (Tsurimoto and Stillman 1989). Human 293 cell extracts were fractionated by phosphocellulose column and the fractions that increased the *in vitro* replication were purified further with various columns. After extensive biochemical purification and glycerol fractionation of protein complex, it was shown that RFC is composed of one large (140 kDa) and four small subunits (37-41 kDa). In a reconstitution experiment with or without the purified RFC complex, the *in vitro* replication reaction without RFC generated short DNA fragments while full-length DNA fragments were acquired from the RFC added reaction. Further incubation of reaction mixture lacking RFC failed to extend the short fragments suggesting the blockage of elongation step. In a binding assay, RFC was hybridised with both leading and lagging strand probes suggesting RFC is required for coordinated synthesis of leading and lagging strand DNA. Interestingly, these properties of replication product were similar with that of replication products from reactions with or without PCNA (Tsurimoto and Stillman 1989).

The replication proteins in bacteriophage T4 have elucidated the general mechanism of replication. The phage polymerase encoded by gene 43 synthesizes the DNA strands at the replication fork. Polymerase accessory proteins encoded by gene 44 and 62 functions as a DNA dependent ATPase and primer recognition protein complex. The DNA dependent ATPase activity is stimulated by the gene 45 encoded protein. In order to know whether RFC and bacteriophage 44/62 proteins play a similar role, the RFC was purified from human 293 cell nuclear extracts and its characteristics were examined. In a DNA binding assay, RFC showed specific binding activity to primer-template DNA, while it did not bind to single-strand or double-strand DNA. *In vitro* ATPase activity assay showed that RFC displays a low level of DNA-independent ATPase activity that was stimulated several fold by the single-stand DNA, double-strand DNA and to the great extent primer-template DNA. The ATPase activity of RFC was further stimulated by PCNA in the presence of primer-template DNA. The DNA synthesis by DNA polymerase δ was examined in

the presence of RPA, RFC and PCNA either with ATP or ATPγS, a non-hydrolyzable analogue of ATP. In the presence of RFC, RPA and PCNA, DNA synthesis by polymerase δ was stimulated about 3 fold when ATP was added in the reaction while addition of ATPγS completely abolished the stimulation by the DNA polymerase accessory proteins. This suggests that the hydrolysis of ATP is required for processive DNA synthesis (Tsurimoto and Stillman 1990).

In the binding assay, RFC formed a complex with PCNA and DNA polymerase δ in the presence of ATP and primed DNA. However, after the formation of complex, the ATP was not required for the synthesis of DNA. RFC formed a complex with PCNA in the presence of primed DNA but it did not form complex with DNA polymerase δ alone, suggesting the complex formation of RFC with DNA polymerase δ requires PCNA. The PCNA and DNA polymerase δ did not form a complex in the presence of primed DNA (Lee and Hurwitz 1990). In the study using purified human RFC complex with DNA polymerase δ and PCNA, RFC reduced the amount of PCNA required for DNA polymerase δ-catalyzed DNA synthesis. First, the requirement of RFC in the primer template was examined. In the reaction with multiply primed template, PCNA and DNA polymerase δ did not require RFC for the processive DNA synthesis. But when singly-primed ϕX174 DNA or low ratio of primer:template oligomer were used as a template, DNA synthesis by DNA polymerase δ and PCNA was totally dependent on RFC. In a reaction with fixed amount of RFC, the amount of PCNA required to stimulate the DNA polymerase δ has remarkably decreased (Lee *et al.*, 1991). These results indicate that RFC functions as a primer recognition factor for DNA polymerase δ and PCNA, increasing the ability to use low levels of primer ends. It also suggests that PCNA interacts with RFC at the primer ends.

1.2.2 RFC structure

The crystal structure of *S.cerevisiae* RFC-PCNA complex has been solved (Bowman *et al.*, 2004). Modified RFC was used to facilitate the crystallization. The N- and C-terminal region of Rfc1 was deleted and the arginine residues in Ser-Arg-Cys (SRC) motif of each small subunit were replaced with alanine because the hydrolysis of

ATP weakens the interaction between clamp and clamp loader. The overall structure showed that hetero-pentameric RFC complex is seated on top of a closed PCNA ring, but tipped away from it (Figure 1.5 left panel). Like δ' subunit of *E.coli* γ complex (Guenther et al., 1997) and the small subunit of *P. furiosus* RFC (Oyama *et al.*, 2001), each RFC subunit is divided into three domains. Domain I of each subunit is a RecA-type ATPase domain, which is followed by a small helical domain (domain II) that is characteristic of AAA^+ ATPase. Domain I and II together form the AAA^+ module that is connected by flexible linker to another helical domain (domain III). The helical third domains of all the RFC subunits pack together to form a stable cylindrical structure called the 'collar'. The five AAA^+ modules of RFC assemble into a right-handed spiral so that three of RFC subunits (Rfc1, Rfc3 and Rfc4) make contact with PCNA. ATPγS is bound to each subunit of RFC although the Rfc5 does not contain the conserved amino acids at the ATPase active site suggesting it cannot hydrolyse ATP. The ATPγSs are the key elements that hold the spiral assembly together by anchoring intersubunits interaction through hydrogen bonds to the phosphate groups.

The right-handed arrangement of the five AAA^+ domains of RFC display roughly the same angle as that of double stranded B-form DNA. The axis of ideal B-form DNA fits with the screw axis of RFC (Figure 1.5 right panel). Each of RFC subunits tracks the minor groove of the double helix suggesting that the RFC:PCNA complex fits tightly around the double helix while allowing the preceding segment of DNA to be positioned comfortably within the PCNA ring (Bowman *et al.*, 2004).

The crystal structure of the *E.coli* δ subunit of γ complex in a complex with mutant β subunit has suggested the clamp opening mechanism (Zeruzalmi *et al.*, 2001). In the crystal structure, only the N-terminal region of δ subunit was involved in the interaction with β subunit. The N-terminus of δ subunit formed wedge-like structure that inserts into a cleft between domains 2 and 3 of β subunit when viewed along the edge of central β sheet. Comparison of the structure of the β dimer with that of β:δ complex has revealed that the β subunit shows more relaxed structure in the β:δ complex than in the β dimer. Structural comparison of the δ subunit in β:δ dimer with that of γ complex also revealed that the δ subunit shows large structural change after the binding to β subunit. From these observations, the clamp opening

mechanism was suggested. The clamp loader opens the clamp not by physically pulling apart the subunit interface but rather by trapping one subunit of clamp in a conformation that works against ring closure. The clamp seems exist under some strain and the interaction with the clamp induce the conformational change that allows the ring to spring open (Jeruzalmi *et al.*, 2001).

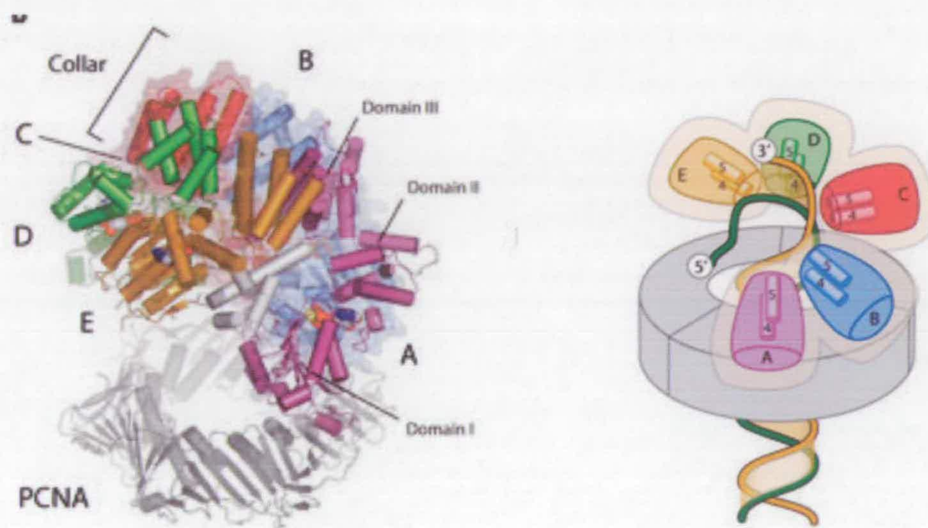


Figure 1.5 Crystal structure of *S.cerevisiae* RFC. Left panel : An overview of RFC:PCNA complex. Each RFC subunits are distinguished by different colors. A: Rfc1 B: Rfc4 C; Rfc3 D: Rfc2 E: Rfc5. Right panel: Schematic representation of the DNA:RFC:PCNA model. Alignment of Rfc1 with the minor groove of the double helix positions the 5' terminus of the template strand near the opening between Rfc5 and Rfc1.

1.2.3 Primary structure of RFC

Genes encoding RFC subunits have been cloned from human (Bunz *et al.*, 1993), mouse (Burbelo *et al.*, 1993) *S.cerevisiae* (Li and Burgers 1994, Gary and Burgers 1995, Cullmann *et al.*, 1995) and *S.pombe* (Reynolds *et al.*, 1999, Gray and MacNeill 2000, Tanaka *et al.*, 1999, Shimada *et al.*, 1999). In tetrad analysis of *S.cerevisiae* RFC, the *rfc* deletion mutant cells showed lethal phenotype indicating all five

subunits of RFC are essential for cell viability (Howell *et al.*, 1994, Cullmann *et al.*, 1995).

Comparison of amino acid sequences of RFC subunits revealed that all five subunits contain seven well-conserved amino acid sequences called RFC boxes II-VIII. One of the most obvious features of RFC protein is that all the subunits contain an ATP binding domain in the N-terminal domain. This consists of several motifs in the N-terminal domain of the small subunits and the equivalent region of the large subunit. The most conserved motif is in RFC box III that is phosphate-binding loop (P loop) with the consensus sequences of GxxxxGK(T/S) that interacts with the phosphate tail of the nucleotide (Cullmann *et al.*, 1995). The crystal structure of γ complex of *E.coli* polymerase III holoenzyme has shown that the P loop in γ complex resides in domain I of each γ complex subunit. Binding of ATP generates a conformational change of sensor 1 and sensor 2 region of the proteins resulting in an overall structural change of each subunit except δ' subunit which does not contain P loop (Guenther *et al.*, 1997, Jeruzalmi *et al.*, 2001).

The second most conserved domain is RFC box V that contains DEAD sequence motif. It is involved in metal binding and catalytic mechanism. RFC box VI is different in the small and the large subunits. RFC box VIa is present in the large subunit while RFC box VIb is present in small subunits. RFC box VIb is also somewhat conserved in the prokaryotic accessory proteins such as bacteriophage T4 gp44. Interestingly, the large subunit of archaeal RFC large subunits contains RFC box VIa while the small subunit contains RFC box VIb (Kelman and Hurwitz 2000, Henneke *et al.*, 2002). RFC box VII, SRC (Ser-Arg-Cys), is conserved in the small subunits but only Cys is present in the large subunit. RFC box I is present only in the large subunits. It consists of about 90 amino acids that show homology with the C-terminus of BASC1 (BRCT domain)(Burbelo *et al.*, 1993, Cullmann *et al.*, 1995, Wu *et al.*, 1996).

1.2.4 DNA binding activity

RFC shows DNA binding activity that is stimulated by PCNA. Early gel mobility shift assays using human or *Drosophila* Rfc1 proteins revealed that the N-terminal

regions including the BRCT domain in Rfc1 were responsible for the DNA binding (Fotedar *et al.*, 1996, Allen *et al.*, 1998, Burbelo *et al.*, 1993).

However, *in vitro* translation analysis has shown that the human RFC complex with N-terminal deleted Rfc1 (N-terminal 687 amino acids deletion) showed moderate DNA binding activity. RFC boxes I-IV were deleted in this experiment suggesting that the BRCT domain is not essential for DNA binding activity of RFC. Further deletion of RFC box V-VII abolished the DNA binding ability of RFC (Uhlmann *et al.*, 1997). Similar results were acquired in surface plasma resonance analysis using recombinant *S.cerevisiae* RFC in which the large subunit is replaced with BRCT domain deleted Rfc1 (RFC-ΔN). In the presence of ATPγS, both wild-type RFC and RFC-ΔN showed high affinity to primed DNA suggesting that the BRCT domain is not involved in DNA binding (Gomes *et al.*, 2000).

The crystal structure of *S.cerevisiae* RFC revealed the DNA binding domains (Bowman *et al.*, 2004). Three α-helices of domain I (α4, α5 and α6) interact with the backbone of modelled double helix. When the primary sequences of Rfc1 was compared with the secondary structure, these α-helices correspond the regions containing RFC boxes IV-VII (Cullmann *et al.*, 1995, Guenther *et al.*, 1997). This result supports the biochemical data that RFC boxes IV-VII is important for DNA binding.

The different results about the DNA binding domain in Rfc1 may be caused from the different method used to examine the DNA binding activity of Rfc1 or from the proteins used for analysis. The early gel mobility shift assays were performed with an Rfc1 fragment alone (Fotedar *et al.*, 1996, Allen *et al.*, 1998, Burbelo *et al.*, 1993) while later gel mobility shift assay or surface plasma resonance was performed with RFC complex in which the part of Rfc1 is deleted (Uhlmann *et al.*, 1997, Gomes *et al.*, 2000).

In an early study, purified human RFC showed binding activity to primer DNA but not to single-stranded or double-stranded DNA (Tsurimoto and Stillman 1990). However, in their later study, Tsurimoto and Stillman showed the single- and double-strand DNA binding activity of RFC (Tsurimoto and Stillman 1991a). Moreover, RFC showed high affinity to both single- and double-stranded DNA in addition to the primed DNA in recent studies (Gomes *et al.*, 2000, Gomes and Burgers 2001,

Hingorani and Coman 2002). Therefore, RFC contains single and double-strand DNA binding activity in addition to primed DNA binding activity.

There is a question to be answered about the DNA selection by RFC caused by the high affinity to both single and double-stranded DNA and relatively low concentration of primer end at the replication fork. The relatively low concentration of primed DNA and high concentration of single and double-strand DNA will reduce the chance of primer DNA-RFC interaction. In the presence of RPA, the affinity for single-stranded DNA decreases remarkably resulting in the increased affinity for the primer-template junction (Tsurimoto and Stillman 1991, Gomes and Burgers 2001). However, there are still high concentrations of double-strand DNA at the replication fork that will inhibit the primer recognition by RFC. From their RFC–DNA interaction study using single, double-stranded and primed DNA, Hingorani and Coman have suggested that the RFC binds single and double-strand DNA with high affinity but rapidly scans to find the primer end and forms a stable complex. When unlabeled primed DNA was used as a competitor of both single stranded and double stranded DNA–RFC interaction, it showed moderate competition while both single and double-stranded DNA were poor inhibitors of primed DNA-RFC interaction even in 50 fold excess amount. Moreover, the half lives of single and double stranded DNA-RFC interaction were 10 fold lower than that of primed DNA-RFC interaction suggesting that the rapid binding and release of RFC to single and double strand DNA can provide scanning mechanism of the chromosome to find the primer end (Hingorani and Coman 2001).

It seems likely that the overall structure of primed DNA is important for the RFC binding rather than specific sequences in the primer end. DNAse footprinting assay using human RFC and hairpin DNA has revealed that the DNA sequences protected by RFC moved to 5' end exactly the same size with the newly added nucleotides at the 5' end. The movement of protected area happened regardless of the DNA sequences added at the 5' end (Tsurimoto and Stillman 1991).

1.2.5 ATPase activity

RFC shows ATPase activity that is stimulated by PCNA and DNA (Tsurimoto and Stillman 1990, Lee *et al.*, 1991). The ATPase activity has shown to be essential to cell viability. Cells carrying mutant RFC with modified ATP binding motif displayed a lethal phenotype (Schmidt *et al.*, 2001). Consistent with its importance, all RFC subunits except Rfc5 contain the AAA^+ ATP binding motif that is required for the binding and hydrolysis of ATP (Cullmann *et al.*, 1995).

However, the hydrolysis of ATP seems to be unimportant for loading of PCNA on DNA because RFC can load PCNA in the presence of ATPγS, a non-hydrolysable ATP homologue (Burgers 1991, Waga and Stillman 1998, Gomes and Burgers 2001). The hydrolysis of ATP is thought to be required for the release of RFC from the DNA after the loading of PCNA. Actually, ATP does not stimulate DNA binding by RFC *in vitro* although the interaction between RFC and PCNA has occurred in the presence of ATP. When ATPγS was used in the binding reaction, the RFC-PCNA interaction stayed stable suggesting that the RFC-PCNA complex is arrested on the DNA (Gomes and Burgers 2001).

The crystal structure of *E.coli* γ complex suggested that the binding of ATP to γ complex generate conformational change in the γ complex resulting in the binding and opening of δ subunit, a clamp in *E.coli* (Jeruzalmi *et al.*, 2001a, Jeruzalmi *et al.*, 2001b). This is also true for human RFC. ATP-bound RFC showed different protease digestion pattern to that seen with RFC alone suggesting that there was a conformational change after the ATP binding (Shiomi *et al.*, 2000). Therefore binding of ATP to RFC cause conformational change that drives opening of PCNA.

In vitro binding assay using ATPγS suggested that four ATP molecules bind to RFC and generate sequential conformational change (Gomes *et al.*, 2001). However, this result is different from the crystal structure data of either *E.coli* γ complex or *S.cerevisiae* RFC. Three ATP molecules were bound to the clamp in the crystal structure of *E.coli* γ complex (Jeruzalmi *et al.*, 2001), while five molecules of ATP were bound to *S.cerevisiae* RFC. In the case of *S.cerevisiae* RFC, the fifth ATP was bound in Rfc5 although Rfc5 does not contain the conserved amino acid residues that are found in the ATPase motif (Bowman *et al.* 2004).

Reconstitution study of recombinant human RFC has revealed that the small subunits of RFC form an alternative RFC complex lacking Rfc1. The alternative RFC complex showed DNA dependent ATPase activity indicating that the small subunits contain intrinsic ATPase activity (Ellison and Stillman 1998). This intrinsic ATPase activity in RFC small subunits seems important for function of RFC, because the mutants that carrying mutation in the ATP binding domain show lethal phenotype (Schmidt *et al.*, 2001).

1.2.6 RFC-PCNA interaction

The primary role of RFC is the PCNA loading onto DNA at the replication fork. For the loading of PCNA, RFC interacts with PCNA and opens the circular structure of PCNA. The interaction between RFC and PCNA has been confirmed in many biochemical studies by either *in vivo* complex formation (Ohta *et al.*, 2002) or the loading of PCNA onto DNA by RFC *in vitro* (Gomes *et al.*, 2001 Uhlmann *et al.*, 1997).

The importance of RFC-PCNA interaction was confirmed by the mutational analysis of both RFC and PCNA. Cold-sensitive phenotype and sensitivity to DNA damaging agents of *rfc1-1* mutant was suppressed by the *Pol30* mutants in *S.cerevisiae.* The *rfc1-1* allele encodes a proteins that is mutated within the well-conserved RFC box VIII and shows cold-sensitive and MMS and UV sensitive phenotypes although the mutant protein shows no difference in ATPase activity, DNA binding activity and slightly reduced processivity in the presence of low molecules of ATP. The *pcna-32* and *pcna-37* mutant proteins contain mutations in the opposite end of monomeric PCNA polypeptide and destabilise the trimeric complex of PCNA (Beckwith *et al.*, 1998). In accordance with that, mutation in RFC subunits has suppressed the cold-sensitive phenotype of pcna mutants (Amin *et al.*, 1999).

In genetic screening, *rfc1*, *rfc2* and *rfc3* mutant proteins were isolated as a suppressor of *Pol30* mutant cells suggesting that Rfc1, Rfc2 and Rfc3 are involved in the PCNA interaction (Amin *et al.*, 1999). However, the crystal structure of *S.cerevisiae* RFC in complex with PCNA revealed that Rfc1, Rfc3 and Rfc4 contact with PCNA (Bowman *et al.*, 2004).

The fact that all *rfc1*, *rfc2* and *rfc3* mutant cells were carrying mutation near the RFC box IV suggests that the regions around RFC box IV are important for PCNA interaction (Amin *et al* 1999). Early gel mobility shift assay also supports the idea. The domain containing RFC box II-VIII in Rfc1 showed retarded mobility in the presence of PCNA suggesting the conserved RFC boxes are important for PCNA binding (Fotedar *et al.*, 1996). The important role of the RFC box in the interaction with PCNA was emphasized in another biochemical study with truncated proteins of RFC small subunits. The deletion of N-terminal domain including the RFC box II from each subunit did not affect the formation of RFC complex but did affect its function. A mutation in any one of the RFC subunits resulted in severe defect in the loading of PCNA onto DNA (Uhlmann *et al.*, 1997). These results were confirmed by the crystal structure of *S.cerevisiae* RFC-PCNA complex (Bowman *et al.*, 2004). In the structure, the interaction between RFC and PCNA took place via α4-helix of Rfc1, Rfc3 and Rfc4. The α4-helix resides proximal to RFC box IV (Cullmann *et al.*, 1995, Guenther *et al.*, 1997) indicating that the domains including RFC box IV are important for PCNA binding.

The domain important for RFC interaction in PCNA was mapped by *in vitro* mutational analysis. The analysis of domain deletion mutant proteins or single mutant proteins with point mutation of conserved amino acids within PCNA has revealed that the C-terminal domain is important for interaction with RFC (Fukuda *et al.*, 1995). *In vitro* binding assay using RFC and the PCNA peptides comprising 20 amino acids of sequences has shown that the surface exposed regions of PCNA interact with RFC (Zhang *et al.*, 1999). In both analysis, Glu41Ala mutation in human PCNA abolished the interaction between RFC and PCNA suggesting that this amino acid is critical for interaction with RFC.

The consensus PCNA binding motif that plays important role in the interaction with PCNA has been identified in Rfc1 (Montecucco *et al.*, 1998). It exists at the N-termini of Rfc1 but in *S.cerevisiae*, the deletion of N-terminus including the PCNA binding domain does not show any defect in the PCNA binding and DNA replication *in vitro* (Uhlmann *et al.*, 1997, Gomes *et al.*, 2000) and DNA damage response and telomere length maintenance *in vivo* (Gomes *et al.*, 2000). It will be interesting to study the function of PCNA binding motif in Rfc1.

1.2.7 Four clamp loaders

Recently, three alternative RFC complexes containing RFC small subunits have been identified. From the structure of these new complexes, they are called RFC-like complex (RLC). These three RLCs are composed of small subunits of RFC but the large subunit is replaced with Rad24, Elg1 or Ctf18. The alternative large subunits of RLCs share sequence similarity between them and with all RFC subunits in an aspect that all of these subunits contain well-conserved RFC boxes II-VIII (figure 1.6). Different from Rfc1, all three other large subunits of RLCs are not essential for cell viability (reviewed in Kim and MacNeill 2003).

The Rad24-RLC functions in the DNA damage checkpoint. The interaction between Rad24 and the small subunit of RFC has been reported (Shimomura *et al.*, 1998, Naiki *et al.*, 2000). The formation of complex between Rad24 and the small subunits of RFC has confirmed by the co-immunoprecipitation assay that all the small subunits but not the large subunit form a complex with Rad24 (Green *et al.*, 2000). The formation of complex between Rad24 and small subunits of RFC explains the involvement of RFC small subunits in the checkpoint pathway that has been identified. The Rad24-RLC interacts and loads Rad17-Mec3-Ddc1 complex (*S.cerevisiae* Rad9-Rad1-Hus1 (9-1-1) homologue) functioning as a clamp in the checkpoint (Majka and Burgers 2003, Rauen *et al.*, 2000, Zou *et al.*, 2002).

Ctf18 is a protein involved in the sister chromatid cohesion, an association of sister chromatid prior to their segregation at mitosis or meiosis. *S.cerevisiae* cells lacking Ctf18 show sister chromatid cohesion failure but are viable (Hanna *et al.*, 2001). The Ctf18-RLC was identified in the study of Ctf8, a protein involved in chromatid cohesion. The CTF8 deletion strain exhibited chromosome loss and sensitivity to microtubule depolymerizing drug. In order to identify the protein interacting with Ctf8, immunoprecipitation was performed with anti-Myc antibody in the Ctf8-13Myc strain. Four discrete bands were identified and the two bands were revealed as Rfc4 and comigration of Rfc2, Rfc3 and Rfc5. The most slowly migrating protein was Ctf18 and the fourth band was unknown protein named as Dcc1. The coimmunoprecipitation experiment in Ctf18 or Dcc1 deletion strain has shown that

Ctf18 binds directly with RFC small subunits and association of Ctf8 with this complex is mediated by the Dcc1 (Mayer *et al.*, 2001).

In the experiment to know the function of RFC small subunits in chromatid cohesion, the *rfc4-20* temperature sensitive mutant strain was arrested in G2/M phase after the incubation at restrictive temperature for 3 hours. It suggests that the Ctf18-RLC is involved in the chromatid cohesion (Mayer *et al.*, 2001). Another possible function of Ctf18-RLC was suggested from deletion analysis of CTF18 and RAD24. The *rad24Δ ctf18Δ* double mutant was sensitive to HU while neither *rad24Δ* nor *ctf18Δ* single mutant was sensitive. In addition, phosphorylation of Rad53 was significantly reduced in the *rad24Δ ctf18Δ* double mutant in the presence of HU. These results suggest that Ctf18 plays redundant role in DNA replication block checkpoint (Naiki *et al.*, 2001). In another study, the *ctf18* deletion strain showed short telemeres suggesting the role of Ctf18 in telomere length maintenance (Hanna *et al.*, 2001).

The biochemical properties of Ctf18-RLC have been studied with recombinant proteins purified from baculovirus system. In a filter binding assay, the Ctf18-RLC showed affinity to single-stranded DNA and primed DNA but not to double-stranded DNA. The Ctf18-RLC contains weak intrinsic ATPase activity that was stimulated by both PCNA and RPA. PCNA alone stimulated only 1.5 fold of ATPase while 9-1-1 complex, clamp loader for DNA damage checkpoint, did not stimulate. In the gel filtration analysis Ctf18-RLC loaded PCNA onto primed and gapped circular DNA in the presence of ATP. The Ctf18-RLC stimulated the DNA synthesis of DNA polymerase δ *in vitro* although the stimulation by Ctf18-RLC was 7-fold less efficient than that of RFC (Bermudez *et al.*, 2003). These results indicate that the Ctf18-RLC can load PCNA onto primed DNA in a similar way as RFC does and stimulate the DNA synthesis of DNA polymerase δ. The loading of PCNA was supported by the proteomic analysis of PCNA binding proteins that Ctf18 was identified as one of the PCNA binding proteins (Ohta *et al.*, 2002).

Elg1 was identified by three different groups. Two groups identified Elg1 as a gene that increases the frequency of Ty elements recombination. Ty elements are the largest family of naturally occurring repeated sequence in yeast. Despite their abundance, Ty elements spontaneously recombine at remarkably low rate. In

addition Ty recombination is not increased by the DNA damaging agents such as MMS and UV. Ben-Aroya *et al.* screened for mutants exhibiting increased frequency of Ty recombination. One of the mutants in which transposon has inserted at nucleotide 260 of ELG1, showed 30 fold increased LTR recombination, five fold increase in Ty recombination and 5 fold elevation in direct-repeat recombination in HIS4 locus. The mutant was named as Elg1 (enhanced level of genomic instability) (Ben-Aroya *et al.*, 2003). In a related study, Kanellis *et al.* generated a gross chromosomal rearrangement (GCR) reporter strain for the characterization of 9 *RTT* genes that were previously isolated but poorly characterized Ty1 regulators. When *RTT101* (encode cullin homologue) or *RTT110* (renamed Elg1) were deleted the mutant strains showed elevated GCR rates (25 or 65 fold respectively) while deletion of other *RTT* genes caused less than 5 fold increase in GCR rate (Kanellis *et al.*, 2003).

In another study, Elg1 was identified from genome-wide synthetic lethal screen with *mus81Δ* and *mms4Δ*. Mus81 and Mms4 are thought to be involved in the processing of stalled replication forks. In order to identify novel genes that function to stabilize replication forks, Bellaoui *et al.* carried out a genome-wide synthetic genetic interaction screen with *MUS81* and *MMS4* deletion strains. ELG1 was one of the seven genes that deletion of the gene showed synthetic lethal phenotype when combined with both *MUS81* and *MMS4* deletion mutation (Bellaoui *et al.*, 2003).

Cells lacking Elg1 showed slow progression through S phase and defects in recovery from MMS induced replication fork stalling. The MMS sensitivity of ELG1 deletion strain was suppressed by the overexpression of PCNA suggesting that Elg1 plays a role in DNA metabolism (Bellaoui *et al.*, 2003, Kanellis *et al.*, 2003). In addition to that, the *elg1* deletion strain showed a synthetic lethal phenotype when combined with the *dna2-4* mutation. Moreover, the minimal permissive temperature or minimal restrictive temperature of the double mutant strains increased or decreased when *elg1Δ* combined with the mutant either the large subunit of DNA polymerase δ, Rfc1, Fen-1 or DNA ligase I suggesting Elg1 plays a role in Okazaki fragment maturation (Kanellis *et al.*, 2003). The genetic and physical interaction between Elg1 and PCNA also suggest the role of Elg1-RLC as a PCNA loader in the stalled replication fork or onto the damaged DNA.

Elg1 is not essential for cell viability and the deletion of *ELG1* does not show defect in the checkpoint. But when the *ELG1* deletion was combined with the deletion of Ctf18 and Rad24, the triple mutant cells showed severe defect in the phosphorylation of Rad53 and sensitive phenotype in response to HU and MMS. These results suggest that like Ctf18, Elg1 plays redundant role in DNA damage checkpoint with Rad24 and Ctf18.

Consistent with the process of identification of Elg1, the *ELG1* deletion strain showed an increased rate of direct-repeat recombination, gene conversion, crossing-over, chromosome loss and sister chromatid recombination (Ben-Aroya *et al.* 2003). The double mutant cells between *ELG1* deletion and the genes involved in the DNA repair show increased rate of direct–repeat recombination. Double mutants cells of *elg1Δ* mutation in genes involved in recombinational repair (*mre11*, *rad52*) form very small colonies and show extremely reduced viability. In the genome wide synthetic lethal screen with Elg1 deletion strain also showed that the double mutants between *elg1Δ* and the Rad52 epistasis group (*rad51*, *rad52*, *rad54*, *rad55* and *rad57*) result in synthetic lethal phenotype. These results strongly suggest that the Elg1 is involved in the recombination pathway (Bellaoui *et al.*, 2003).

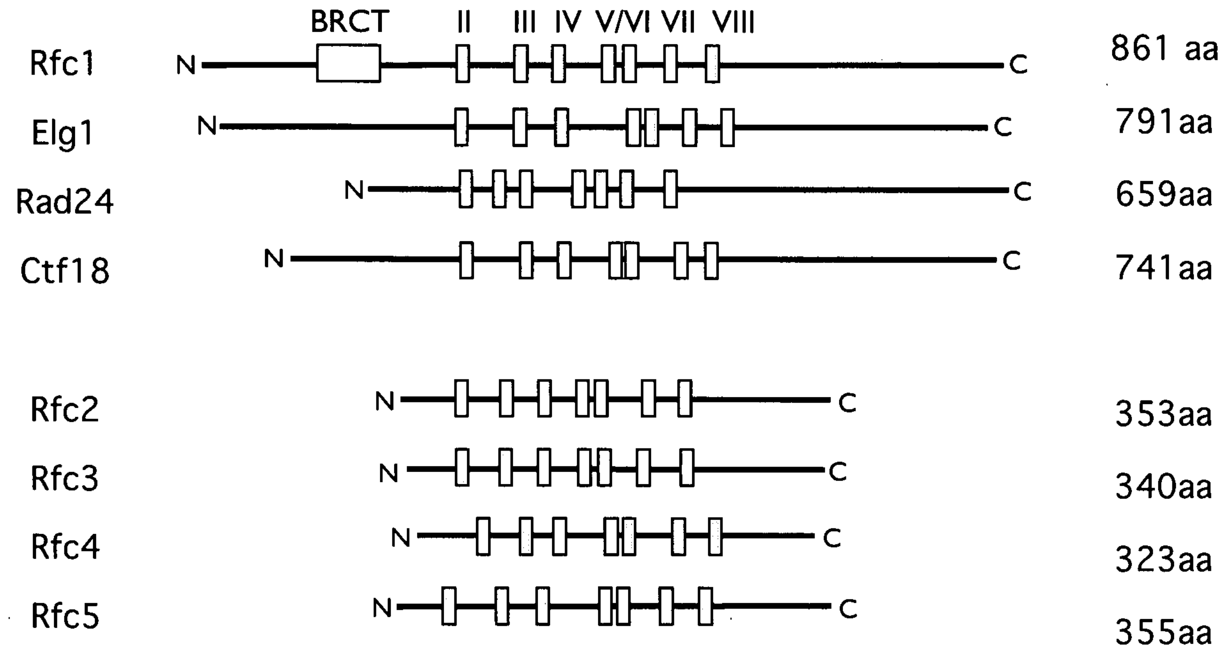


Figure 1.6 The large subunits of RLC share sequence homology with RFC. Rad24, Elg1 and Ctf18 contain well-conserved RFC boxes II-VIII that is found in all RFC subunits. Similar to Rfc1, the large subunit of RFC, the large subunits of RLC have long stretch of amino acids at the C-terminal domain.

1.2.8 RFC in DNA polymerase switching

After the synthesis of RNA/DNA primer by DNA polymerase α/primase complex, the pol α/primase complex is dissociated from the template DNA. The DNA polymerase α/primase is replaced with another polymerase, DNA polymerase δ, which continues and finishes the replication (Tsurimoto and Stillman 1991b, Yuzhakov *et al.*, 1999, Mossi *et al.*, 2000, Maga *et al.*, 2000).

Two protein complexes play essential roles in DNA polymerase switching: RFC and RPA. The involvement of RPA and RFC in DNA polymerase switching was first suggested from the *in vitro* analysis of the activity of DNA polymerase α and DNA polymerase δ. Both RPA and RFC-PCNA showed inhibitory effect on the DNA polymerase α activity while these proteins stimulated the activity of DNA polymerase δ (Tsurimoto and Stillman 1991b) suggesting that the DNA polymerase δ is required for processive synthesis while the DNA polymerase α/primase complex is responsible for short DNA synthesis.

The polymerase switching mechanism was further studied by binding assay of DNA polymerases and accessory proteins on primed circular DNA. In binding assays with various combinations of proteins, it was shown that RFC-PCNA competes with DNA polymerase α for binding to RPA resulting in the displacement of DNA polymerase α from the primer end (Yuzhakov *et al.*, 1999). Similar results were acquired by another group that examined the mechanism of DNA polymerase α displacement from the primer end with RFC and PCNA (Maga *et al.*, 2000).

From the DNA synthesis analysis using a primer that result in different size of product according to the nucleotides added, it was shown that the RFC-PCNA limit the length of primer end synthesized by DNA polymerase α/primase. After the synthesis of certain length (30 mer) of primer, RFC-PCNA switches the DNA polymerase (Mossi *et al.*, 2000).

1.2.9 Archaeal RFC

The characteristics of recombinant RFCs from *Sulfolobus solfataricus* (Pisani *et al.*, 2000), *Methanobacterium thermoautotrophicum ΔH* (Kelman and Hurwitz 2000,), *Pyrococcus furiosus* (Cann *et al.*, 2001), *Archaeoglobus fulgidus* (Seybert *et al.*,

2002) and *Pyrococcus abyssi* (Henneke *et al.*, 2002) have been studied. With the completion of archaeal genome sequencing the archaeal RFCs were identified by the homology searching.

The identified archaeal RFCs are composed of two subunits, large and small. The amino acids sequences of large and small subunit of archaeal RFC showed that the archaeal RFC contains highly conserved RFC box II-VIII. The RFC box I, conserved only in the large subunit of eukaryotic RFC was not found in the large subunit of archaeal RFC. Recombinant archaeal RFC proteins has formed 1 large subunit : 4 small subunits complex in most cases when the stoichiometry was examined by the glycerol gradient or gel filtration column (Cann *et al.*, 2001, Seybert *et al.*, 2002, Henneke *et al.*, 2002). In the case of *M. thermoautotrophicum ΔH*, the large and small subunit formed 2 large subunts : 4 small subunits complex (Kelman and Hurwitz 2000).

The archaeal RFC showed intrinsic ATPase activity that was stimulated by PCNA and DNA. The requirement for the stimulation of intrinsic ATPase of RFC was different from species to species. Although the characteristics of ATPase activity stimulation were different from species to species, all archaeal RFCs interacted with PCNA in DNA dependent manner and RFC and PCNA stimulated the DNA synthesis by DNA polymerase II (or DNA polymerase B) in the presence of ATP. The only exception was RFC and PCNA of *A.fulgidus* which was stimulated the DNA synthesis by DNA polymerases I and II in the absence of ATP (Seybert *et al.*, 2002).

The crystal structure of the small subunit of RFC (RFCS) of *P.furiosus* was solved. The solved crystal contained six RFCS and each subunit folded into three domains to form a "crescent". The N-terminal domain 1 contains canonical nucleotide binding domain and the basic residues of domain 1 assembles to form a remarkable strong positive surface implying its critical role in DNA binding. The overall structure of RFCS was similar with that of *E.coli* δ' and like the δ', RFCS contains nucleotide binding site and sensor 1 and sensor 2 motif. This result suggests that the clamp loading mechanism is evolutionary conserved (Oyama *et al.*, 2001).

The PCNA binding motif that play important role in the interaction with PCNA was found in the C-terminus of the large subunit of RFC in archaea. A peptide of 11

amino acids containing the PCNA binding motif inhibited DNA synthesis by *P.furiosus* polymerase I in the presence of RFC indicating that the PCNA and RFC interact via this motif. The crystal structure of *P.furiosus* PCNA complexed with the PCNA binding peptide has confirmed that the N-terminus of the peptide is connected with the C-terminus of PCNA through hydrogen bonds. The interaction mode of *P.furiosus* PCNA with the peptide containing the PCNA binding motif was remarkably similar with that of human PCNA and a peptide containing PIP-box of $p21^{WAF}$ (Matsumiya *et al.*, 2002).

1.2.10 Rfc1

The large subunit of RFC is a 95-140 kDa protein that is evolutionary conserved. It contains extra N- and C-terminal domain that do not exist in all other small subunits (Cullmann *et al.*, 1995). At its N-terminus Rfc1 contains a consensus PCNA binding motif (Montecucco *et al.*, 1998). Rfc1 contains one more homology domain in its N-terminal domain called BRCT domain. (Bunz *et al.*, 1993, Cullmann *et al.*, 1995). The BRCT domain of human Rfc1 may be involved in the DNA binding activity of Rfc1 (Fotedar *et al.*, 1996, Allen *et al.*, 1998) but *in vitro* domain deletion assays indicate that the large subunit without the BRCT domain could bind DNA (Uhlmann *et al.*, 1997). Moreover, in *S.cerevisiae*, deletion of the N-terminal domain including the BRCT domain did not show any defect with regard to DNA replication, telomere length maintenance, interchromosomal recombination and damage response (Gomes *et al.*, 2000). At the C-terminal domain, Rfc1 contains one more extra domain that does not exist in the small subunits. The function of the C-terminal domain is not yet known. Interestingly, all the large subunits of the RLCs (Rad24, Ctf18 and Elg1) contain extra N- and C-terminal domains that the function is not known.

The most well known function of RFC is in PCNA loading at the replication fork (Tsurimoto and Stillman 1990). In addition to that, several lines of evidence suggest that the small subunit of RFC is also involved in the checkpoint and DNA damage repair pathway (Kim and Brill 2001, Sugimoto *et al.*, 1997, Naiki *et al.*, 2000). Rfc1 is also known to play a role in DNA repair from the study of *cdc44* mutant cells (McAlear *et al.*, 1996). The telomere binding property of Rfc1 (Uchiumi *et al.*, 1996)

and the inhibition of telomerase activity by the C-terminal deleted human Rfc1 (Uchiumi *et al.*, 1999) suggest the involvement of Rfc1 in the telomere length maintenance.

Interactions between Rfc1 and proteins playing important roles in cell cycle regulation have been reported. Rfc1 is a component of the super complex of BRCA1-associated proteins. BRCA1 is a protein that plays important role in the G2/M phase checkpoint and repair. In order to find out the proteins forming complex with BRCA1, the BRCA1-associated proteins were purified from the HeLa nuclear extracts with two different BRCA1 antibodies. Rfc1 was one of the 7 proteins identified. Interestingly, two other proteins were Rfc2 (p37) and Rfc4 (p40). The function of Rfc1 in the BASC complex is not known. Other proteins form complex with BRCA1 were MSH6, MSH2, MLH1, ATM and Rad50 complex that involved in the checkpoint and repair of aberrant DNA suggesting the involvement of Rfc1 in the repair pathway (Wang *et al.*, 2000).

The involvement of Rfc1 in the repair of DNA damage was supported by a study that aimed to understand the role of RFC in response to DNA damage in mammalian cells. The cells carrying ectopic Rfc1 expression construct have shown dramatic effect on promoting cell survival after UV irradiation. In an effort to identify the protein interacting with the Rfc1 in the UV-irradiated cells, Rb (retinoblastoma), cell cycle regulator was identified. The interaction between Rb and Rfc1 is LxCxE motif dependent. The LxCxE motif is conserved in viral oncoproteins and is critical for their binding to Rb. Only Rfc1 in RFC complex contains LxCxE consensus sequence and the promotion of survival by Rfc1 was LxCxE sequence dependent (Pennaneach *et al.*, 2001).

Interactions with the proteins involved in the transcription suggest the function of Rfc1 in transcription regulation. Human Rfc1 was isolated as a protein associating with C/EBPα, a transcription factor, in the screening of C/EBPα interacting proteins. The interaction was confirmed in the MBP-Rfc1 affinity chromatography with the rat liver nuclear extracts that the presence of C/EBPα was checked with the western blot analysis with appropriate antibody. Rfc1 stimulated 6 to 14 fold the transcription activity of C/EBPα when co-expressed in human cell line. The stimulation of transcription was dependent on the binding of Rfc1 to C/EBPα (Hong *et al.*, 2001).

The role of Rfc1 in transcription regulation was supported by the study that Rfc1 showed HDAC1 dependent transcription repression. The idea of interaction between Rfc1 and HDAC1 (histone deacetylase 1) was based on the previous reports that Rfc1 is interacting with BRCA1 and Rb, the proteins known to interact with HDAC1. In order to determine whether Rfc1 associated with HDAC, endogenous Rfc1 was immunoprecipitated from either HeLa or 293 cell lines and the deacetylase activity of the precipitated fractions was examined. The deacetylase activity was associated with the presence of Rfc1. Subsequent immunoprecipitation of Rfc1 from the Rfc1 and HDAC1 over-expression strain revealed that Rfc1 interacts with HDAC1. In the transcriptional activation and repression assay by using GAL4-Rfc1 and the vectors containing either minimal or strong promoters, Rfc1 showed repression of the transcription activity of strong promoter. In the presence of HDAC1 inhibitor, the repression of transcription activity was abolished suggesting that the repression of transcription activity by Rfc1 is HDAC1 dependent (Anderson and Perkins 2002). The interaction of Rfc1 with HDAC1 and HDAC1-dependent repression of transcription suggest the potential role of Rfc1 in the regulation of chromatin structure.

1.3 Introduction to *S.pombe*

1.3.1 Introduction to *S.pombe*

The fission yeast *Schizosaccharomyces pombe* was first isolated from 'an exceedingly over-sulfurized grape juice' in 1921 by A. Osterwalder and was first characterized genetically by Urs Leopold in the late 1940s (Egel 2004). It started to be used in the studies of the cell cycle in 1950's by J.M. Mitchison. *S.pombe* is a unicellular ascomycete that is rod-shaped, grows by apical extension and divides by medial fission. The genome size is 13.8 Mb that is contained in three chromosomes, chromosome I, II, III with size of 5.7, 4.6 and 3.5Mb respectively. The sequencing of genome has been completed. The *S.pombe* genome contains 4,940 predicted open reading frames (Egel 2004).

1.3.2 *S.pombe* life cycle

Under conditions where there is plenty of nutrition, *S.pombe* cells grow and enter mitotic cell cycle (Figure 1.7). In a poor nutrition conditoin, *S.pombe* cells choose alternative cycle dependent on the other condition. When there is only single mating type haploid, cells exit the mitotic cell cycle, arrest growth and wait until the supply of nutrition. If both mating type of haploid are present, the two haploid cells conjugate and form a diploid zygote that can undergo meiosis and sporulation to produce four haploid spores. The spores wait in dormant condition until the re-supply of nutrition. With the re-supply of nutrition, the spores germinate and enter the mitotic cell cycle again. However, if the diploid zygotes are re-supplied with nutrients, the cells enter the mitotic cycle and reproduce as diploid cells. Again, the deprivation of nutrients from the mitotic diploid zygote forces the cell to undergo meiosis and sporulation to form spores (reviewed in MacNeill and Nurse 1997).

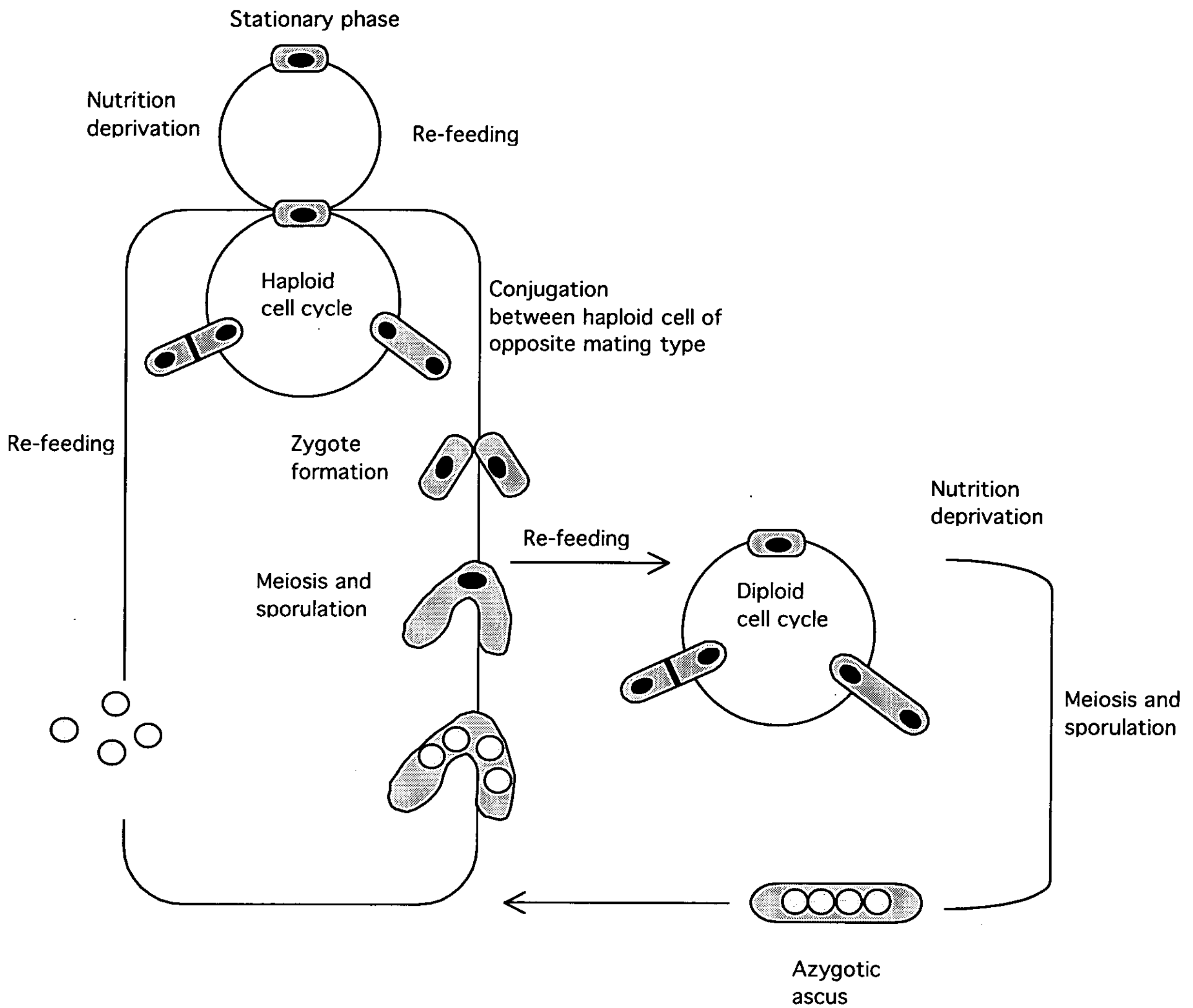


Figure 1.7 Life cycle of the fission yeast *S.pombe.*

1.3.3 *S.pombe* cell cycle

S.pombe undergo a typical eukaryotic cell cycle with distinct G1, S, G2 and M phases. G1, S and M phase is short while the G2 phase is very long. So in exponential culture most of the cells are in their G2 phase. The replication of DNA occurs in a short S phase. The term START is used to indicate the point or period in late G1 when a cell becomes committed to the mitotic cell cycle. To pass through the

START, *S.pombe* cells express many genes involved in the G1/S transition that is regulated by Cdc10 protein. Upon completion of START, cells proceed through late G1 and into S phase. A large number of mutants that show defect in cell cycle progression have been identified in genetic screen. The cell cycle mutant cells continue to grow but do not divide, resulting in a highly elongated phenotype. This phenotype is used as the identification method of cdc (cell division cycle) mutant. In contrast to cdc mutant, the mutant cells showing defect in mitosis do not elongate.

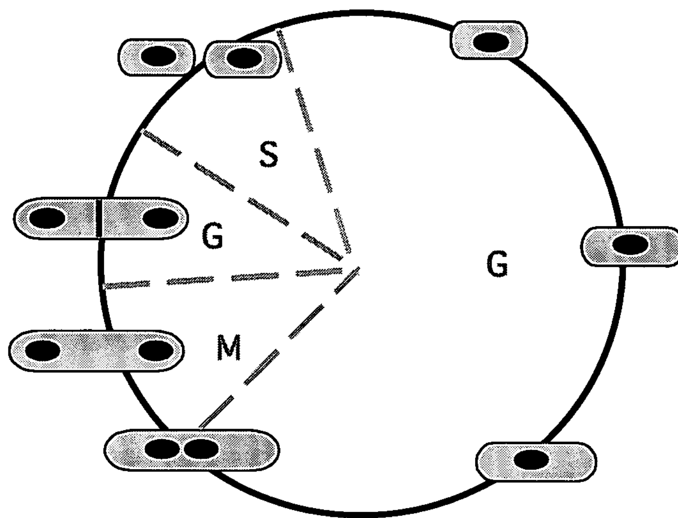


Figure 1.8 Schematic representation of the *S.pombe* cell cycle. The relative duration of the cell cycle and morphological changes are indicated.

1.3.4 Replication factors in *S.pombe*

In *S.pombe*, the DNA replication starts from the activation of ORC that is constitutively bound on chromatin by Cdc2 protein kinase (Leatherwood *et al.*, 1996). ORC is composed of six subunits (Orc1-Orc6) and the DNA binding is mediated by the N-terminal domain of Orc4 that binds on A/T-rich DNA region through the repeat sequences (Moon *et al.*, 1999). Orc2 interact with Cdc18 and

recruits it onto the replication origin (Leatherwood *et al.*, 1996). In addition to Cdc18, *S.pombe* homologue of Cdc6 (Muzi-Falconi *et al.*, 1996), Cdt1, a replication factor whose expression is regulated by Cdc10 transcription factor, is recruited on chromatin (Hofmann and Beach 1994). *S.pombe* MCM is composed of six subunits (Miyake *et al.*, 1993, Takahashi *et al.*, 1994, Sherman *et al.*, 1998, Sherman and Forsburg 1998, Coxon *et al.*, 1992) and the recruitment of MCM complex at the replication fork is mediated by the interaction with Cdc18 and Cdt1.

Once MCM proteins are loaded onto the DNA at the replication origin the cell is 'licensed' for replication. Activation of the origin from a prereplicative to replicative state is dependent on CDK and Hsk1/Dfp1 kinase, *S.pombe* homologue of Cdc7/Dbf4 (Brown and Kelly 1998, Masai *et al.*, 1995). Licensed DNA replication machinery recruits the Sna41 protein, *S.pombe* Cdc45, on the replication origin through interaction with MCM (Miyake and Yamashita 1998). Through the interaction with Sna41, both RPA (the large subunit of *S.pombe* RPA is encoded in *rad11*$^{+}$), (Parker *et al.*, 1997) and DNA polymerase α /primase complex are recruited on the replication fork. In *S.pombe*, the DNA polymerase α /primase complex is composed of four subunits called Pol1, Spb70, Spp1 and Spp2 (D'Urso and Nurse 1995, Tan and Wang 2000). The DNA polymerase α /primase complex then synthesises short RNA/DNA primer for the duplication of DNA.

Short RNA/DNA primer is extended by DNA polymerase δ (Pignede *et al.*, 1991, MacNeill *et al.*, 1996, Reynolds *et al.*, 1998) that is composed of Pol3, Cdc1, Cdc27 and Cdm1. For the processivity of DNA polymerase δ, *S.pombe* requires two accessory proteins. The first protein is PCNA that is encoded in *pcn1*$^{+}$. PCNA encircles the double-stranded DNA and interacts with DNA polymerase δ resulting in the tethering of the DNA polymerase δ on DNA. For the loading of PCNA onto DNA the second accessory protein, RFC is required. There is a possibility that the leading strand synthesis is performed by DNA polymerase ε comprising Cdc20 (D'Urso and Nurse 1997), Dpb2 (Feng *et al.*, 2003), Dpb3 and Dpb4 (Spiga and D'Urso 2004) while lagging strand synthesis is performed by the DNA polymerase δ.

For the maturation of lagging strand, *S.pombe* requires Rad2, Dna2 and Cdc17. Rad2 is *S.pombe* homologue of Fen-1 that removes the RNA primer from the Okazaki fragment (Jacquier *et al.*, 1992). The DNA primer that is left from Rad2 excision is cleaved by Dna2 endonuclease (Gould *et al.*, 1998) and the Okazaki fragment is ligated by DNA ligase I that is encoded in *cdc17*$^+$ (Johnston *et al.*, 1986).

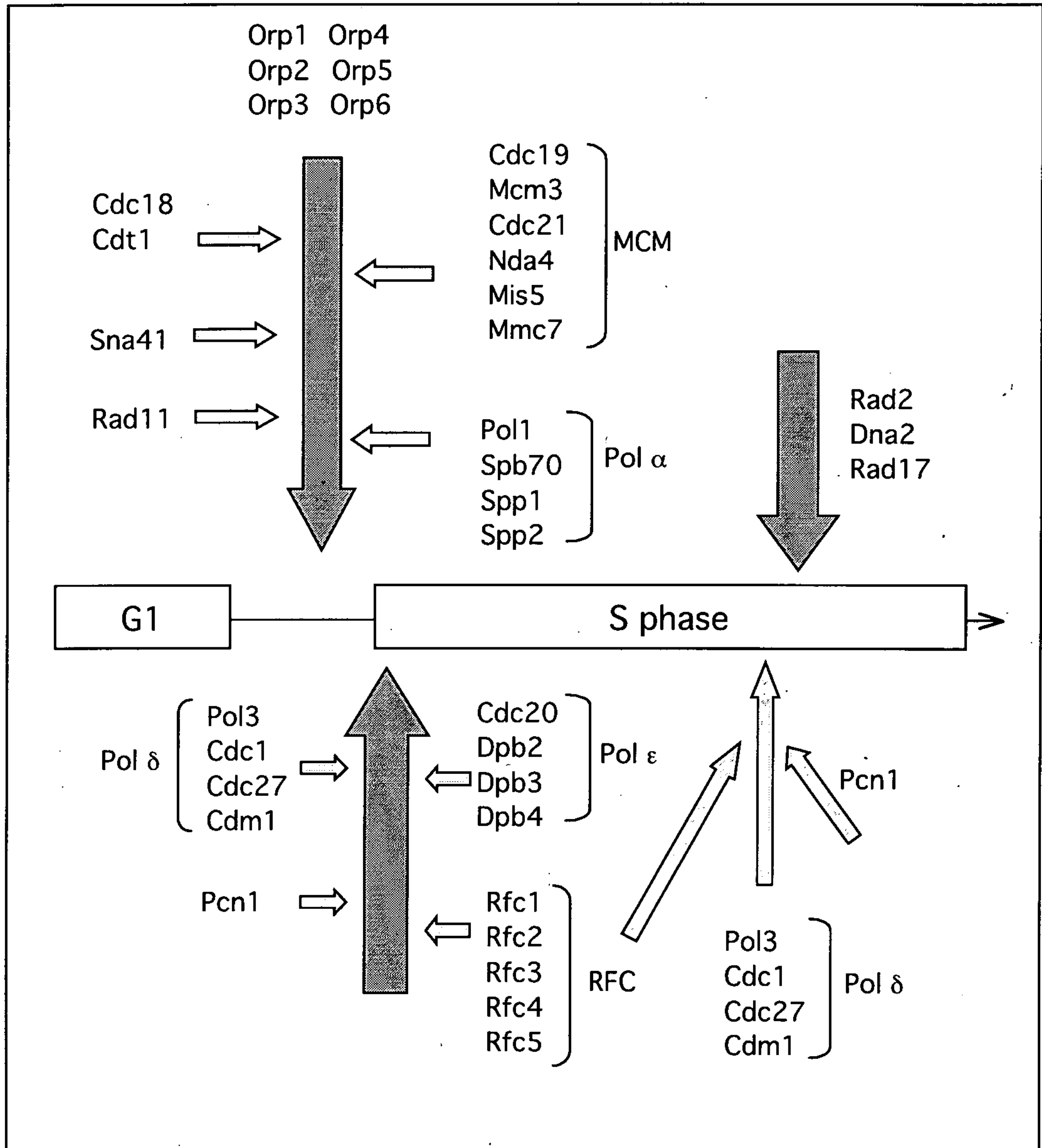


Figure 1.9 Schematic representation of *S.pombe* replication factors in S phase. The cell cycle progression is indicated. Large black arrow was used to indicate the cell cycle progression and small white arrows are used to indicate the order of protein role in the DNA replication. The DNA polymerases δ and accessory factors are indicated again in the middle of S phase to explain its role in Okazaki fragment maturation.

1.4 Aim of this work

This study initially aimed to examine the functions of Rfc1-C terminal domain in *S.pombe*. In particular, whether the C-terminal domain of Rfc1 is essential for cell viability and the effects of mutational analysis in the C-terminal domain is examined (Chapter 2). The characteristics of the mutant cells are examined using biochemical and genetic methods (Chapter3). As an effort to understand the role of Rfc1 C-terminal domain, suppressors for the mutant phenotype are screened (Chapter 5). Using *rfc1* mutant cells, the roles of three clamp loaders (RFC, Ctf18-RLC and Elg1-RLC) were also investigated (Chapter 4).

Chapter 2. C-Terminal domain of Rfc1 is essential for cell viability

2.1 Introduction

Like the small subunits, the large subunit of RFC contains well-conserved amino acids sequences called RFC boxes (see section 1.2.3). In addition to these, Rfc1 contains extended N- and C-terminal regions that are not found in the small subunits. The PCNA binding motif and BRCT domain are found in the N-terminal domain of Rfc1. Biochemical data suggest that the PCNA interaction takes place through the well-conserved RFC boxes II-VIII (Fotedar *et al.*, 1996) while the DNA binding occurs through the BRCT domain (Fotedar *et al.*, 1996, Allen *et al.*, 1998). However, in *S. cerevisiae*, the N-terminal domain is non-essential: deletion of amino acid 3-277 resulted in no apparent differences in comparison to wild-type Rfc1 with regard to DNA replication, damage susceptibility, telomere length maintenance and interchromosomal recombination (Gomes and Burgers 2001).

Extended C-terminal regions are also found in the large subunit of RFC-like complexes (RLC), Ctf18, Rad17 and Elg1. This extended C-terminal domain is evolutionary conserved. The conservation of the C-terminal domain in Rfc1 across species and within the large subunit of RLCs, suggests that the C-terminal domain possesses functions essential for the role of RFC or RLCs in DNA metabolism.

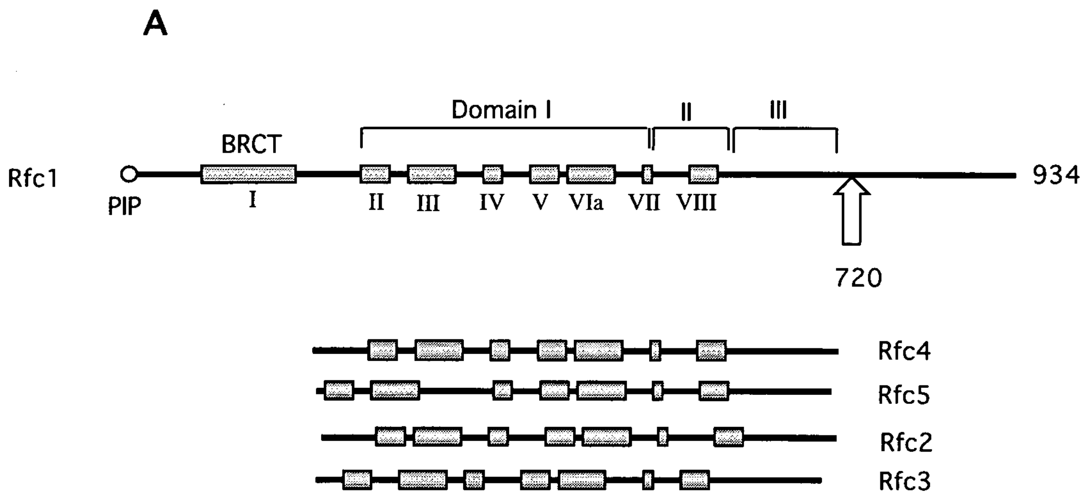
In this chapter, the role of the C-terminal domain in the function of RFC is examined in *S.pombe*. In an effort to understand this role, cells harbouring a mutation in the C-terminal domain were generated and their phenotypes examined.

2.2 C-terminal domain of Rfc1 is essential for cell viability

In order to know the function of C-terminal domain of Rfc1, the amino acids corresponding to C-terminal domain was determined by the sequence alignment of RFC subunits (Figure 2.1). The C-terminal domain was considered to begin at Ala720 because it lies immediately outside of any sequence overlap with the smaller subunits and because it follows after a Pro719 which may function as a molecular hinge. The sequences are also outside of the domain III, the domain forms 'collar" in association with the corresponding domain of Rfc2-Rfc5 in RFC complex (Bowman *et al.*, 2004, Guenther *et al.*, 1997). Sequences alignment of *S.pombe* and *S.cerevisiae* Rfc1 revealed that the selected domains included the Rfc1-Rfc5 interacting regions (Gregory Bowman, personal communication). Conserved RFC boxes are illustrated in Figure 2.1 and the position of Ala720 is indicated by arrow.

To examine the requirement of Rfc1 C-terminal domain for cell viability, three consecutive stop codons were inserted at the starting point of the C-terminal region by PCR-based site-directed mutagenesis (see Materials and Methods). The *rfc1* gene containing the mutation was subcloned into a vector containing a $Ura4^+$ auxotroph marker and transformed into diploid *S.pombe* cells (*leu1-32/leu1-32 ura4-D18/ura4-D18 ade6-M210/ade6-M216* h^+/h^-). These cells were then patched on YE medium and transferred onto EMM plates to isolate stable integrants. The cells harbouring mutant *rfc1* gene containing the stop codons were identified by sequencing of the *rfc1* C-terminal region after amplification by PCR. PCR was performed with primers that amplify the genomic *rfc1* sequence containing Rfc1 C-terminal domain. The cells were then patched on ME plates and incubated for 2 days at 28°C to allow sporulation to take place. Asci were separated by micromanipulator and incubated at 28°C until colonies formed.

A



B

```
                                                   CTD
Sp 678 LELISSAANSFSDSDLVDSMIHGPQQHWSLMPTHALMSCVRPASFVAGSGSRQIRFTNWLGNNSKTNKLYRMLREIQVHM
Sc 612 -EAVAEAANCISLGDIVEKKIRSSEQLWSLLPLHAVLSSVYPASKVAGHMAGRINFTAWLGQNSKSAKYYRLLQEIHYHT
                                                                            rfc1-44

Sp 758 RLKVSANKLDLRQHYIPILYESLPVKLSTGHSDVVPEIIELMDEYYLNREDFDS ITELVLPADAGEKLMKTIPTAAKSAF
Sc 691 RLGTSTDKIGLRLDYLPTFRKRLLDPFLKQGADAISSVIEVMDDYYLTKEDWDS IMEFFVGPDVTTAIIKKIPATVKSGF
```

Figure 2.1 The comparison of the amino acid sequences of RFC subunits. **A**. The highly conserved RFC boxes are indicated as a box. The starting point for the C-terminal domain of Rfc1 is indicated by arrow. Three structural domains that are important for the RFC complex formation are indicated above the Rfc1 protein. **B**. Sequence alignment of *S.pombe* and *S.cerevisiae* Rfc1 C-terminal region. The starting point of C-terminal domain is indicated by arrow. The position of *rfc1-44* pentapeptide insertion is indicated by inverted triangle (see section 2.3.2). Amino acids involved in the Rfc1-Rfc5 interaction are boxed.

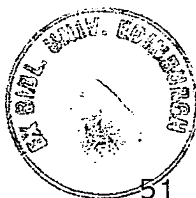
Many spores were unable to germinate and did not form colonies. Among those tetrads which did form colonies, half of the asci (14/28) formed only two colonies (Figure 2.2 Lanes 1, 3, 5, 7 and 8). Ten of the asci formed one viable and three non-viable colonies (Figure 2.2 Lanes 2, 6 and 9). Two of the asci did not form any colony although the spores have germinated (Figure 2.2 Lane 4). One ascus formed three colonies. The results of the tetrad analysis are summarized in Table 2.1.

Table2.1. The phenotype of spores from *rfc1⁺/rfc1-ΔC*. The phenotype represents the phenotypes of asci carrying Rfc1 C-terminal domain deletion construct. It represents the numbers of viable : non-viable cells / four spores.

Phenotype	2 : 2	1 : 3	0 : 4	3: 1
Number of asci	14	10	2	1

The germinated spores that did not form colonies showed highly elongated phenotype under the microscope. The cells had undergone one to five rounds of division and some of the cells showed a branched and sick phenotype. The colonies formed were replica plated on an EMM plate that did not contain uracil. None of them was able to grow suggesting that the colonies contained wild-type *rfc1*. None of the cells from three viable spores was able to grow in the uracil lacking plate indicating that all these colonies contain wild-type *rfc1* (Figure 2.2).

In order to confirm that the lethal phenotype was caused by the deletion of the C-terminal domain, a plasmid containing either wild-type Rfc1 (pREP1-Rfc1) or control vector (pREP3XH6) was transformed into the mutant *S.pombe* cells (*rfc1*$^{+}$*/rfc1-ΔC leu1-32/leu1-32 ura4-D18/ura4-D18 ade6-M210/ade6-M216h*$^{-}$*/h*$^{+}$) where mutant *rfc1* was inserted onto the chromosome. Cells were sporulated, treated with helicase (*Helix pomatia* juice) to disrupt the ascus wall and plated on minimal media containing appropriate nutritional markers.



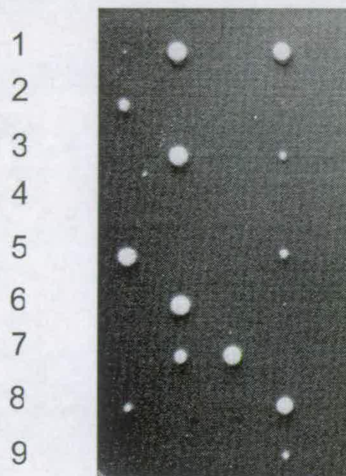


Figure 2.2 The C-terminal domain of Rfc1 is essential for cell viability. The plasmid containing stop-codon inserted Rfc1 was inserted into chromosome of diploid cells by linearizing with *NcoI* enzyme and transforming into the cells. The cells are incubated for 2 days at 28°C on malt extract plate. The asci were dissected by micromanipulator.

On the EMM plate containing adenine, leucine and uracil, thousands of colonies were formed in both wild-type *rfc1*$^+$ and vector transformed cells. However, in EMM medium which was supplemented with adenine only, the mutant cells carrying wild-type *rfc1*$^+$ formed about 500 colonies while the mutant cells carrying control vector formed only a few colonies (Figure 2.3). There were some large colonies formed on all plates regardless of which nutritional marker was present. These grew better under all conditions and formed spores when incubated more than 4 days. Most of the cells could grow in the absence of adenine and the cell size was larger than that of the cells from small colonies when examined under the microscope. These facts indicated that these large colonies were diploid cells. The several colonies formed on the EMM + adenine plate containing the *rfc1-ΔC* mutant cells carrying control vector

showed the same phenotype as the larger colonies indicating that these too are diploid.

The restoration of viability in the *rfc1-ΔC* mutant cells by wild-type *rfc1*$^{+}$ indicates that the lethal phenotype of the Rfc1 mutant indeed comes from the deletion of the C-terminal domain of Rfc1. The viability of the *rfc1-ΔC* mutant was restored when transformed with a plasmid expressing Cdc44 (pREP3X-Cdc44), the *S.cerevisiae* homologue of Rfc1 (data not shown), suggesting that *S.pombe* and *S.cerevisiae* Rfc1 are functionally similar. These results show that the C-terminal domain of Rfc1 is essential for the viability of *S.pombe* cells.

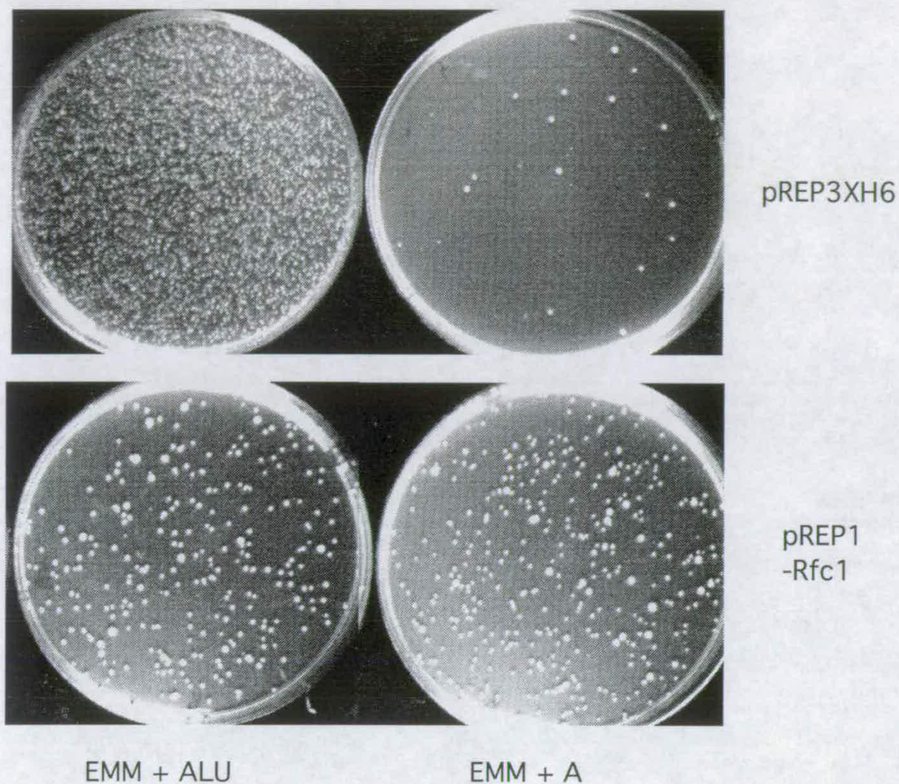


Figure 2.3 *rfc1* mutant cells in which one wild-type Rfc1 gene is replaced with *rfc1-ΔC* and the strain transformed with either pREP3XH6 or pREP1-Rfc1. The transformants were patched on ME plate and incubated for 2 days at 28°C. A loopful of cells were resuspended in 500μl of DW and 10μl of 10 fold diluted Helicase were added. The cells were incubated overnight at 28°C. The spores were plated on either EMM+ adenine or EMM+adenine, leucine and uracil plates and incubated for 3 days at 32°C.

2.3 Mutational analysis of the Rfc1 C-terminal domain

2.3.1 Strategy for mutagenesis

Tetrad analysis and random spore analysis showed that the C-terminal domain of Rfc1 is essential for viability. In an effort to understand more fully the role of the C-terminal domain of Rfc1, a series of mutations were generated in the domain by both random and site-directed mutagenesis. Fasta33 (Pearson and Lipman 1988) protein sequence alignment software (http://www.ebi.ac.uk/fasta33) was used in order to reveal the regions displaying high homology across species in the Rfc1 C-terminal domain. There were amino acids that are highly conserved across species were replaced with alanine (Figure 2.5). To facilitate the homologous recombination and integration of the plasmid into the chromosome, the DNA encoding amino acids 590-719 as well as the C-terminal domain of Rfc1 was subcloned into the backbone vector (Figure 2.5). Point mutants of *rfc1*$^{+}$ were constructed by PCR overlap extension mutagenesis (see Materials and Methods).

The transposon based pentapeptide insertion method (Hallet *et al.*, 1997, Hayes *et al.*, 1997) was used to generate random mutations in Rfc1 C-terminal domain. Transposons are discrete sequences in the genome that are able to transpose to other locations within the genome. During integration, the transposon cleaves the target sequence at a specific point leaving staggered ends in the cleaved DNA. The transposon efficiently inserts between protruding single-stranded ends generated by the staggered cuts. The single stranded regions are filled after the integration generating repeat sequences in the target gene.

Transposon Tn4430Ω has been used to insert random pentapeptide in target proteins (Hallet *et al.*, 1997, Hayes *et al.*, 1997). The Tn4430Ω transposon is a Tn3 related transposon carrying a kanamycin resistant gene. During integration into the target gene, Tn4430Ω duplicates five nucleotides of the target gene. Tn4430Ω also contains two *KpnI* restriction enzyme sites 5 bp from the outer end of its terminal inverted repeats. The *KpnI* restriction enzyme digestion and re-ligation of the plasmid containing the transposon results in a 15 bp insertion in the target gene.

A plasmid encoding the Rfc1 C-terminal domain was transformed into FH1046 *E.coli* strain that carried the Tn4430Ω transposon. The transformants were mated

with DS941 recipient cells to select for the plasmid which contains integrated transposons. These plasmids were purified from DS941 cells and digested with *KpnI* restriction enzyme to remove the transposon. The plasmid was then re-ligated and transformed into DH5α *E.coli* cells. The mutated DNA by transposon was amplified and the position and sequence of inserted nucleotides were examined by DNA sequencing. The transposon-based pentapeptide insertion method is summarised in Figure 2.4.

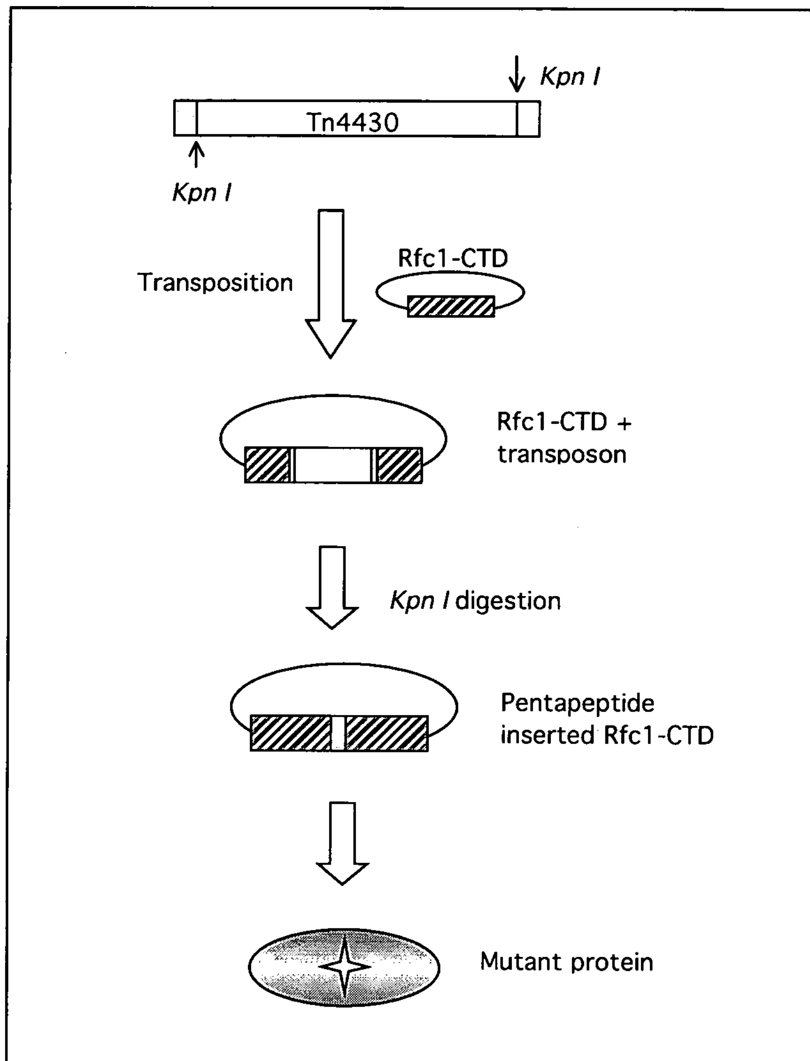


Figure 2.4 Schematic diagram of pentapeptide insertion mutagenesis. The plasmid carrying Rfc1-CTD was transformed in *E.coli* cells containing Tn4430Ω transposon to render the integration. After the isolation of transposon-integrated plasmid, transposon was removed by digestion of plasmid with *Kpn I* restriction enzyme and re-ligation.

2.3.2 Sequence alignment and the site of mutation

Fasta33 sequence alignment program was used to align the C-terminal domain of Rfc1 from various species. The C-terminal domain of Rfc1 shows high percentage of charged amino acids either positive or negative that are highly conserved across the species. The aligned sequences showed a tendency that the middle and N-terminal part of C-terminal domain show homology across the species while the C-terminal part does not. The first amino acid that was changed to Ala is Asn739 that is Lys in most of other species but Asn in *S.pombe* and Gln in *S.cerevisiae.* All other amino acids were highly conserved in all species examined.

The insertion of pentapeptide by transposon insertion took place at random positions throughout the C-terminal domain and the additional sequence upstream of the C-terminal domain (amino acids 590-719). Seventeen different DNAs were purified from *E.coli* cells and the insertion of transposon into the plasmid was checked. Of those three plasmids contained an insertion of same sequences and two plasmids contained identical mutations. Five mutations lay within the upstream sequences of the Rfc1 C-terminal domain and seven mutations lay within the Rfc1 C-terminal domain itself. The sequence alignment of Rfc1 C-terminal domains and the positions of both point and pentapeptide insertion mutations are summarized in Figure 2.5

```
        45            48                49              53                   47
Sp  597 NSQA VIKNSEKHIVMKPWDICSRYL HGGMFHPSSKSTINDK LELYFNDHEFSY LMVQENYLNTTPDRIRQEPPKMSHLK H
Sc      NINE ISKAWEKNIALKPFDIAHKML DGQIYSdsRNFTLNDK IALYFDDFDFTP LMIQENYLSTRPSVLK---PGQSHL- -
Hs      QAKA DSHRAKKDIKMGPFDVARKVF AAG--EETAHMSLVDK SDLFFHDYSIAP LFVQENYIHVKPVAAGGD---MK--K H
Mm      QAKA DSQRAKKDIRLGPFDVTRKVF AAG--EETAHMSLMDK SDLFFHDYSIAP LFVQENYLHVKPVAAGGD---MK--K H
Dm      KSGQ QV--ATKDLKLGPWEVVRKVF TADEHKHMS---FADK SDLFFHDYSLAP LFVQQNYLQVLPQGNKKDV------- -

                                                      CTD →       33    34 35    43
Sp  678 LELISSAANSFSDSDLVDSMIHGPQQHWSLMPTHALMSCVRPASFVAGSGSRQIRFTNWLGNNSKTNKLYRMLREI QVHM
Sc      -EAVAEAANCISLGDIVEKKIRSSEQLWSLLPLHAVLSSVYPASKVAGHMAGRINFTAWLGQNSKSAKYYRLLQEI HYHT
Hs      LMLLSRAADSICDGDLVDSQIRS-KQNWSLLPAQAIYASVLPGELMRGYMTQFPTFPSWLGKHSSTGKHDRIVQDL ALHM
Mm      LMLLSRAADSICDGDLVDNQIRS-KQNWSLLPTQAIYASVLPGELMRGYMTQFPSFPSWLGKHSSTGKHDRIVQDL SLHM
Dm      LAKVAATADALSLGDLVEKRIRA-NSAWSLLPTQAFFSSVLPGEHMCGHFTGQINFPGWLGKNSKSGKRARLAQEL HDHT

                              54                    36 37 44 38                    39
Sp  758 RLKVSANKLDLRQHYIPILYESL PVKLSTGHSDVVPEIIELMDEYYLNREDFDS ITELVLPADAGEKLMKTIPTAAKSAF
Sc      RLGTSTDKIGLRLDYLPTFRKRL LDPFLKQGADAISSVIEVMDDYYLTKEDWDS IMEFFVGPDVTTAIIKKIPATVKSGF
Hs      SLRTYSSKRTVNMDYLSLLRDAL VQPLTSQGVDGVQDVVALMDTYYLMKEDFEN IMEISSWGGKPSPFSKLDPKV-KAAF
Mm      SLRTYSSKRTVNMDYLSHIRDAL VRPLTSQGVEGAQHVIKLMDTYYLMKEDFEN IMEVSSWGGKPSAFSKLDPKV-KAAF
Dm      RVCTSGSRLSVRLDYAPFLLDNI VRPLAKDGQEGVPAALDVMKDYHLLREDLDS LVELT--SWPGKKslDAVDGRVKAAL

        40 41           46     51          50           52
Sp  838 TRKYNSSSHPIAFFG SSDVLP MKGSAQREVPD VEDAIEAEDEM LEEASDSEAANEEDIDLSKDKFISVPKKPKKRTKAKA
Sc      TRKYNSMTHPVAIYR TGSTIG GGggTSTSTPD FEDVVDADDNP VP--ADDEETQDSSTDLKKDKLIKQKAKPTKR---KT
Hs      TRAYNKEAhpYAL-- ----QA IKASRHSTSPS LDS--EYNEEL NEDDSQSDeaIETDAMIKKKTKSSKPSKPEKDKEPRK
Mm      TRAYNKEAHLTPY-- SLQVV- -KTSRLSTGPA LDS--EYSEEF QED--DTQSEKEQDA-VETDAMIKKKTRSSKPSKSER
Dm      TRSYNKEVMAYSYSA QAGIKK KKSEAAGADDD YLDEGPGE--- -EDGAGGHLSSEEDEDKDNLELDSLIKAKKRTTTSKA

Sp  918 EASSSSSTSRRSRKKTA
Sc      ATSKPGGSKKRKTKA--
Hs      GKGKSSKK---------
Mm      EKESKKGKGKNWKK---
Dm      SGGSKKATSSTASKSKA
```

★ Point mutation

▼ Penta-peptide insertion

Figure 2.5 Mutagenesis within the Rfc1 C-terminal domain. The amino acids in chromosome integration constructs are aligned with the same regions of Rfc1 from various eukaryotes. Point mutated amino acids are indicated by star. The poistion of penta-peptide insertion are indicaed by an inverted triangle.

2.3.3 Results of tetrad analysis

Plasmid DNA containing either a point mutation or a penta-peptide insertion mutation was transformed into a diploid strain (*leu1-32/leu1-32 ura4-D18/ura4-D18 ade6-M210/ade6-M216* h^-/h^+) which had been prepared by mating two haploid cells Sp292 (*leu1-32 ura4-D18 ade6-M210* h^-) and Sp295 (*leu1-32 ura4-D18 ade6-M216* h^+). The integration of the mutation into the chromosome was checked by PCR of *rfc1* with primer sets in which one primer binds to the plasmid vector and the other primer binds to the upstream sequence of the subcloned DNA. The integration was confirmed by sequencing the DNA acquired from the PCR. The mutant cells were patched on an ME plate and the formation of spores was checked under the microscope. The asci were separated by micromanipulator on YE plates and spores were incubated at 32°C until colony formation had occurred. In order to identify genotype of each colonies, the colonies formed were replica-plated on EMM + adenine + leucine plate.

Most of the asci from mutant cells formed four viable colonies suggesting that the mutant cells are viable. Spores from both *rfc1-43* mutant and *rfc1-44* mutant formed only two colonies. Three asci from *rfc1-54* mutant formed four viable colonies, eleven asci formed only two colonies and three asci formed three viable colonies. Two colonies from four viable cells were tiny while the other two were normal size. Tiny colonies were also formed in the asci showing two or three viable cells. In order to know whether the mutant cells show temperature-sensitive phenotype, the tetrad was separated and incubated at 25°C. Spores from *rfc1-43* mutant still formed only two colonies. Spores from *rfc1-44* mutant and *rfc1-54* mutant formed four viable spores at 25°C.

Spores from *rfc1-35, rfc1-40* and *rfc1-41* mutants formed two viable colonies suggesting that the mutant cells show lethal phenotype. However, one of them or both of the colonies grew well on the EMM + adenine + leucine plate indicating that the mutant cells are viable.

At least 36 asci were dissected for the analysis of each mutant phenotype. Four viable spores were formed and two of them could survive in the EMM + adenine + leucine plate suggesting that the mutant cells are viable. The results of tetrad analysis

are summarized in Table 2.2

Table 2.2. The phenotype of mutations. Three-letter code was used to indicate the position of amino acids that was changed. Single-letter code was used to indicate the amino acids that are inserted.

Mutant	Mutation	Phenotype
Point mutation		
rfc1-33	Asp739Ala	Viable
rfc1-34	Lys745Ala	Viable
rfc1-35	Arg748Ala	Viable
rfc1-36	Glu807Ala	Viable
rfc1-37	Asp808Ala	Viable
rfc1-38	Glu814Ala	Viable
rfc1-39	Lys834Ala	Viable
rfc1-40	Arg839Ala	Viable
rfc1-41	Asp842Ala	Viable
Penta-peptide insertion		
rfc1-43	Ile753QGVPLGln754	Lethal
rfc1-44	Ser811IRGTPIle812	Temperature-sensitive
rfc1-45	Ala601VRGTPVal602	Viable
rfc1-46	Gly852SRGTPSer853	Viable
rfc1-47	Lys677HLGAPLeu678	Viable
rfc1-48	Leu622HGGVPHis623	Viable
rfc1-49	Lys638LGVPQLeu639	Viable
rfc1-50	Glu870VGVPHVal871	Viable
rfc1-51	Pro858MKGYPMet859	Viable
rfc1-52	Met880LGVPLLeu881	Viable
rfc1-53	Tyr650LMGVPLeu651	Viable
rfc1-54	Leu780PVGVPPro781	Temperature-sensitive

In order to examine the defects of viable mutant cells more carefully, the cells were plated on YE plates after 10-fold serial dilution. The plates were then incubated at

18°C, 25°C or 35°C until colonies had formed. To test sensitivity to DNA damaging agents, the viable mutant cells were spotted on YE plate containing 5, 7.5 or 10mM hydroxyurea (HU), 0.0025, 0.005 or 0.01% methyl methansulfonate (MMS) or 6, 6.5 or 7μM camptothecin (CPT) and incubated at 25°C. To test the effect of UV irradiation, cells were spotted on YE plates and irradiated with 100, 200, 300, 400 or 500 J/m^2 of UV and incubated at 25°C in the dark.

Cells carrying most of the point mutation did not show any defect in cell viability at both high and low temperature or sensitivity to DNA damaging agents. *rfc1-33* mutant cells grew well at high temperature but showed a weak cold-sensitive phenotype. They also showed increased sensitivity to 0.01% MMS, 7.5mM of HU and 6μM of CPT.

rfc1-46 mutant cells showed a weak cold-sensitive phenotype and growth defects in the presence of 0.01% MMS, 10mM HU and 6.5μM CPT. *rfc1-49* mutant showed growth defects in the presence of 0.005% MMS and 7.5mm HU. Both *rfc1-52* and *rfc1*-53 showed growth defects in the presence of 0.01% MMS and 10mM HU. The characteristics of each mutant cells are summarized in Table 2.3.

In summary, with regard to the point mutations, only *rfc1-33* showed a weak cold-sensitive phenotype and sensitivity to DNA damaging agents. For the penta-peptide insertion mutations one lethal, two temperature-sensitive and four drug-sensitive strains were isolated.

Table 2.3 Phenotypes of mutants. Three-letter code was used to indicate the position of amino acids that was changed. Single-letter code was used to indicate the amino acids that are inserted

Mutant	35°C	18°C	UV	MMS	HU	CPT
Point mutation						
rfc1-33	+++	++	+++	++	+	-
rfc1-34	+++	+++	+++	+++	+++	+++
rfc1-35	+++	+++	+++	+++	+++	+++
rfc1-36	+++	+++	+++	+++	+++	+++
rfc1-37	+++	+++	+++	+++	+++	+++
rfc1-38	+++	+++	+++	+++	+++	+++
rfc1-39	+++	+++	+++	+++	+++	+++
rfc1-40	+++	+++	+++	+++	+++	+++
rfc1-41	+++	+++	+++	+++	+++	+++
Pentapeptide insertion						
rfc1-43	lethal					
rfc1-44	-	+++	-	-	-	+++
rfc1-45	+++	+++	+++	+++	+++	+++
rfc1-46	+++	++	+++	++	++	+
rfc1-47	+++	+++	+++	+++	+++	+++
rfc1-48	+++	+++	+++	+++	+++	+++
rfc1-49	+++	+++	+++	+	+	+++
rfc1-50	+++	+++	+++	+++	+++	+++
rfc1-51	+++	+++	+++	+++	+++	+++
rfc1-52	+++	+++	+++	++	++	+++
rfc1-53	+++	+++	+++	++	++	+++
rfc1-54	-	++	-	-	-	++

2.4 Characterization of two temperature-sensitive mutants

Temperature-sensitive mutant strains can be a useful genetic tool and in this case can be used for examination of the function of the Rfc1 C-terminal domain. The characteristics of two temperature-sensitive mutants *rfc1-44* and *rfc1-54* were examined by spotting assay (Figure 2.6). *rfc1-44* did not show any difference in growth at 18°C and 25°C when compared with wild-type cells. However, at 35°C the cells showed severe growth defects and did not form colonies. When *rfc1-44* was spotted on a YE plate containing DNA damaging agents it exhibited growth defects in the presence of 5mM HU and 0.005% MMS while the wild-type cells grew normally. *rfc1-44* growth was highly sensitive to 300 J/m^2 of UV irradiation while wild-type cells remained unaffected. Neither wild-type cells nor *rfc1-44* was affected by the presence of 10μM of CPT. *rfc1-54* did not grow well at either 18°C or 35°C. In the presence of 5mM HU and 0.005% MMS, *rfc1-54* showed more severe growth defects than *rfc1-44* and in contrast to *rfc1-44, rfc1-54* showed a weak sensitivity at 10μM CPT. *rfc1-54* showed a highly sensitive phenotype to UV irradiation such that almost all the cells were dead after irradiation with 300 J/m^2 of UV.

In summary, *rfc1-44* showed tighter temperature-sensitive phenotype while *rfc1-54* showed both cold-sensitive and temperature-sensitive phenotype. *rfc1-54* also showed more severe defects in response to DNA damaging agents than *rfc1-44*.

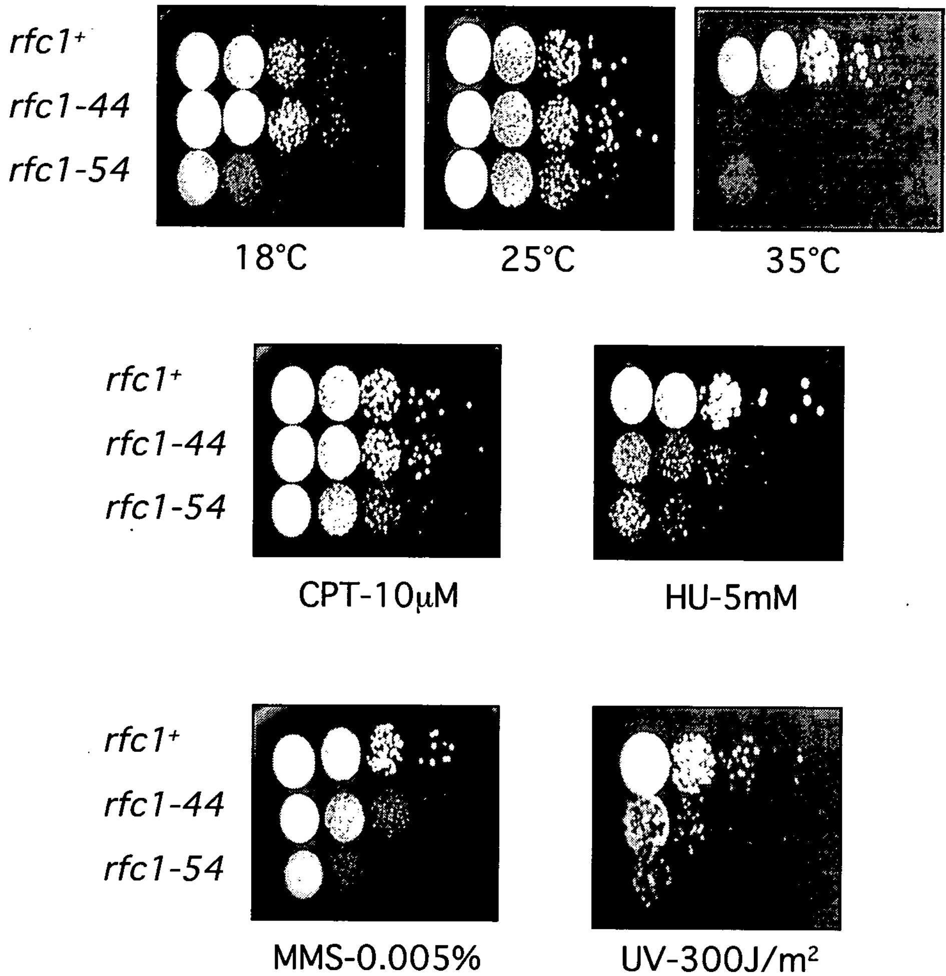


Figure 2.6 Characteristics of *rfc1-44* and *rfc1-54*. The mutant cells were serially diluted ten-fold and spotted on YE plate and incubated at 18°C, 25°C or 35°C. To test UV sensitivity, 1 x 10^5 to 10 cells were spotted on YE and were irradiated with 300J/m^2 by UV-cross linker and incubated at 25°C in the dark. To test sensitivity to DNA damaging agents, cells were spotted on YE containing 5mM HU, 0.005% MMS or 10μM of CTP and incubated at 25°C.

2.5 Discussion

The role of the C-terminal domain of Rfc1 in cell viability was examined by tetrad analysis of asci from cells in which two of the four spores contained C-terminal deleted Rfc1. The lethal phenotype of *rfc1-ΔC* mutant cells obtained through tetrad analysis was confirmed by the transformation with wild-type Rfc1which rescued the lethal phenotype. In order to understand the role of the Rfc1 C-terminal domain, 16 mutations were generated in the C-terminal domain of Rfc1 by either PCR-based point mutation or by the transposon-based pentapeptide insertion method. The phenotype of each mutation was examined by tetrad analysis and subsequent spotting assay. One lethal, two temperature-sensitive and four mutants sensitive to DNA damaging agents were obtained from the pentapeptide insertion mutagenesis. One of the point mutations, *rfc1-33* resulted in weak cold-sensitive phenotype, sensitive to DNA damaging agents HU, MMS and CPT. Among the two temperature sensitive mutants, *rfc1-44* showed tighter temperature-sensitive phenotype while *rfc1-54* showed both temperature-sensitive and cold-sensitive phenotype. Both mutants were highly sensitive to DNA damaging agents HU, MMS and UV. In all cases *rfc1-54* showed a greater sensitivity to DNA damaging agents than did *rfc1-44*. The *rfc1-54* showed weak sensitivity to CPT while *rfc1-44* was not sensitive. The phenotype of each mutation in the Rfc1 C-terminal domain is summarized in figure 2.5

The lethal or temperature-sensitive phenotype of the three pentapeptide insertion mutants *rfc1-43*, *rfc1-44* and *rfc1-54*, confirmed the previous results of the stop-codon insertion experiment that the C-terminal domain of Rfc1 is essential for cell viability.

The difference in sensitivity to temperature and DNA damaging agents seen between *rfc1-44* and *rfc1-54* suggests a variety of role for the C-terminal domain of Rfc1 in DNA metabolism. RFC is known to be involved in the DNA repair pathway in addition to its role as a clamp loader (Wang *et al.*, 2000, Pennaneach *et al.*, 2001). Rfc1 is one member of a multi-protein complex that associates with BRCA1, a protein that recognizes DNA damage. The majority of the proteins found in the same complex are proteins known to play important roles in the homologous recombination or mismatch repair such as Rad50 or Msh6 (Wang *et al.*, 2000). The

fact that RFC forms a complex with proteins involved in the DNA repair pathway suggests that it plays a role in DNA repair. More direct evidence for the involvement of Rfc1 in repair came from analysis of UV-damaged mammalian cells which showed that Rfc1 interacts with Rb and promotes cell survival in a Rb dependent manner (Pennaneach *et al.* 2001). The fact that the Rb binding site in human Rfc1 contains RFC boxes II-VIII suggests that the DNA damaging agent sensitive phenotype of *rfc1-44* and *rfc1-54* is not caused by a defect in the association between the DNA damage repair proteins.

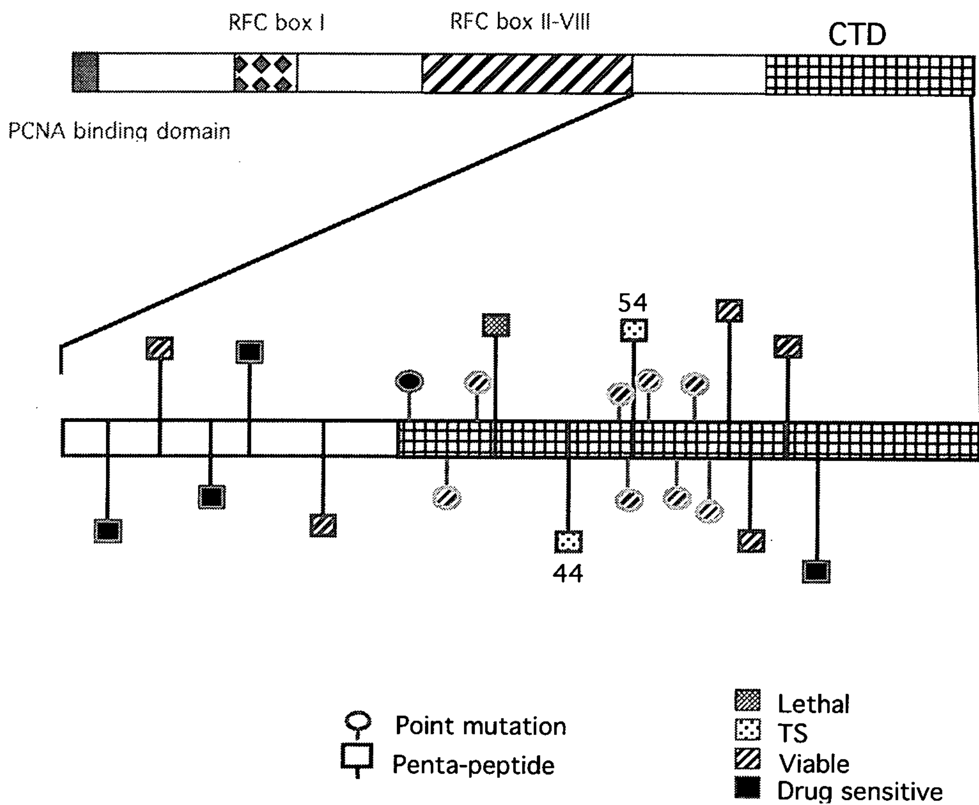


Figure 2.7 Results of tetrad analysis. One lethal, two temperature sensitive and four DNA damaging agents sensitive mutants were isolated from the tetrad analysis of Rfc1-CTD mutants. The position of mutation, type of mutation and the phenotype of each mutation are indicated. Oval sign represents the point mutation and square sign indicates the penta-peptide insertion mutation.

Many of the pentapeptides inserted in random positions within the Rfc1 C-terminal domain contained proline an amino acid that can function as a hinge in the three dimensional structure of proteins. In many cases, the structure of the protein dramatically changes after a proline, so it is reasonable to suggest that the insertion of the pentapeptide has changed the three-dimensional structure of Rfc1 or that of the C-terminal domain. No amino acid was consistently found before or after the pentapeptide insertion nor were the amino acid sequences around the pentapeptide insertions that caused lethal or temperature-sensitive phenotypes conserved. Therefore, it seems likely that the pentapeptide insertion has altered the environment in which the interaction of critical amino acids takes place rather than the amino acids around the pentapeptide insertion have a critical role in either DNA replication or DNA repair.

It is interesting that *rfc1-33* Asp739Ala shows a weak cold-sensitive phenotype and sensitivity to DNA damaging agents HU, MMS and UV while all the other point mutants within the C-terminal domain of Rfc1 did not show any defect with regard to growth and sensitivity to DNA damaging agent. All other amino acids substituted to Ala were highly conserved in all organisms examined while the Asp739 was not conserved in *S. pombe* and *S. cerevisiae*. In higher eukaryotes the amino acid found at this position is Lys but is Asp in *S.pombe* and Gln in *S.cerevisiae*. Asp or Gln is also found in some fungi.

In the tetrad analysis of C-terminal domain deleted Rfc1, a high population of the spores displayed a lethal phenotype. More than one third of the asci showed one viable : three lethal phenotype and the cells that did not form colonies were all highly elongated when examined under the microscope. The reason for this abnormal phenotype is not known but one possible explanation is that the deletion of C-terminal domain causes the mis-segregation or abnormal segregation of the chromosome resulting in an abnormal phenotype.

2.6 Summary

The essential role of Rfc1 C-terminal domain for cell viability was examined. Stop codons were inserted at the starting point of C-terminal domain and the phenotype of

spores carrying the mutation examined. The spores with mutant Rfc1 did not form colonies. When wild-type *rfc1*$^{+}$ was transformed into the mutant cells, the lethal phenotype was suppressed, while transformation with the control vector failed to rescue the viability of mutant cells, indicating that Rfc1 C-terminal domain has an essential role in cell viability.

Mutational analysis was performed in order to further elucidate the role of Rfc1-C-terminal domain. Two different methods were used to generate mutations at the C-terminal domain of Rfc1. First, amino acids which were well-conserved between various organisms and charged amino acids were substituted to Ala by PCR- based site-directed mutagenesis. Secondly, random pentapeptides sequences were inserted into the Rfc1 C-terminal domain by transposon based pentapeptide insertion method. The phenotypes of these mutations were examined by tetrad analysis yielding one lethal, two temperature-sensitive and five DNA damaging agents sensitive mutants. Two temperature sensitive mutants also showed highly sensitive phenotype to DNA damaging agents such as HU, MMS and UV suggesting an important role for Rfc1 C-terminal domain in DNA metabolism. Five drug sensitive mutants showed a broad range of sensitivity to DNA damaging agents.

Chapter 3. Characterisation of *rfc1-44*

3.1 Introduction

In the previous chapter, two temperature-sensitive mutants were generated from pentapeptide insertion mutation of the Rfc1 C-terminal domain. *rfc1-44* showed a tighter temperature-sensitive phenotype than *rfc1-54,* while *rfc1-54* was more sensitive than *rfc1-44* to DNA damaging agents.

For the functional analysis of the Rfc1 C-terminal domain, *rfc1-44* was selected because a mutant showing tighter temperature-sensitive phenotype would display clearer results. The characteristics of *rfc1-44* were examined before the functional analysis of Rfc1 C-terminal domain was performed.

In order to understand the growth arrest of *rfc1-44* in greater detail, the cell division cycle of the mutant cells was examined. In an effort to identify the possible reason for the temperature-sensitive phenotype, the DNA content and chromosome integrity of the mutant cells were examined. In order to know whether the mutant cells have defects in DNA replication, the DNA replication proteins in *rfc1-44* were examined. First, the DNA replication mutant cells were crossed with *rfc1-44* and the phenotype of double mutants examined by tetrad analysis. Second, the DNA replication proteins were over-expressed in *rfc1-44* and the suppression of the temperature-sensitive phenotype of *rfc1-44* by replication proteins was examined.

3.2 Growth curve of *rfc1-44*

The growth of *rfc1-44* cells after shift to the restrictive temperature was examined. Exponentially-growing *rfc1-44* cells in EMM were shifted to 36.5°C and the number of cells per ml were counted every 30 minutes by Coulter counter. As a control, wild-type cells were incubated at 25°C in EMM media and divided into two fractions. One of the fractions was shifted to 36.5°C. HU was added in the other fraction of wild-type cells (to a final concentration of 12mM) and the temperature was shifted to 36.5°C. Cell number was counted in duplicate and average cell number was calculated (Figure 3.1).

Wild-type cells showed normal growth at the restrictive temperature. The cell number increased over time, with a doubling time of approximately two hours. In contrast, the HU-treated wild-type cells arrested after four hours of incubation. Until two hours the HU-treated cells showed normal rate of division with the doubling time of two hours. After two hours the rate of division of HU-treated wild-type cells started to decline and finally arrested. *rfc1-44* showed normal growth until two hours and the division rate started to decrease after two hours. However, *rfc1-44* showed continuous, albeit slow increase in cell number until eight hours with the doubling time of five hours. Most of *rfc1-44* cells showed a highly elongated phenotype after five hours of incubation at restrictive temperature when they were examined under the microscope.

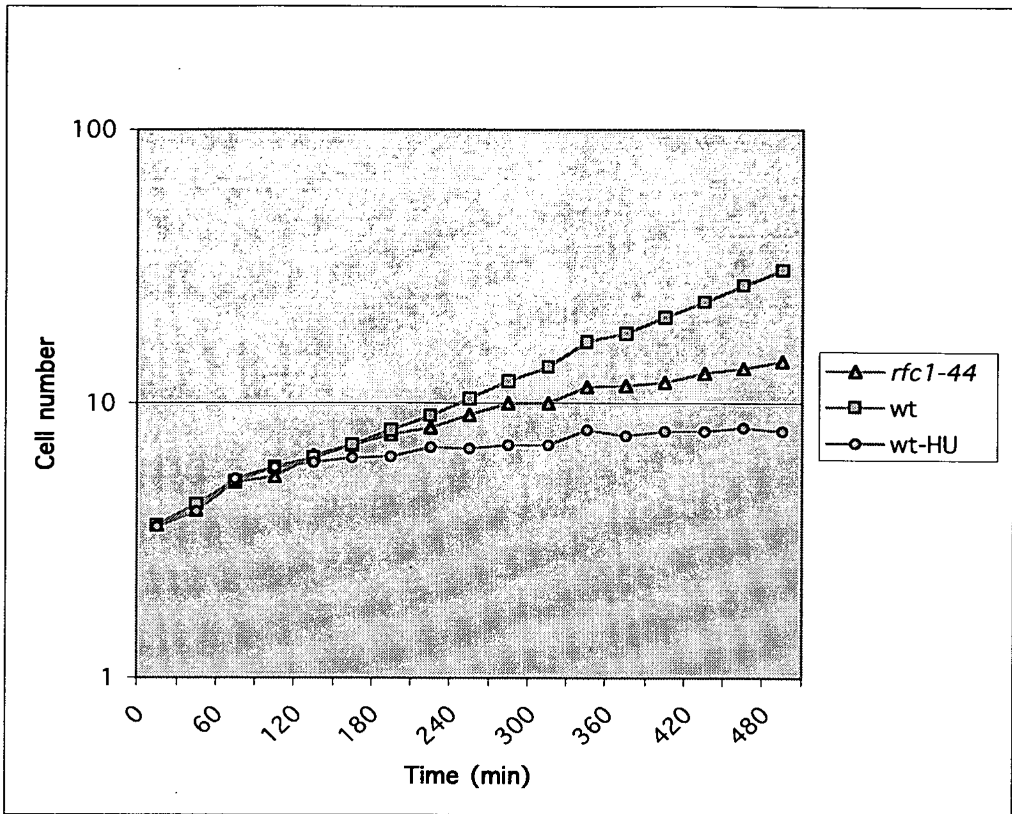


Figure 3.1 Cell number increase of *rfc1-44*. Wild-type, HU-treated wild-type and *rfc1-44* cells were shifted to 36.5°C and the number of cells per ml were counted by Coulter Counter. 100μl of cells suspension were diluted in 10ml of Isoton and the cell number was counted according to the manufacturers' instruction

3.3 DNA content by Flow Cytometric analysis

rfc1-44 shows a highly elongated phenotype at the restrictive temperature suggesting that the *rfc1-44* has defect in cell cycle progression. In order to know whether the highly elongated phenotype of *rfc1-44* resulted from DNA replication defect, the DNA content of the mutant strain was examined following shift to the restrictive temperature. To check the DNA content of *rfc1-44* mutant strain, exponentially-growing wild-type or *rfc1-44* cells in EMM were shifted to the restrictive temperature of 36.5°C. Wild-type cells were split into two fractions and final concentration of 12mM of HU were added to one of them. Cells were collected every hour for eight hours and processed for flow cytometry.

At both permissive and restrictive temperature, wild-type cells showed 2C DNA content (Figure 3.2 left panel). The HU-treated wild-type cells started to show 1C DNA content from one hour after addition of drug and after four hours most of the cells showed 1C DNA content. The HU-arrested cells started to escape from the arrest after five hours and started to show 2C DNA. After eight hours, large populations of the cells were escaped from the arrest and showed 2C DNA content (Figure 3.2 middle panel). Typically, the DNA content peaks for *rfc1-44* were wider than the peaks for wild-type cells and HU-treated wild-type cells. *rfc1-44* showed 2C DNA content when the cells were in permissive temperature. After the shift to restrictive temperature, the peak indicating the DNA content started to widen suggesting that the DNA content of the cells is various. However, there was no obvious accumulation of 1C DNA in the *rfc1-44* cells until eight hours after temperature shift (Figure 3.2 right panel). Apparent DNA content is affected by cell size-bigger and longer cells fluoresce more than shorter ones- a wide peak may mean a wide range of cell sizes.

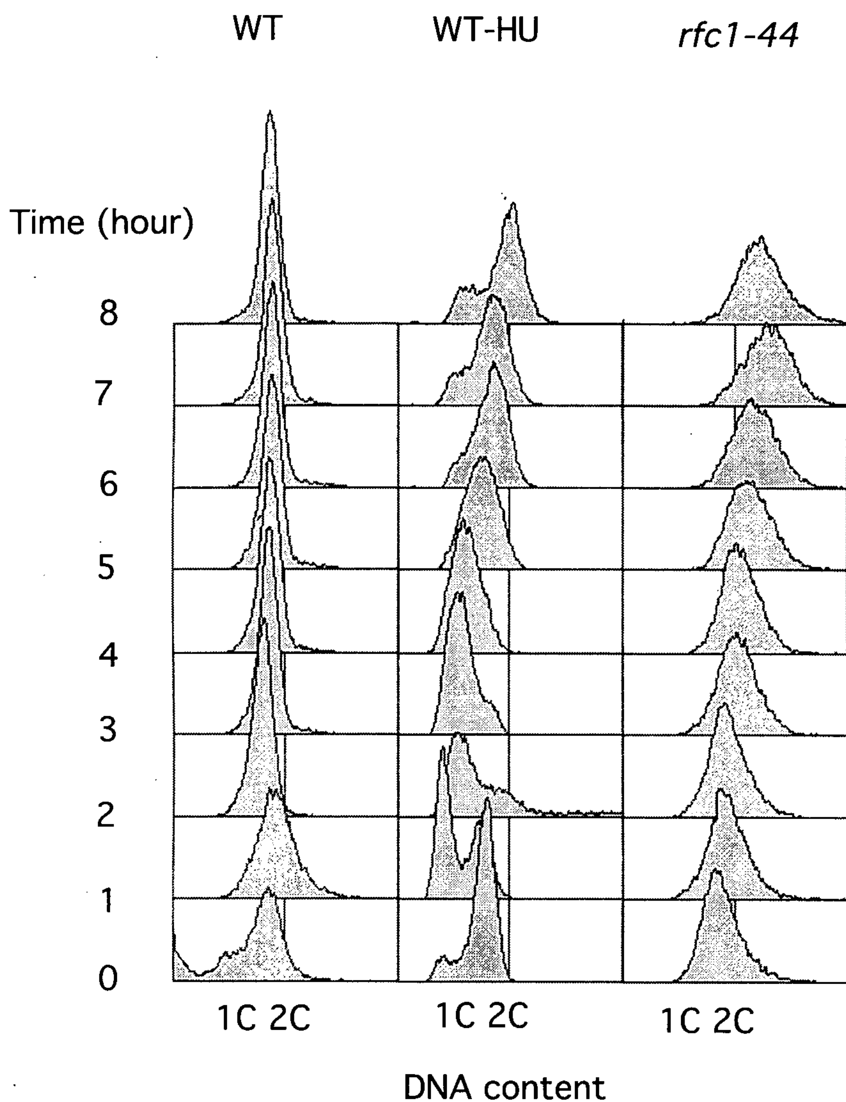


Figure 3.2 Flow cytometric analysis. Wild-type and *rfc1-44* were cultured at 25°C in EMM to mid-logarithmic phase before being shifted to 36.5°C. Before the wild-type cells were shifted to the restrictive temperature, they were split into two fractions and a final concentration of 12mM HU was added to one of them. The cells were harvested and processed for flow cytometry.

3.4 Chromosome stability by PFGE

The previous flow cytometry analysis result suggests that *rfc1-44* does not have a defect in DNA replication. One of the possible reasons that *rfc1-44* did not show a defect in flow cytometry analysis is that this method only can detect gross replication defects. To address this, a more sophisticated method to detect the DNA defects Pulse Field Gel Electrophoresis (PFGE), was performed.

Both exponentially-growing wild-type and *rfc1-44* cells were shifted to 36.5°C. Cells were taken at 0, 2, 4, 6 and 8 hours after the temperature shift and processed for PFGE. The chromosomes from wild-type cells migrated through the gel and discrete chromosome I, II and III bands were observed. There was no difference in the length and intensity in the chromosomes from wild-type cells at permissive and restrictive temperature (Figure 3.3 Lanes 1-5). At permissive temperature, the chromosomes from *rfc1-44* did not show any apparent defect and separated on the gel as discrete bands (Figure 3.3 Lane 6). At restrictive temperature, the chromosomes from *rfc1-44* started to show a smeared band pattern after four hours of the temperature shift (Figure 3.3 Lane 8). No discrete bands were observed after eight hours. In addition, there were some small smeared DNA bands 4, 6 and 8 hours after the temperature shift. These DNAs migrated faster than the chromosome III, the smallest chromosome in *S.pombe* suggesting the presence of fragmented DNA or incompletely processed Okazaki fragment.

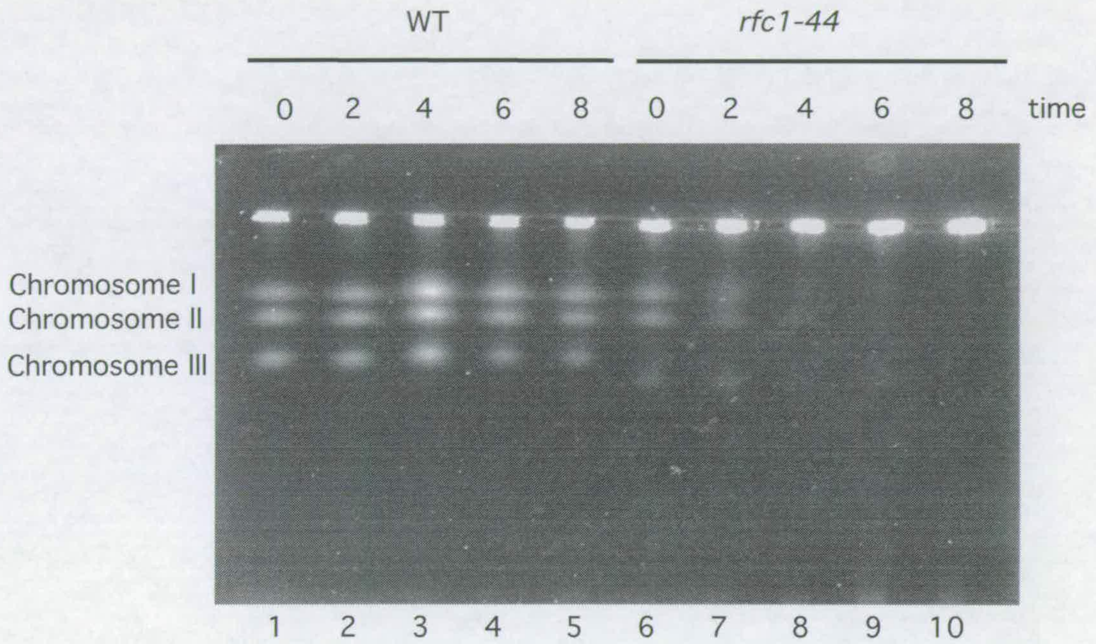


Figure 3.3 Pulse Field Gel Electrophoresis of wild-type and *rfc1-44*. Exponentially-growing wild-type and *rfc1-44* cells were shifted to 36.5°C and cells were harvested at 0, 2, 4, 6 or 8 hours after the temperature shift. The cell wall was disrupted and the resulting spherplasts were embedded in the low melting porint agarose gel. Proteins from the gel was removed and the gels were run in chromosome grade agarose gel.

3.5 Genetic analysis

In order to investigate potential genetic interactions between *rfc1-44* and other components of the DNA replication apparatus, *rfc1-44* was crossed with mutant strains that have mutations or deletions in genes that are involved in DNA replication. Four strains carrying mutations in one of the DNA polymerase δ subunits were used for genetic crosses. Three of them show a temperature-sensitive phenotype and one *(cdc27-D1)* mutant shows cold-sensitivity. In addition to these DNA polymerase δ subunits, mutant strains carrying mutation in the gene encoding Cdc24 or Dna2 were used for the genetic cross with *rfc1-44.*

rfc1-44 and other DNA replication-related mutant strains were mixed and spotted on ME medium. The cells were incubated at 28°C and the formation of spores was checked under the microscope. Asci were separated from other cells and incubated for the breakdown of the cell wall. Spores were separated by micromanipulator and incubated at 28°C until colonies formed. The mutant strains used and the phenotype of double mutant cells are listed in Table 3.1.

It was impossible to get double mutant cells from the cross between *rfc1-44* and *pol3-ts3*, *cdc1-P13*, *cdc27-P11* or *Dna2-C2*. The asci formed from the cross between *rfc1-44* and *pol3-ts3*, *cdc1-P13*, *cdc27-P11* or *Dna2-C2* formed only three colonies at permissive temperature of 28°C. The double mutant spores were germinated but the cells were highly elongated and could not form colonies. Only *rfc1-44 cdc24-38* was able to form colonies at permissive temperature of 28°C and formed four viable colonies.

Table 3.1 The results of genetic cross between *rfc1-44* and the replication mutant cells. Mutant strains used and their genotypes are listed. Number of asci formed three viable colonies or four viable colonies (only in the case of *rfc1-44 cdc24-M38*) are indicated. The mutant cells were crossed and by plating on ME medium and the phenotype of each spore was examine by tetrad analysis.

Mutant strain used	**No. of asci**	**Double mutant phenotype**
pol3-ts3 leu1-32 ura4-D18 ade6-M210 h^+	9	Synthetic lethal
cdc1-P13 h^-	11	Synthetic lethal
cdc27-P11 h^-	11	Synthetic lethal
cdc27-D1 leu1-32 h^-	16	Synthetic lethal
dna2-20 h^-	12	Synthetic lethal
cdc24-M38 leu1-32 h^+	24	Viable
rfc1-44 h^+		
rfc1-44 h^-		

3.6 Over-expression of the proteins involved in the DNA replication

The chromosome mobility defect of *rfc1-44* indicates that the mutant cells have a defect in chromosome integrity when incubated at restrictive temperature. The synthetic lethal phenotype of double mutant cells between *rfc1-44* and the replication mutants indicates that *rfc1-44* cells require intact function of replication proteins for viability. Together, these results suggest that *rfc1-44* has defect in DNA replication. In order to confirm the replication defect in *rfc1-44* and to get a clue about the function of Rfc1 C-terminal domain, various replication proteins were over-expressed in *rfc1-44*.

The proteins examined were: all five RFC subunits, all four DNA polymerase δ subunits, DNA ligase I, Dna2, Cdc24, Pfh1, Rad17 and PCNA. In addition to that, the effects of N-terminal deleted Rfc1 were tested too. To test the effect of over-expression, the plasmids that encoded the replication proteins were transformed into *rfc1-44* (*rfc1-44 leu1-32 ura4-D18*) by electroporation. The transformants were streaked on EMM + supplements plate and incubated either at 25°C or at 35°C.

Expression of Rfc1 in *rfc1-44* rescued the temperature-sensitive phenotype. Over-expression of *S.cerevis*iae Cdc44 also suppressed the mutant phenotype (data not shown), consistent with the previous data that the lethal phenotype of C-terminal deleted Rfc1 was rescued by the expression of *S.cerevisiae* Rfc1 (see chapter 2). None of other replication related protein rescued the temperature-sensitive phenotype of *rfc1-44*. Over-expression of mutant Rfc1 proteins with either the last 30, 60 or 109 amino acids were deleted from the N-terminus did not suppress *rfc1-44* suggesting that the N-terminal amino acids are required for the function of Rfc1. When Dna2, DNA ligase I and Rfc3 were expressed in *rfc1-44* the cells were sick and highly elongated at permissive temperature.

The results of over-expression of proteins are listed in Table 3.2

Table 3.3 Over-expression of proteins involved in the DNA replication in *rfc1-44*.

Protein	Plasmid used	Description	Suppression	phenotype
Rfc1	pUR19-Rfc1	Large subunit of RFC	Yes	
	pCL-Rfc1C30	N-terminal 30 a.a deletion	NO	
	pCL-Rfc1C60	N-terminal 60 a.a deletion	NO	
	pCL-Rfc1C109	N-terminal 109 a.a deletion	NO	
Rfc2	pREP3XH6-Rfc2	Small subunit of RFC	NO	
Rfc3	pUR19-Rfc3	Small subunit of RFC	NO	Sick
Rfc4	pUR19-Rfc4	Small subunit of RFC	NO	
Rfc5	pUR19-Rfc5	Small subunit of RFC	NO	
Pol3	pREP41X-Pol3	The large subunit of DNA polymerase δ	NO	
Cdc1	pREP3X-Cdc1	The B subunit of DNA polymerase δ	NO	
Cdc27	pREP3X-Cdc27	The third subunit of DNA polymerase δ	NO	
Cdm1	pREP3X-Cdm1	The fourth subunit of DNA polymerase δ	NO	
PCNA	pIRT2-Pcn1	Clamp for DNA polymerase δ	NO	
Cdc17	pREP3X-Cdc17	DNA ligase I	NO	Sick
Dna2	pREP1-Dna2	Endo/exonuclease	NO	Sick
Cdc24	pAL-Cdc24	Rfc1 binding protein	NO	
Pfh1	pCL-pfh1	DNA helicase	NO	
Rad17	pCL-Rad17	DNA damage checkpoint	NO	

3.7 Discussion

In this chapter, the genetic characteristics of *rfc1-44* were examined. The cell number increase of *rfc1-44* at restrictive temperature showed that complete growth arrest in mutant cell does not takes place until eight hours of incubation. No obvious gross replication defect was identified in the analysis of DNA content by flow cytometry in mutant cells. The examination of chromosome integrity by PFGE showed the opposite result. The chromosome from *rfc1-44* displayed defects after the shift to restrictive temperature. Chromosomes from *rfc1-44* displayed abnormal mobility. They either did not enter the gel or migrated faster than the smallest chromosome, chromosome III. The results of genetic crosses between *rfc1-44* and the replication mutants supported the results of chromosome structure study. Most of the double mutants showed synthetic lethal phenotype except the double mutant generated from *rfc1-44* x *cdc24-M38*. Over-expression of proteins involved in DNA replication did not rescue the temperature-sensitive mutant phenotype of *rfc1-44* while wild-type Rfc1 did.

The cell number increase curve result and the DNA content of the mutant cell suggest that there is no defect in DNA replication. The abnormal chromosomal structure of *rfc1-44* resulted in abnormal gel mobility and the synthetic lethal phenotype of double mutants between *rfc1-44* and the mutant strain in DNA replication indicates severe defect in DNA replication.

Rfc1-44 cells showed wide peak pattern at the restrictive temperature when the DNA content of the cells were examined by flow cytometry. The wide peak is a result of checkpoint function in the mutant cells that inhibit the progression of DNA replication. However, cell growth does not stop even when the DNA replication is blocked by checkpoint resulting in the large single nucleated cells. These large single nucleated cells results in the wide peak in flow cytomertry analysis that the ratio between the cell size and amount of labelled DNA is a major factor for the analysis of DNA content.

There are two possible reasons about the slow but constant cell number increase and the failure of DNA replication defect detection by flow cytometry analysis if the *rfc1-44* has defect in DNA replication. First, the method used to detect DNA replication defect was not sensitive enough to detect the replication defects caused in

the *rfc1-44*. Often the DNA replication defects caused by replication protein defect are not detected by flow cytometry analysis in *S.pombe*. For example, the mutant cells that have mutation in *cdc1*, *cdc27* and *cdc17* did not show accumulation of 1C DNA although the mutant cells have defect in DNA replication (Stuart MacNeill personal communication).

The second possibility is that there are some other factors that compensate the defect of RFC. Because RFC is essential for cell viability and for DNA replication, the other factors could not replace the function of RFC. But they should have similar function with RFC or play a role in the same process with RFC. In fact, there are two proteins that can form complex with RFC small subunits and are considered to load PCNA. They are Elg1 (Kanellis *et al.*, 2003, Ben-Aroya *et al.*, 2003, Bellaoui *et al.*, 2003) and Ctf18 (Mayer *et al.*, 2001) that are known to play a role in the chromosome integrity and sister chromatid cohesion respectively (see section 1.2.7). The fact that Ctf18-RLC (RFC-like complex) loads PCNA onto DNA and Elg1 interacts genetically and physically with PCNA support the idea of Elg1 and Ctf18 as a supporter of RFC.

If the other clamp loaders cannot substitute the role of RFC then why there are two more clamp loaders in eukaryotic cells? The simple answer is that although these protein complexes cannot replace the RFC, they can provide enough time to repair damage caused by abnormal RFC. In fact the *rfc1-44* cells showed growth defect at restrictive temperature although the growth rate was not completely arrested. However, the cells eventually stopped growth and did not form colonies when *rfc1-44* was plated on YE plate (Figure 2.4). These results suggest that mutation in Rfc1 C-terminal domain causes severe defect in DNA replication although the growth of the cells are maintained by other RLCs for a while.

In the analysis of the chromosome integrity using PFGE, the chromosome III from *rfc1-44* migrated faster than the chromosome III from wild-type cells suggesting that there was a chromosome loss in *rfc1-44*. However, the chromosome I and II from *rfc1-44* did not show size difference with the wild-type chromosomes. Therefore it is possible that the fast migrating chromosome III in rfc1-44 is resulted from the short rDNA repeat that affect on the chromosome size rather than chromosome loss (Hiroyuki Tanaka personal communication).

Over-expression of the replication proteins did not suppress the temperature-sensitive phenotype of *rfc1-44*. It suggests that there is no direct interaction between the C-terminal domain of Rfc1 and the proteins examined. Another possibility is that the method used was not appropriate for the detection of any interaction. For example, the mutant phenotype of Cdc44, *S. cerevisiae* homologue of Rfc1, was suppressed by the expression of mutant PCNA and *vice versa* (Beckwith *et al.*, 1998, Amin *et al.*, 1999). If it is true in *S.pombe* too, then there is a possibility that the temperature-sensitive phenotype of *rfc1-44* can be suppressed by over-expression of mutant proteins, rather than wild-type proteins. Over-expression of mutant proteins in *rfc1-44* or screening of *S.pombe* mutation library will be required to know if there is any mutant protein that suppress the temperature-sensitive phenotype of *rfc1-44*.
It is interesting that none of the N-terminal deletion mutant of Rfc1 suppress the temperature sensitive phenotype of *rfc1-44*. These results suggest that the N-terminal domain of Rfc1 is required for optimal function of this protein. In the *in vivo* expression experiment, strains in which the Rfc1 was replaced with the N-terminal deletion mutant of Rfc1 (*rfc1-ΔN*) did not show any difference in activity with wild-type cells in regard to the DNA replication, telomere length maintenance, damage susceptibility and interchromosomal recombination in *S.cerevisiae* (Gomes *et al.*, 2001). In another *in vitro* experiment, the deletion of N-terminal domain of human Rfc1 did not affect on the RFC complex formation and the PCNA loading (Uhlmann *et al.*, 1997). These data suggest that the N-terminal domain of Rfc1 is not required for the function of Rfc1. However, the result acquired in this chapter indicates that the N-terminal domain of Rfc1 is required for the function of protein in *S.pombe*.
There are two possible reasons for this difference. The simple explanation is that the N-terminal domain of Rfc1 in *S.pombe* plays an important role in DNA metabolism while N-terminal domain of *S.cerevisiae* Rfc1 does not. Second possibility is that N-terminal domains of both *S.pombe* and *S.cerevisiae* Rfc1 have an important role for DNA metabolism. More studies are required to understand whether the N-terminal domain of Rfc1 plays a role in DNA metabolism. If it is required for the function of Rfc1 then it will be an intriguing subject to study.

3.8 Summary

In this study the characteristics of *rfc1-44* were examined. *rfc1-44* showed slow but steady growth compared with wild-type cells at restrictive temperature. No obvious accumulation of 1C DNA was observed when the DNA content of *rfc1-44* was examined at restrictive temperature. However, the chromosomes from *rfc1-44* showed structural defect resulting in abnormal migration in the gel. The chromosomes from the mutant cells did not enter the gel or some chromosomes migrated faster than the smallest chromosome at restrictive temperature. In addition to that, the cells resulted from genetic crosses between *rfc1-44* and the temperature-sensitive mutant strains that the DNA replication proteins are mutated showed synthetic lethal phenotype. Over-expression of replication proteins or N-terminal deletion mutant *rfc1* in *rfc1-44* did not rescue the temperature-sensitive mutant phenotype of *rfc1-44*. The growth curve and the flow cytometry analysis suggest that *rfc1-44* does not have replication defect although the chromosome structure of the mutant and the double mutant phenotype strongly suggest that the temperature-sensitive phenotype of *rfc1-44* resulted from DNA replication defect. From the fact that many DNA replication mutant cells does not show accumulation of 1C DNA at restrictive temperature and the results acquired in this chapter, *rfc1-44* seems have defect in DNA replication at restrictive temperature.

Chapter 4. The role of three clamp loaders

4.1 Introduction

Recent biochemical and genetic studies identified the presence of three RFC-like complexes (RLCs) in eukaryotic cells. The three RLCs are composed of the four small subunits of RFC but the large subunit is replaced with Rad24, Elg1 or Ctf18 (see section 1.2.7). The roles of the large subunits of the RLCs are suggested from biochemical and genetic analysis. Rad24 play an important role in DNA damage checkpoint, Ctf18 plays a role in sister chromatid cohesion while Elg1 plays roles in genome stability. Biochemical and genetics analysis suggested that Rad24-RLC loads the Rad9-Hus1-Rad1 (9-1-1) complex, a clamp playing a role in DNA checkpoint, onto DNA. It is suggested that Ctf18-RLC and Elg1-RLC load PCNA onto DNA (Bermudez *et al.*, 2003, Bellaoui *et al.*, 2003, Kanellis *et al.*, 2003). If it is true then there is a question to be answered: why do the cells require three PCNA loaders? What is the role of each RLC in the DNA metabolism or cell cycle progression?

In order to understand the role of three PCNA loaders, double or triple mutant cells of *rfc1-44*, *ctf18Δ* and *elg1Δ* were generated. The characteristics of each mutant cells were determined in this chapter.

4.2 Failure to get *rfc1-44 ctf18Δ*, *rfc1-44 dcc1Δ* and *rfc1-44 ctf8Δ*

In order to study the role of three PCNA loaders, genes encoding *S.pombe* Ctf18, Dcc1 and Ctf8 were identified by homology search with the *S. cerevisiae* Ctf18, Dcc1 and Ctf8 (S. A. MacNeill unpublished data). The *dcc1$^+$*, *ctf8$^+$* or *ctf18$^+$* deletion strains showed no defect in viability (Mylonas and MacNeill, unpublished data) consistent with the results of *S.cerevisiae DCC1, CTF8* or *CTF18* deletion strains that the genes were not essential for cell viability (Mayer *et al.*, 2001). Although the mutant cells with *dcc1$^+$*, *ctf8$^+$* or *ctf18$^+$* deletion construction did not show a defect in viability, they showed highly sensitive phenotype to the microtubule inhibitor, benomyl suggesting the essential role of these proteins in chromatid cohesion (Mylonas and MacNeill, unpublished data).

In order to understand the role of Ctf18 in the Rfc1 deficient cell, *ctf18Δ* (*ctf18::natMX6 h$^-$*) was crossed with *rfc1-44* (*rfc1-44 h$^+$*) and the meiotic products were examined by tetrad analysis.

Most of the asci formed three viable colonies (Figure 4.1 lanes 1, 6, 7, 8 and 9). In order to know the reason of colony formation failure, the cells were examined under the microscope. The spores that did not form colony germinated and underwent two or three rounds of cell division. Two asci formed four viable colonies and two of them formed only two colonies. The results of tetrad analysis of *rfc1-44* x *ctf18Δ* are summarized in Table 4.1.

To determine the genotype of each of the colonies that formed, the cells were replica plated on a YE plate containing 10μg/ml ClonNat and incubated at permissive temperature of 25°C. The natMX6 cassette carrying ClonNat resistant gene was used to delete the *ctf18$^+$* gene, thus the *ctf18Δ* cells show resistance against the drug. The cells were also replica plated on a YE plate and incubated at 35°C to identify those cells displaying the temperature-sensitive characteristic of *rfc1-44*.

Among three viable colonies, one colony showed growth defect at high temperature and one was resistant to ClonNat. The last one did not form colony in the presence of ClonNat although it formed colony at high temperature. No colony was temperature-

sensitive and ClonNat-resistant suggesting that the double mutant showed the synthetic lethal phenotype (tetra-type).

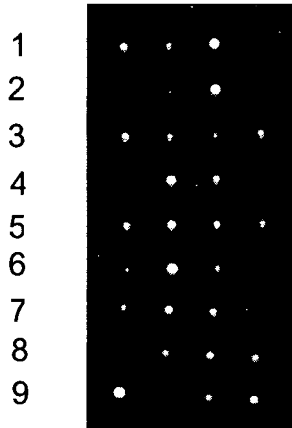


Figure 4.1 The tetrad analysis of *rfc1-44* x *ctf18Δ*. For the analysis of double mutant phenotype, *rfc1-44* and *ctf18Δ* were mixed in 20μl of DW and spotted on ME plate. The cells are incubated for 2 days at 28°C. The formation of ascus was examined under the microscope and the spores were dissected by micromanipulator. The spores are incubated at 25°C until the colony formation.

Two of the four viable colonies showed temperature-sensitive phenotype and the other two showed ClonNat resistant phenotype suggesting that this high rate of four viable phenotype resulted from the parental di-type. Two asci showed two viable : two lethal phenotype. The colonies were viable at high temperature but could not survive in the presence of ClonNat, suggesting these colonies are wild-type cells (non-parental di-type). These results suggest that the function of Ctf18 is essential for the viability of *rfc1-44*.

In *S.cerevisiae*, two additional proteins, Dcc1 and Ctf8 are required to form intact Ctf18-RLC. These two proteins are required for the function of Ctf18 as a key component of sister chromatid cohesion (Mayer *et al.*, 2001). In order to know whether these proteins are required for the viability of *rfc1-44,* the phenotype of double mutant cells was examined by genetic cross and tetrad analysis.

Table 4.1 Results of *rfc1-44* x *ctf18Δ*. The results of tetrad analysis and replica-plating are listed. The genotype of formed colonies were examined by replica-plating the cells on YE medium or YE containing 10μg/ml ClonNat and incubating the cells at 25°C or 35°C.

Phenotype	Number of asci	*rfc1-44*	*ctf18Δ*	*rfc1-44 ctf18Δ*	Wild-type
TT	10	1	1	0	1
PD	5	2	2	0	0
NPD	2	0	0	0	2

TT: Tetra type PD: parental di-type NPD: non-parental di-type

The genetic crosses were performed as previously. Double mutant between *rfc1-44* (*rfc1-44 h⁺*) and *dcc1Δ* (*dcc1::natMX6 h⁻*) showed synthetic lethal phenotype. Seventeen out of twenty-three asci showed three viable: one lethal phenotype (Figure 4.2 Left panel Lanes 1, 2, 4, 6, 7, 8, 9). Three out of twenty-three showed four viable : zero lethal phenotype (Figure 4.2 Left panel Lane 3) and two showed two viable : two lethal phenotype (Figure 4.2 Left panel Lane 5). Again, the cells from lethal phenotype showed highly elongated phenotype with two or three rounds of replication. No temperature-sensitive and ClonNat resistant cells were acquired from tetra type colonies when the genotypes of the cells were examined by the replica plating. Four viable: zero lethal cells were revealed as parental di-type and two viable: two lethal cells were revealed as non-parental di-type in the replica plate examination. This result indicates that the Dcc1 is required for the viability of *rfc1-44* (Figure 4.2). The results of *rfc1-44* x *dcc1Δ* are summarized in Table 4.2

When *rfc1-44* (*rfc1-44 h⁺*) was crossed with *ctf8Δ (ctf8::natMX6 h⁻)* mutant strain the resulting double mutant was lethal. Fourteen out of twenty-six asci showed (three viable: one lethal) phenotype (Figure 4.2 Right panel Lanes 1,3, 4, 7, and 8). Six were (four viable: zero lethal) and six were (two viable: two lethal) (Figure 4.2 Right panel lanes 5, 6, and 9). The genotype of each colony was examined by replica-plating and there was no temperature sensitive and ClonNat resistant colony. The genotype of parental and non-parental di-type cells were also tested by replica-

plating the colonies that formed in tetrad analysis (Figure 4.2). The results of *rfc1-44* x *ctf8Δ* are summarized in Table 4.2

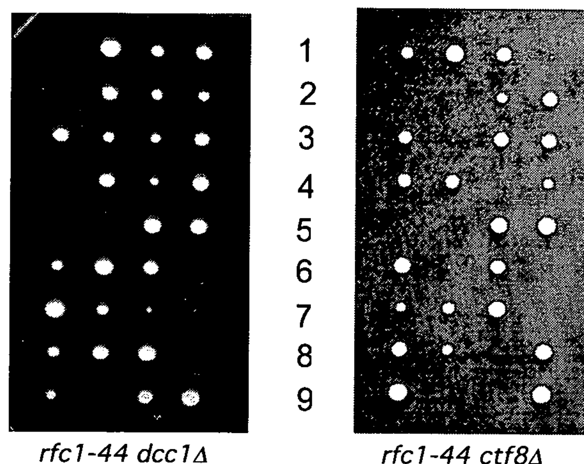


Figure 4.2 The tetrad analysis of *rfc1-44* x *dcc1Δ and rfc1-44* x *ctf8Δ*. For the analysis of double mutant phenotype, *rfc1-44* and *dcc1Δ* or *ctf8Δ* were mixed in 20μl of DW and spotted on ME plate. The cells are incubated for 2 days at 28°C. The formation of ascus was examined under the microscope and the spores were dissected by micromanipulator. The spores are incubated at 25°C until the colony forms.

Table 4.2 results of *rfc1-44* x *dcc1Δ* and *rfc1-44* x *ctf8Δ*. The results of tetrad analysis and replica-plating are listed. The genetic background of formed colonies were examined by replica-plating the cells on YE medium or YE containing 10μg/ml ClonNat and incubating the cells at 25°C or 35°C.

Phenotype	Number of asci	*rfc1-44*	ClonNat^{res}	*rfc1-44* ClonNat^{res}	Wild-type
rfc1-44 x *dcc1Δ*					
TT	17	1	1	0	1
PD	3	2	2	0	0
NPD	2	0	0	0	2
rfc1-44 x *ctf8Δ*					
TT	14	1	1	0	1
PD	6	2	2	0	0
NPD	6	0	0	0	2

TT : tetra type PD : parental di-type NPD : non-parental di-type

4.3 Generation of *rfc1-44 elg1Δ*, *rfc1-44 elg1Δ ctf18Δ*

The genetic cross between *rfc1-44* and *elg1Δ* was performed and the result of double mutation was examined by tetrad analysis. The results of cross between *rfc1-44* and *elg1Δ* are summarized in table 4.3.

Table 4.3 the results of genetic cross between *rfc1-44* and *elg1Δ*. Germ: the number of spore showing germination problem.

Phenotype	No of asci	*rfc1-44*	*elg1Δ*	*rfc1-44 elg1Δ*	Wild-type
4 : 0	23	1	1	1	1

All the asci formed four viable colonies at restrictive temperature. In order to examine the genotype of the colonies formed, the colonies were replica plated on a YE plate containing 10μg/ml ClonNat and incubated at 25°C. Colonies were also replicated on YE medium and incubated at 35°C to identify those cells displaying the temperature-sensitive phenotype of *rfc1-44*. Among four viable colonies one colony showed growth defect at high temperature. One was resistant to ClonNat and one was able to grow at high temperature but could not grow in the presence of ClonNat. The last one was able to grow in the presence of ClonNat although it could not grow at high temperature suggesting that the *rfc1-44 elg1Δ* double mutant cells are viable. This result suggests that the function of Elg1 is not essential for the viability of *rfc1-44*.

When *elg1Δ* cells were serially spotted on the plate containing high concentration of HU (12mM), it showed better growth than wild-type cells. This result suggests that Elg1 may play a negative role in normal cells (Mylonas and MacNeill, unpublished data). If Elg1 plays negative role in the DNA replication in normal cells, deletion of *elg1*$^+$ in *rfc1-44 ctf18Δ* cells may restore the viability of the double mutant. To examine this possibility, *rfc1-44 elg1Δ* (*rfc1-44 elg1::natMX6 h*$^+$) double mutant was crossed with *ctf18Δ elg1Δ* (*ctf18::kanMX6 elg1::natMX6 h*$^-$) double mutant, as described previously and the phenotype of triple mutant cells was examined following tetrad analysis.

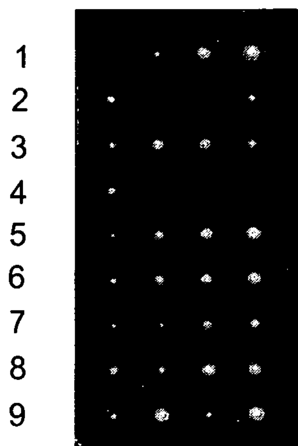


Figure 4.4 The tetrad analysis of *rfc1-44* X *elg1Δ*. For the analysis of double mutant phenotype, *rfc1-44* and *elg1Δ* were mixed in 20μl of DW and spotted on ME plate. The cells are incubated for 2 days at 28°C. The formation of ascus was examined under the microscope and the spores were dissected by micromanipulator. The spores are incubated at 25°C until colonies formed.

The cross of two double mutants has resulted in four viable cells (Figure 4.5). Fourteen asci showed four viable: zero lethal phenotype. The colonies were replica plated on the plate containing either ClonNat or G418 to identify the *elg1Δ* or *ctf18Δ* respectively. Formed colonies were also replica plated on a YE plate and incubated at 35°C to check the *rfc1-44* dependent temperature-sensitive phenotype. Among four viable cells the smallest cell was temperature-sensitive and resistant to ClonNat and G418. The triple mutant was also checked by the PCR with the two sets of primers that amplify either Ctf18 or Elg1. No PCR product was generated from both PCR reactions using Ctf18 and Elg1 primers using genomic DNA from putative *rfc1-44 ctf18Δ elg1Δ* cells as template, while about 1 kb long PCR product was generated using genomic DNA from wild-type cells as template (data not shown).

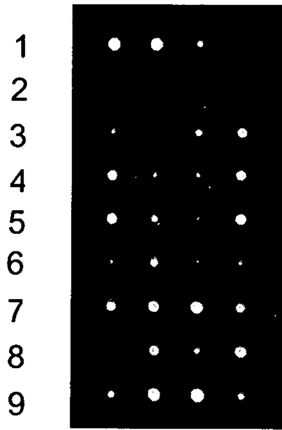


Figure 4.5 Tetrad analysis of *rfc1-44 elg1Δ* X ctf18*Δ elg1Δ*. For the analysis of double mutant phenotype, *rfc1-44 elg1Δ* and *ctf8Δ elg1Δ* were mixed in 20μl of DW and spotted on ME plate. The cells are incubated for 2 days at 28°C. The formation of ascus was examined under the microscope and the spores were dissected by micromanipulator. The spores are incubated at 25°C until colonies formed.

4.4 Drug sensitive and temperature-sensitive phenotype of mutant cells

The restoration of viability in *rfc1-44 elg1Δ ctf18Δ* triple mutant suggests that the Elg1-RLC plays negative role in *rfc1-44 ctf18Δ* cells. To understand further the role of Elg1 in *S.pombe* cells, single, double and triple mutant cells were plated on YE medium and incubated at various temperatures or plated on YE medium containing DNA damaging agents and incubated at 25°C. At 25°C, most of the cells grew well and did not show any difference with the wild-type (Figure 4.6 A). At 36.5°C, *rfc1-44* showed temperature-sensitive phenotype as the previous results. The *rfc1-44 elg1Δ* and *rfc1-44 elg1Δ ctf18Δ* also showed growth defect at restrictive temperature. The *ctf18Δ* strain showed weak growth defect at restrictive temperature. This is different from its *S. cerevisiae* homologue which did not display growth defect at restrictive temperature (Naiki *et al.,* 2001, Bellaoui *et al.*, 2003) (Figure 4.6 B). *elg1Δ* and *elg1Δ ctf18Δ* showed normal growth at 35°C as seen previously in *S. cerevisiae.* In the presence of 5mM HU, *rfc1-44*, *rfc1-44 elg1Δ* and *rfc1-44 elg1Δ ctf18Δ* showed growth defect as expected from the replication defect caused by the *rfc1-44* mutation. *ctf18Δ* also showed weak growth defect in the presence of HU (Figure 4.6 C). The growth defect of *ctf18Δ* was severe in the presence of 7.5mM HU and at this concentration, the growth defect of *ctf18Δ* was more severe than that of *rfc1-44 elg1Δ* and *rfc1-44 ctf18Δ elg1Δ* (Figure 4.6 D). In the presence of 5mM HU, the Elg1 deletion strain and *elg1Δ ctf18Δ* double mutant strain did not show any defect in growth. In the presence of 7.5mM HU, both strains showed better growth than wild-type cells same as the previous data of Mylonas and MacNeill (personal communication). In the presence of 5μM CPT, most of the cells did not show any growth defect but the *rfc1-44 elg1Δ ctf18Δ* mutant cells showed growth defect (Figure 4.6 E) suggesting that the triple mutant cells have problem in re-start of replication after the replication fork stall. CPT is an inhibitor of Topoisomerase I and is required for the CPT-Topoisomerase-DNA complex that results in the single-strand break. Ongoing replication fork collide with the CPT-Topoisomerase-DNA complex that results in the replication fork stall. Normal replication machinery can overcome the replication fork stall by rapid disassembly and re-assembly of the

replication proteins. However, if there is a problem in the initiation of DNA replication or defect in the replication proteins, the replication cannot be started again after the replication fork stall.

Therefore, the sensitive phenotype to CPT of the triple mutant cells suggests that the mutant cells have problem in the re-start of replication.

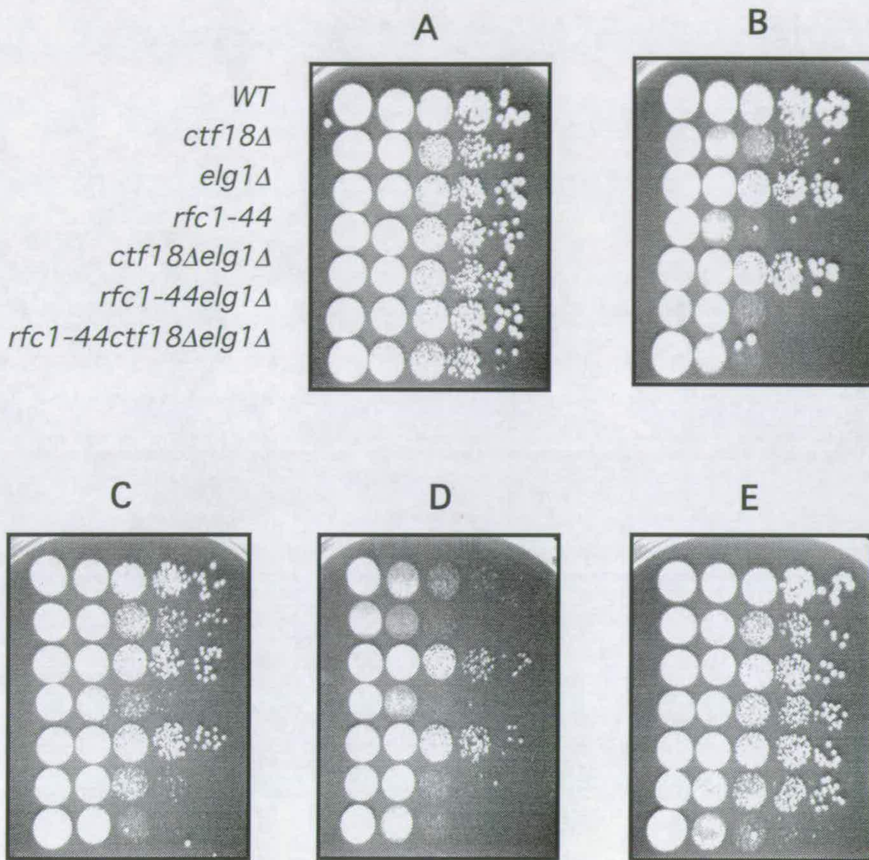


Figure 4.6 Characteristics of mutant cells. Exponentially growing wild type or mutant cells were serially diluted 10 fold from 1 x 10^7/ml with DW. Diluted cells are spotted on YE p late or YE plate containing DNA damaging agents and incubated either 25°C or 36.5°C. **A)** Wild-type and mutants cells that were incubated at 25°C. **B)** Wild-type and mutants cells are incubated at 36.5°C. **C)** The diluted cells are spotted on the YE plate containing 5mM HU and incubated at 25°C. **D)** The diluted cells are spotted on the YE plate containing 7.5mM HU and incubated at 25°C. **E)** The cells are spotted on the YE plate containing 5μM CPT and incubated at 25°C.

4.5 DNA content of mutants by flow cytometric analysis

In the previous study (see section 3.3) *rfc1-44* at restrictive temperature did not show any accumulation of 1C DNA content suggesting *rfc1-44* cells does not have any defect in bulk DNA replication. The presence of other clamp loaders that can load PCNA in the absence of fully-functional RFC was suggested as a possible reason of normal flow cytometry results. To examine whether the double or triple mutant shows DNA replication defect, the DNA content of mutant cells were examined by flow cytometry.

Exponentially-growing single, double and triple mutants were shifted to 36.5°C and DNA content was examined every hour for 6 hours by flow cytometry of propidium iodide stained cells. At the restrictive temperature, wild-type cells showed constant 2C DNA content through the time examined (Figure 4.7 A). *elg1Δ*, *ctf18Δ* and *elg1Δ ctf18Δ* cells showed no difference with wild-type cells with regard to DNA content and the shape of the peak (Figure 4.7 B, C, and E). At permissive temperature (Figure 4.7 D Time 0) *rfc1-44* cells shows no difference with the wild-type cells. After the shift to restrictive temperature, the DNA content peaks became wide but it did not show any 1C DNA content (See chapter 3). At permissive temperature, the triple mutant cells did not show much difference with wild-type cells. After two hours of temperature shift more than half of the cells contained less than 2C DNA suggesting the gross DNA replication defect. From three hours after the shift, the accumulated 1C DNA peak started to decrease and after five hours most of the cells contained 2C DNA again.

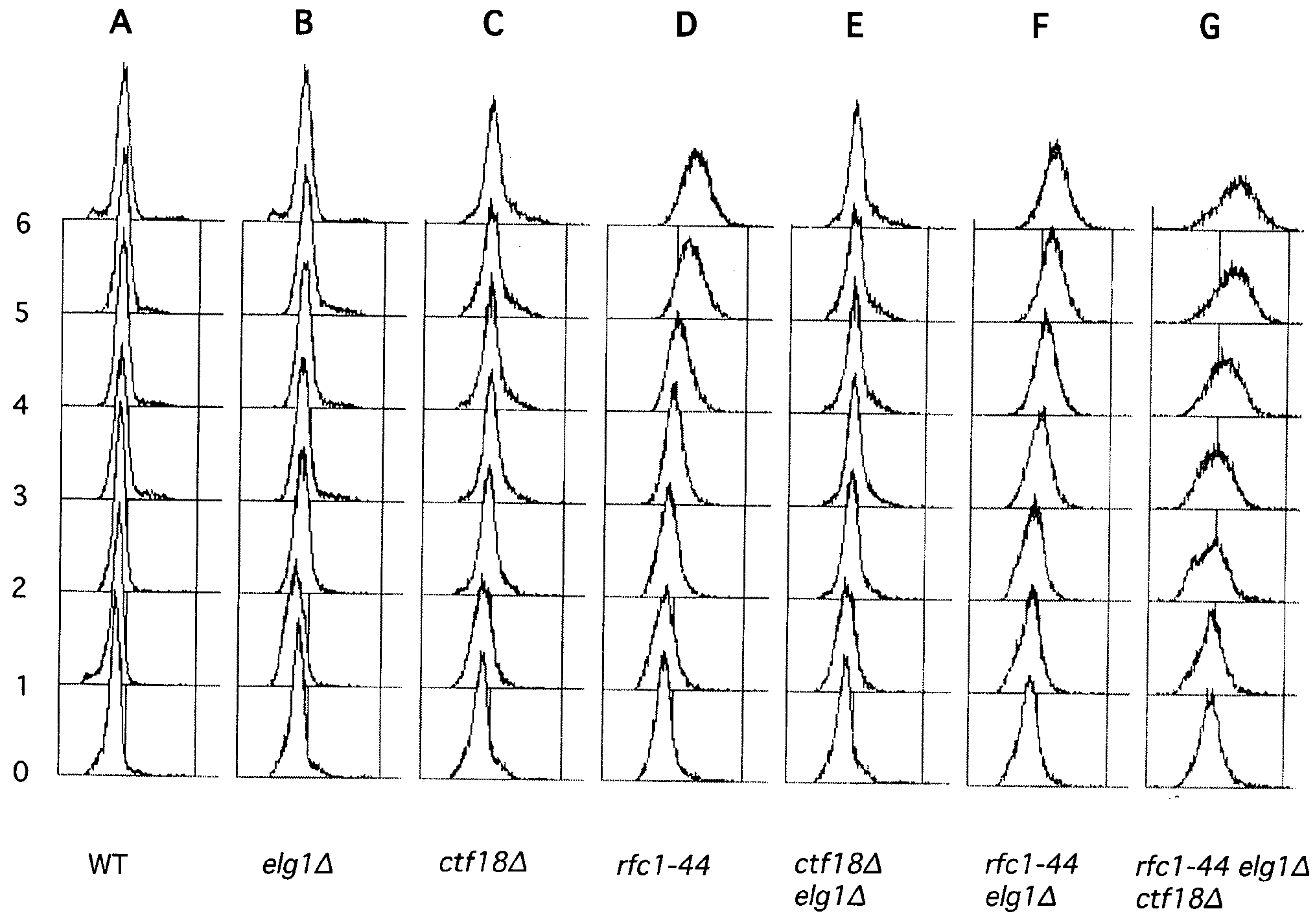


Figure 4.7 The FACS analysis of the mutant cells. The cells are shifted to 36.5°C and treated with Zymolyase to get rid of the cell wall. 1mg/ml of RNAse was treated overnight to remove RNA and the DNA was labeled with propidium iodide for 2 hours at 4°C. The cells were diluted 1/20 and briefly sonicated before the FACS analysis. **A**. wild type **B**. elg1Δ **C**. *ctf18Δ* **D**. *rfc1-44* **E**. *ctf18Δelg1Δ* **F**. *rfc1-44elg1Δ* **G**. *rfc1-44elg1Δctf18Δ*

4.6 Chromosome integrity of mutant cells

Deletion of *elg1*$^{+}$ restored viability to the *rfc1-44 ctf18Δ* double mutant (See section 4.3). *rfc1-44 elg1Δ* cells showed a small difference in growth rate in the presence of HU (See section 4.4). These results suggest that Elg1 plays a weak negative role in normal cells and removal of this negative role recovers the viability of triple mutant. Moreover, the *elg1Δ* cells showed better growth in the presence of high concentration of HU suggesting that the deletion of *elg1*$^{+}$ increases the resistance to DNA damaging agent. To test whether the Elg1 plays negative role, the chromosomal DNA from *rfc1-44* and *rfc1-44 elg1Δ* was prepared and examined by PFGE.

Exponentially-growing *rfc1-44* and *rfc1-44 elg1Δ* were shifted to restrictive temperature and cells were taken away from the culture every hour for four hours. Cell numbers were measured and cells were processed for PFGE.

After two hours of temperature shift, the chromosomes from *rfc1-44* migrated abnormally as shown previously. The bands were not discrete and were smeared (Figure 4.8 lane 3). After three hours of temperature shift, severe chromosome mobility defects were observed. No discrete bands are visible after four hours incubation at restrictive temperature. There were some smeared bands migrating faster than the chromosome III in all the lanes after the temperature shift (compare lane 1 and lanes 2-5). After two hours of temperature shift, the chromosomes from *rfc1-44 elg1Δ* cells started to show smeared bands at restrictive temperature (Figure 4.7 lane 8). However, the chromosomes from *rfc1-44 elg1Δ* did not show further defect in chromosome structure after the three hours of temperature shift (Figure 4.7 lane 9). The greatest difference was observed from the chromosomes incubated for four hours at restrictive temperature that the chromosome from *rfc1-44* showed defect and migrated abnormally while the chromosome from *rfc1-44 elg1Δ* has maintained its integrity although there was still some defect that resulted in the small, smeared band (Figure 4.7 Compare the lane 5 and 10). This experiment was repeated three times and in all case similar results were acquired.

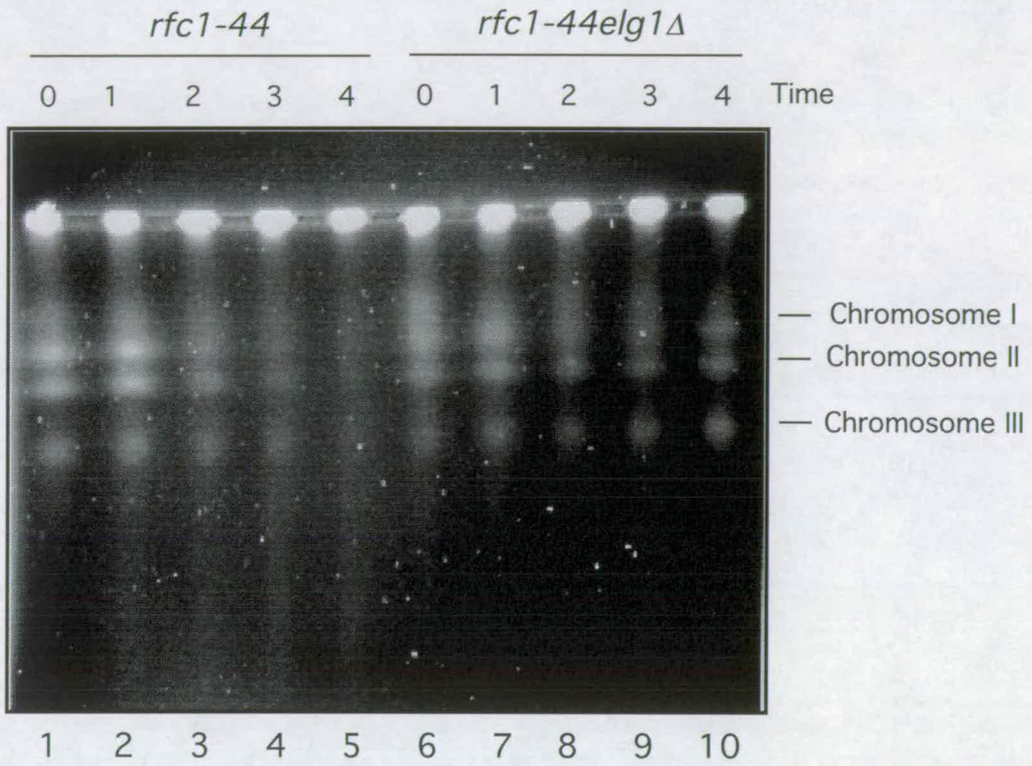


Figure 4.7 The Pulse field gel electrophoresis of *rfc1-44* and *rfc1-44 elg1Δ*. Exponentially growing cells were shifted to 36.5°C and cells were taken away 0, 1, 2, 3 and 4 hours after the temperature shift from the culture. Cell wall was removed by Zymolyase treatment and the resulting spheroplast was embedded in low melting point agarose. Proteins were removed from the plug by the proteinase K treatment the plug was put in chromosomal grade agarose and run for 72 hours. The gel was stained with ethidium bromide and destained with DW.

4.8 Over-expression of Rfc1, Elg1 or Ctf18 in each single mutant.

In the previous section the deletion of *elg1*$^+$ had no effect on the viability of *rfc1-44* while deletion of *ctf18*$^+$ resulted in a synthetic lethal phenotype. In the subsequent experiment, deletion of *elg1*$^+$ in the *rfc1-44 ctf18Δ* recovered the viability of the mutant cells. These results suggest that the Elg1 plays the negative role while the Ctf18 pays positive role for the viability of *rfc1-44*. In order to understand the function of Ctf18 and Elg1 in the cell, the proteins were over-expressed in various genetic backgrounds.

rfc1-44 (*rfc1-44 ura4-18 leu1-32*), *ctf18Δ* (*ctf18::natMX6 ura4-18 leu1-32*), *elg1Δ* (*elg1::natMX6 ura4-18 leu1-32*) and wild-type (*ura4-18 leu1-32*) cells were used for the over-expression of each proteins. The pREP3XH6, pREP1-Rfc1, pREP3X-Elg1 or pREP3X-Ctf18 plasmids were transformed into mutant or wild-type cells by electroporation. Transformants were isolated from the EMM plate containing thiamine. Single colony was inoculated in EMM solution containing thiamine and transferred into either EMM or EMM + thiamine solution. Exponentially-growing cells counted and serially diluted before spotting on EMM or EMM + thiamine plate and incubated at 25 or 35°C. Cells were also exposed to 300 J/m^2 of UV irradiation and incubated at 25°C. The effect of DNA damaging agents was examined by spotting the cells in the plate containing CPT, HU or MMS.

No colonies were formed when pREP3X-Elg1 was transformed into *rfc1-44* at 25°C. The transformed cells were sick and highly elongated even in the presence of thiamine when examined under the microscope. Cells showed extremely slow growth rate and prolonged incubation did not give usable colonies (data not shown).

When Rfc1 was expressed in the wild-type cells in the absence of thiamine, it showed dominant-negative effect on growth at both 25°C and 35°C (Figure 4.9). The cells were elongated and exhibited slower growth than the cells transformed with pREP3X. The dominant-negative effect of Rfc1 over-expression was also found in other mutant cells, *ctf18Δ* and *elg1Δ*, in the absence of thiamine. When the expression of Rfc1 was suppressed by the thiamine, the cells did not show growth defect caused by over-expression of Rfc1. The over-expression of Rfc1 in *rfc1-44*

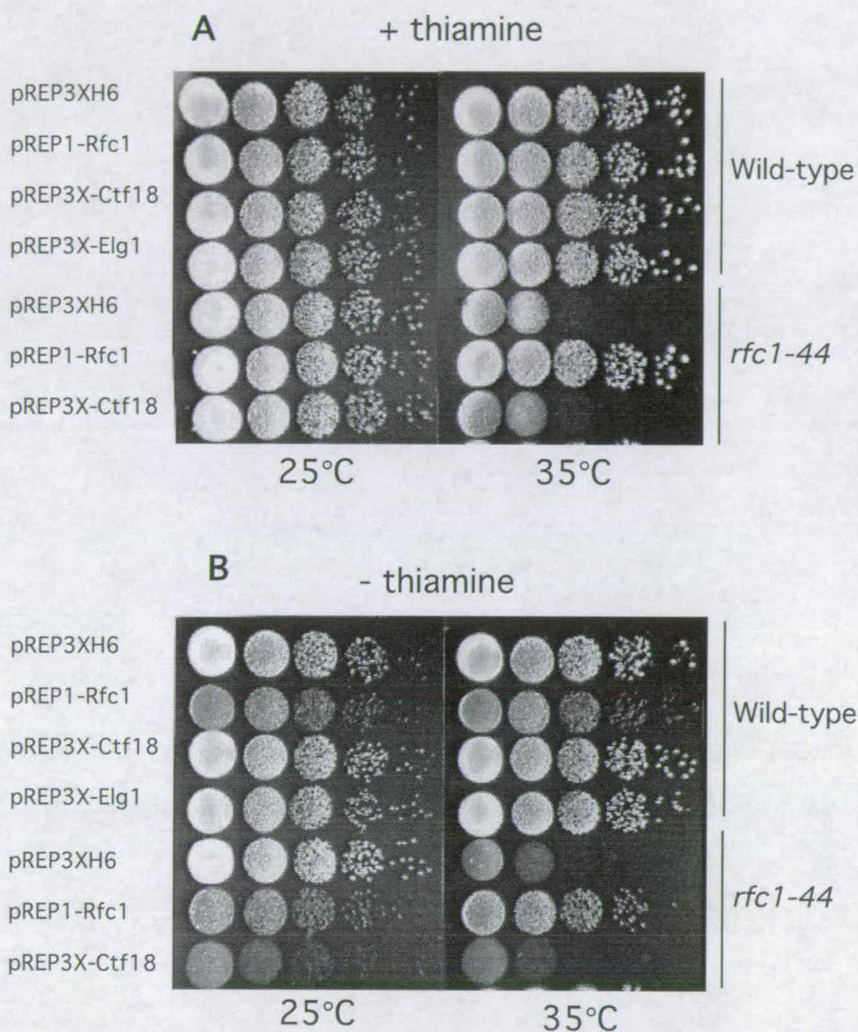


Figure 4.9 The over-expression of the Rfc1, Ctf18 and Elg1 in wild type and *rfc1-44* cells. The plasmid carrying the over-expression construct of Rfc1, Ctf18 or Elg1 was transformed into the wild type or rfc1-44 cells by electroporation. The transformants were cultured in the presence of 5μg/ml thiamine and transferred into the EMM medium with or without 5μg/ml thiamine and incubated for overnight. Exponentially growing cells were serially diluted 10 folds and the diluted cells were spotted on EMM plate with or without 5μg/ml thiamine, the cells were incubated at 25°C or 35°C until colony forms. **A**. The cells spotted on the EMM plate containing 5μg/ml thiamine. **B**. The cells spotted on EMM.

also showed negative effect at 25°C. However, when the over-expression of Rfc1 was repressed by thiamine, the temperature-sensitive mutant phenotype of *rfc1-44* was rescued at the restrictive temperature.

The over-expression of Ctf18 showed the dominant-negative effect in *rfc1-44* at both permissive and restrictive temperature in the absence of thiamine (Figure 4.9 B). The over-expression of Ctf18 did not rescue the temperature sensitive phenotype of *rfc1-44* at the restrictive temperature when the expression of the protein was suppressed by the thiamine (Figure 4.9 A).

The over-expression of Rfc1 in *ctf18Δ* also showed the dominant-negative effect on the growth of mutant cells in the absence of thiamine (Figure 4.10). However, the over-expression of Rfc1 in *elg1Δ* did not show the dominant-negative effect. At restrictive temperature, the *ctf18Δ* cells showed slow growth that was found previously (See section 4.4). The over-expression of Rfc1 did not rescue the growth defect of *ctf18Δ.* The over-expression of Ctf18 or Elg1 did not affect on the growth of the wild-type cells.

Over-expression of the proteins did not affect on cell viability when cells are spotted on plates containing DNA damaging agents in the presence of thiamine (data not shown). In the absence of thiamine, over-expression of the proteins did not affect on the viability of wild-type and mutant cells when the cells are spotted on a plate containing 6μM CPT. Again, high level of Rfc1 expressed in *ctf18Δ* and *elg1Δ* showed dominant-negative phenotype that was found in the earlier assay (Figure 4.11 right panel). The dominant negative effect of high-level of Rfc1 was also observed in the cells that were spotted on the plate containing 7.5mM HU too (Figure 4.11 left panel). The sensitive phenotype of *rfc1-44* to HU was not rescued by the over-expression of Ctf18. Interestingly, the over-expression of Elg1 in *ctf18Δ* cells showed negative effect on the viability of the cells (see Figure 4.10 bottom panel). It also increased the sensitivity of *ctf18Δ* to MMS. *ctf18Δ* showed highly sensitive phenotype to 0.005% MMS when Elg1 was over-expressed (Figure 4.11 Bottom middle panel). However, over-expression of Elg1 in wild-type cells or in *elg1Δ* cells did not show defects in the growth nor the sensitivity to DNA damaging agents.

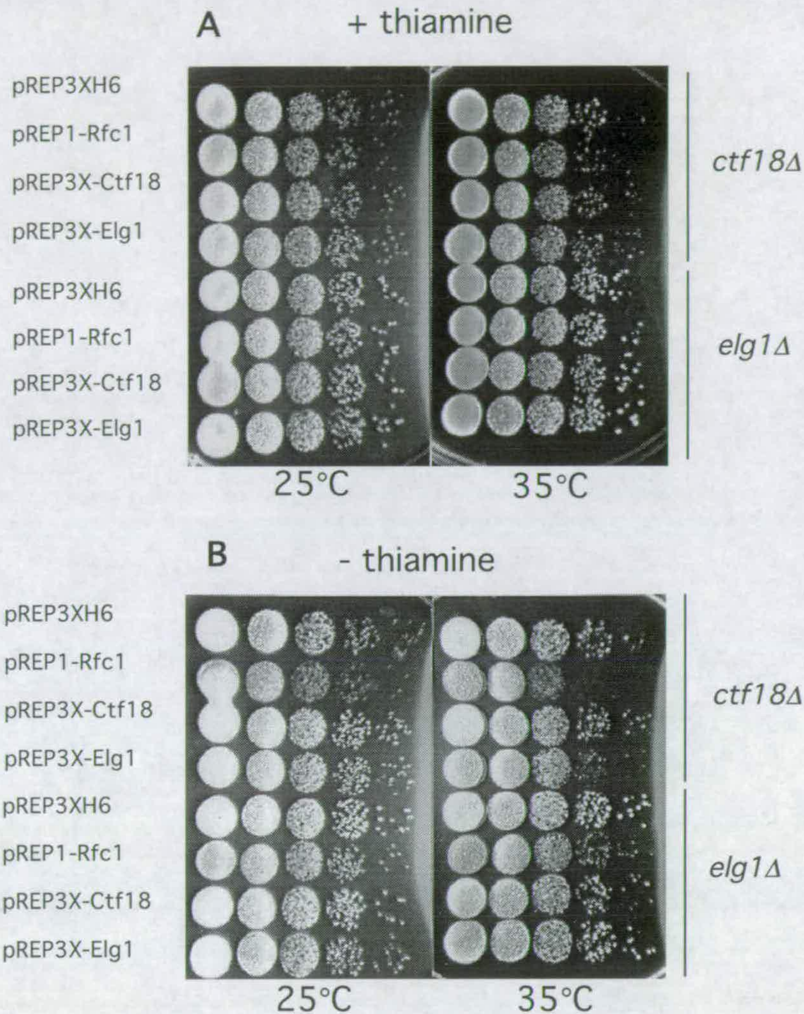


Figure 4.10 Over-expression of the proteins in *ctf18Δ* a nd *elg1Δ* c ells. The plasmid carrying the over-expression construct of Rfc1, Ctf18 or Elg1 was transformed into the wild type or rfc1-44 cells by electrophoration. The transformants were cultured in the presence of 5μg/ml thiamine and transferred into the EMM medium with or without 5μg/ml thiamine and incubated for overnight. Exponentially-growing cells were serially diluted 10 folds and the diluted cells were spotted on EMM plate with or without 5μg/ml thiamine, the cells were incubated at 25°C or 35°C until the colony formation. **A**. The cells spotted on the EMM plate containing 5μg/ml thiamine. **B**. The cells spotted on EMM

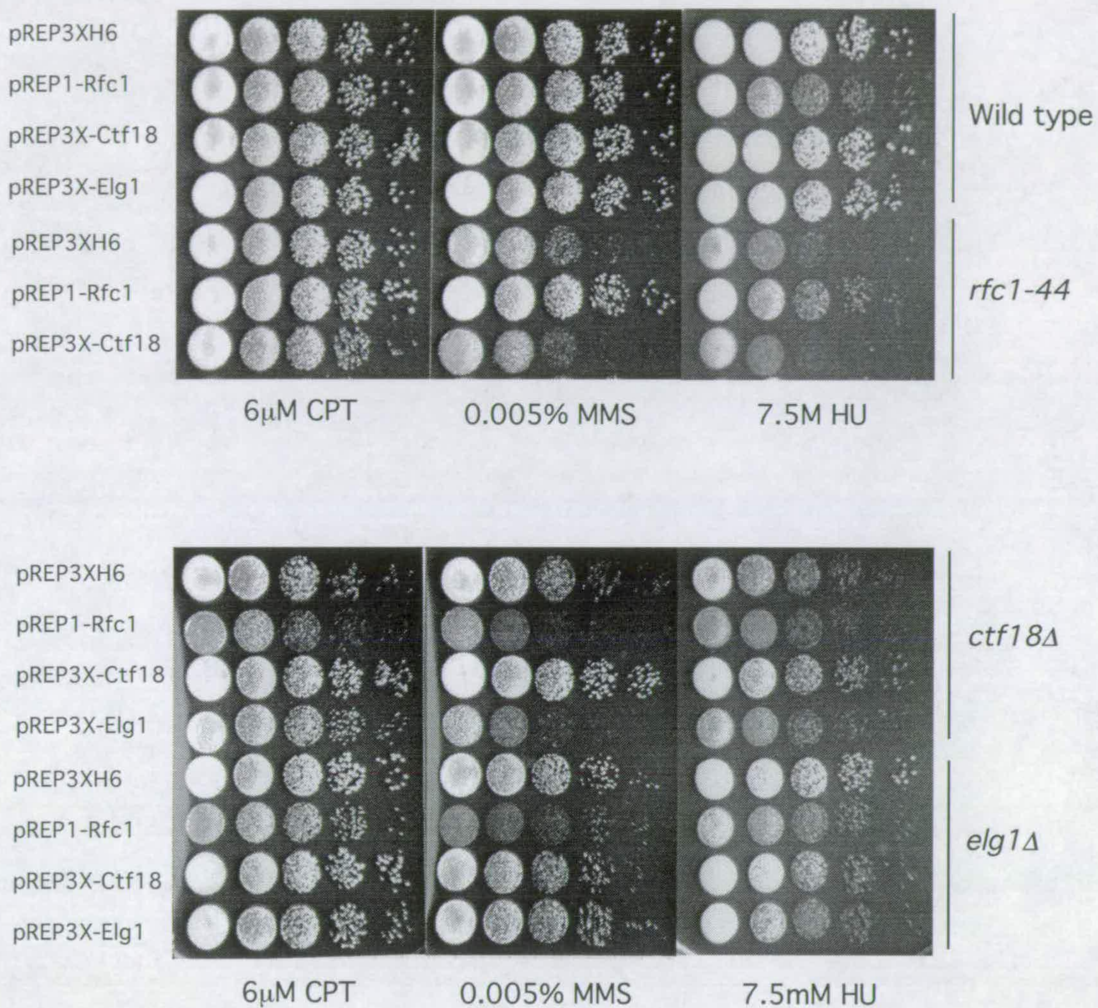


Figure 4.11 The over-expression of the proteins in the mutant cells. The plasmid carrying the over-expression construct of Rfc1, Elg1 and Ctf18 were transformed into wild type, *rfc1-44*, *ctf18Δ* or *elg1Δ* cells by transformation. As a control, the backbone vector was transformed. The isolated transformants are inoculated in EMM medium containing 5μg/ml thiamine and transferred into the EMM media without thiamine. Exponentially-growing cells were serially diluted 10-fold and spotted on the palte containing 6μM CPT, 7.5mM HU or 0.005% MMS. Cells were incubated at 25°C until colony forms.

4.9 Discussion

In this chapter the roles of three clamp loaders were examined. To understand the role of Elg1 and Ctf18 in *rfc1-44*, the genes were deleted in an *rfc1-44* background by genetic cross. Construction of *rfc1-44 ctf18Δ*, *rfc1-44 dcc1Δ* and *rfc1-44 ctf8Δ* failed because the double mutants showed synthetic lethal phenotypes suggesting the positive role of Ctf18 for *rfc1-44* viability. Moreover, the *ctf18Δ* cells showed weak temperature-sensitive phenotype indicating that the function of Ctf18 is required for cell viability. However, over-expression of Ctf18 in *rfc1-44* did not rescue the temperature-sensitive phenotype or sensitivity to DNA damaging agents of the mutant cells. Four viable spores were generated from the genetic cross of *rfc1-44* and *elg1Δ* indicating the *rfc1-44 elg1Δ* cells are viable. *elg1Δ and ctf18Δ elg1Δ* cells showed better growth than wild-type cells in the presence of 7.5mM HU suggesting that Elg1 plays negative role in eukaryotic cells. The possible negative role of Elg1 was supported by the restoration of viability of *rfc1-44 ctf18Δ* in *rfc1-44 ctf18Δ elg1Δ*, the highly elongated and sick phenotype of *rfc1-44* at high level of Elg1, and increased sensitivity to DNA damaging agents in *ctf18Δ* when Elg1 was over-expressed. Comparison of the chromosome structure between *rfc1-44* and *rfc1-44 elg1Δ* suggested that Elg1 plays a negative role though the regulation of chromosome integrity. From the genetic cross between *rfc1-44 elg1Δ* and *ctf18Δ elg1Δ* and subsequent tetrad analysis of the spores, *rfc1-44 ctf18Δ elg1Δ* was generated. The triple mutant cells showed accumulation of 1C content at restrictive temperature and sensitivity to CPT. High-level of Rfc1 in wild-type and mutant cells showed negative effect on the viability of the cells and increased the sensitivity to DNA damaging agents suggesting that high level of Rfc1 is toxic for the cells.

The synthetic lethal phenotype of *rfc1-44 ctf18Δ, rfc1-44 dcc1Δ* and *rfc1-44 ctf8Δ* indicates that the normal functions of Ctf18, Dcc1 and Ctf8 are required for the viability of *rfc1-44*. The fact that all three proteins are required for the viability of *rfc1-44* suggests that the function of Ctf18-RLC, rather than the function of Ctf18 itself, is essential for the viability of *rfc1-44*. Two different groups have demonstrated PCNA loading by Ctf18-RLC *in vitro* (Bermudez *et al.*, 2003, Shiomi *et al.*, 2004). The data acquired in this chapter and the previous biochemical data

suggest that the Ctf18-RLC plays a role in PCNA loading onto DNA when there is a defect in the function of RFC as a clamp loader. It is possible therefore that the Ctf18 plays a role supporting the function of RFC in DNA metabolism.

Another possible reason is that sister chromatid cohesion failure has resulted in the synthetic lethal phenotype. It has been suggested that the DNA replication and sister chromatid cohesion are linked. The proteins involved in DNA replication are thought be required for the sister chromatid cohesion (Mayer *et al.,* 2003, Skibbens *et al.*, 1999). It is possible that the mutation in Rfc1 C-terminal domain has caused defects in the sister chromatid cohesion and further inactivation of proteins involved in the sister chromatid cohesion (Ctf18, Ctf8 and Dcc1) has resulted in the synthetic lethal phenotype.

The fact that *elg1Δ* and *ctf18Δ elg1Δ* cells show better growth in the presence of HU suggests that the Elg1 plays a negative role in cells. The idea is supported by the recovery of the cell viability of *rfc1-44 ctf18Δ* by further deletion of *elg1*$^{+}$ from the mutant cells. Over-expression of Elg1 in *rfc1-44* resulted in no colony formation with highly elongated and sick cells in permissive temperature emphasizes the negative role of Elg1. Over-expression of Elg1 also increased the sensitivity to MMS and HU in *ctf18Δ*. More Stable chromosome structure in *rfc1-44 elg1Δ* than in *rfc1-44* suggests that the negative role of Elg1 in the cells might come from its involvement in recombination pathway. *elg1Δ* mutant cells show increased level of crossing over, chromosome loss and sister chromatid recombination (Ben-Aroya *et al.*, 2003). The *elg1Δ* cells results in synthetic lethal phenotype when crossed with the deletion mutants involved in recombination pathway (Rad50, Rad52, Rad57, Mre11 etc). These results suggest that the Elg1 is involved in the chromosome integrity maintenance through recombination. In wild-type cells, deletion or over-expression of Elg1 does not show any defect in the growth or the sensitivity to DNA damaging agents in *S.cerevisiae*. However, over-expression of Elg1 in *rfc1-44* caused severe growth defect and elongated phenotype suggesting the defect in S phase progression. The stable chromosome integrity in *rfc1-44 elg1Δ* suggests that the normal recombination function of Elg1 can cause defects in chromosome integrity.

Over-expression of Ctf18 did not rescue either the temperature-sensitive phenotype of *rfc1-44* or the sensitivity of *rfc1-44* to DNA damaging agents. At the permissive

temperature the over-expression of Ctf18 in *rfc1-44* resulted in growth defect. This is the opposite of the previous data that the deletion of *ctf18*$^+$ caused a lethal phenotype in *rfc1-44*. It is possible that the over-expression of Ctf18 but without co-over-expression of Dcc1 and Ctf8 results in the formation of inactive and incomplete Ctf18-RLC complex. According to Mayer *et al.*, Ctf18 directly interacts with the RFC small subunits (Mayer *et al.,* 2003). In addition, a five subunits complex composed of Ctf18 and the four small subunits of RFC was purified *in vitro* (Bermudez *et al.,* 2003). However, *rfc1-44 dcc1Δ* and *rfc1-44 ctf8Δ* cells showed synthetic lethal phenotype indicating that *rfc1-44* requires Dcc1 and Ctf8 for viability. Therefore the high ratio of Ctf18 to Dcc1 and Ctf8 can form incomplete Ctf18-RLC that competes with intact Ctf18-RLC for PCNA loading. As a result over-expression of Ctf18 may show negative effect on the viability for the *rfc1-44*. Co-expression of Dcc1 and Ctf8 with Ctf18 is required to know whether the Ctf18-RLC plays a positive role for DNA replication in eukaryotic cells.

When all three clamp loaders were inactive, the mutant cells showed gross replication defect at restrictive temperature. At the permissive temperature, the triple mutant cells showed sensitivity to CPT, an inhibitor of topoisomerase I that causes replication forks to stall. It is possible that the inactivation of all three clamp loaders at the replication fork has caused a defect in the re-start from the stalled replication fork. It also suggests that all three clamp loaders are involved in the S phase progression.

4.10 Summary

In this chapter the role of three clamp loaders were examined. In genetic crosses the *rfc1-44 ctf18Δ* double mutant showed a synthetic lethal phenotype that was recovered by further deletion of *elg1*$^+$. These results suggest that the Ctf18 plays a positive role for the viability of *rfc1-44* while Elg1 plays a negative role. The idea of negative role of Elg1 was supported by the better growth of *elg1Δ* cells in the presence of HU, the lethal phenotype of *rfc1-44* at permissive temperature when Elg1 was over-expressed, and the increase of sensitivity to DNA damaging agents in *ctf18Δ* under the over-expression of Elg1. The pulse field gel electrophoresis result

that the chromosomes from *rfc1-44 elg1Δ* maintain chromosome integrity, while chromosomes from *rfc1-44* do not, suggest that the normal role of Elg1 as a recombination protein might have caused defect in *rfc1-44*.

In the FACS analysis, the *rfc1-44 ctf18Δ elg1Δ* cells showed a gross replication defect and accumulation of 1C DNA content, indicating that there is a severe defect in DNA replication. It suggests that all three PCNA loaders are required for normal S phase progression.

Chapter 5. Function of Rfc1 C-terminal domain

5.1 Introduction

Recently, the structure of *S.cerevisiae* RFC was solved (Bowman *et al.*, 2004). In this structure the C-terminal domain of Rfc1 interacts with the N-terminal domain of Rfc5. From the overall structure of RFC and the B-form DNA structure, a model for the DNA binding of RFC was suggested. In the model, the RFC threads onto the minor groove of double helix like a screw-cap. The model also suggested that the end of primer strand or template strand of DNA positions in near Rfc5 or the gap between Rfc5 and Rfc1 respectively (Bowman *et al.*, 2004).

The suggested DNA binding model of *S.cerevisiae* RFC indicates that the interaction between Rfc5 and Rfc1 is important for the holding of the template strand of primer DNA within RFC. In order to find out whether the Rfc1 C-terminal domain interacts with the N-terminus of Rfc5, mutation analysis of Rfc5 N-terminal domain in *rfc1-44* was performed.

In addition, an *S.pombe* genomic DNA library was screened for suppression of *rfc1-44*.

5.2 Screen for Rfc5 mutants that suppresses *rfc1-44*

5.2.1 Strategy

The crystal structure of *S.cerevisiae* RFC complex revealed that each subunit is composed of three structural domains, domain I, II and III. Domain I and II contain ATPase motif and sensor domain respectively that plays important role for PCNA loading. Together, these two domains form AAA^+ module that is connected by a flexible linker to domain III. Domain III of all RFC subunits pack together to form a stable cylindrical structure, 'collar'. *S.cerevisiae* RFC structure showed that the N-terminal domain of Rfc5 interacts with the C-terminal region of Rfc1 (Bowman *et al.*, 2004).

In order to find out the domains interacting with the Rfc1 C-terminal domain, the DNA corresponding both domains I and II of Rfc5 was subcloned and used for the mutagenesis. The 1070 nucleotides from the N-terminus of *rfc5+* were amplified by PCR. To avoid the removal of promoter during the integration of the mutant DNA into the chromosome, the upstream 200 nucleotides from the open reading frame were amplified and subcloned. Transposon-based pentapeptide insertion mutagenesis was used to generate mutation library of the N-terminus of Rfc5. In order to know the diversity of the mutation library, the plasmids were transformed in *E.coli* cells. The *rfc5* gene was amplified from the *E.coli* cells by colony PCR and digested with two different restriction enzymes in combination with *Kpn* I. There was no same insertion of nucleotides when seven PCR products were examined suggesting that the mutation library contains various mutations.

The regions subjected to mutagenesis are indicated in Figure 5.1. The mutation library was constructed by collecting the mutations generated by transposon method.

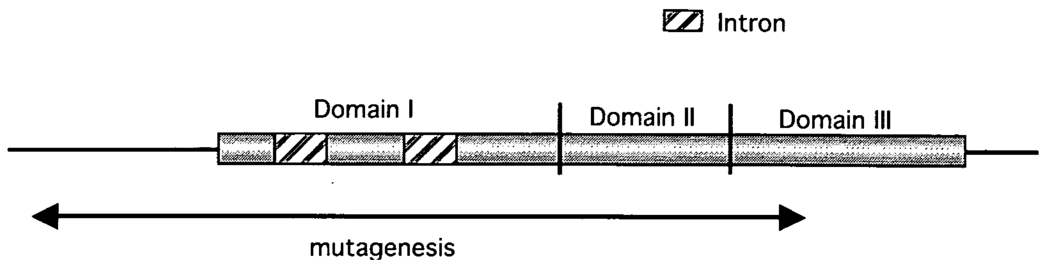


Figure 5.1 The domains used in the mutagenesis. The domains indicated are divided based on the crystal structure of δ' subunit of *E.coli* γ complex (Guenther *et al.*, 1997). The two introns within domain I are indicated by separate box. The regions subjected to the mutagenesis are indicated by arrow.

5.2.2 Screening Rfc5 mutants as suppressors of *rfc1-44*

The Rfc5 mutation library was cut by *PstI* restriction enzyme and the DNAs were transformed into *rfc1-44* (*rfc1-44 ura4-D18 h*⁻). Cells were plated on an EMM plate and incubated overnight at 25°C and transferred to 35°C and further incubated until colonies formed. Ten candidates were acquired from 2,400 transformants. The candidate cells suppressed the temperature sensitive phenotype of *rfc1-44* at high temperature (Figure 5.3). However, the double mutant cells still showed elongated phenotype when examined under the microscope. The genomic DNA from the candidates was amplified by PCR and the PCR products were sequenced to examine the position and sequences of the mutations. Five of the candidates contained pentapeptide insertion at the same position. The sixth contained an insertion four nucleotides downstream from the mutation site of other five candidates. The translation of the DNA revealed that the sequence PSRGT was inserted between Thr75 and Pro76 in five identical mutants (*rfc5-S1*), wherease SRGTP were inserted between Pro76 and Ser77 in the sixth mutant. Both mutations were within the domain I of the crystal structure. The position of penta-peptide insertion was compared with the *S.cerevisiae* Rfc1 sequences that interact with the N-terminal domain of *S.cerevisiae* Rfc5. The information submitted in the Protein Data Bank (http://www.rcsb.org/pdb) was used for the analysis of mutant positions. The

sequence of *S.pombe* Rfc1 was aligned with *S.cerevisiae* Rfc1 and the potential interacting domain was determined. The mutation in Rfc5 was within the sequences that interact with Rfc1 C-terminal domain. Unfortunately, the *rfc1-44* mutation was within the sequences that the density of the crystal was too low to interpret. However, the *rfc1-44* mutation was very close from the amino acids that interact with Rfc5 N-terminal domain (See figure 2.1B). The modelled structure of RFC and the Rfc5-Rfc1 interaction with the position of Rfc5 and Rfc1 mutation are indicated in Figure 5.2. The modelled structure of RFC was copied from Bowman *et al.* and modified to indicate the mutations in Rfc5 and Rfc1.

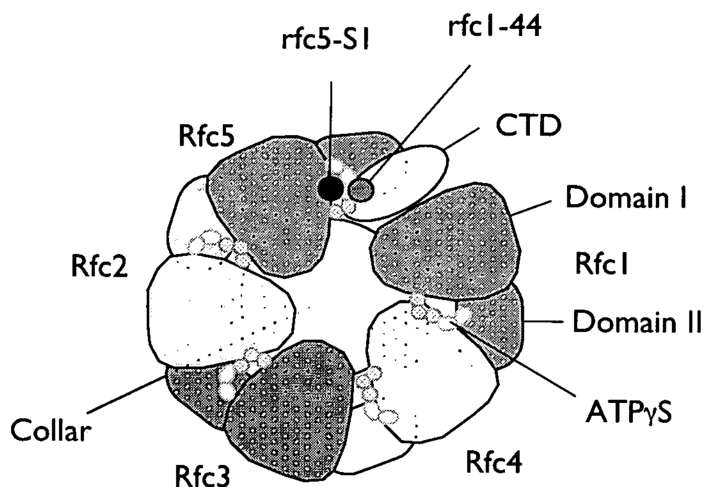


Figure 5.2 The positions of mutations in *rfc1-44* and *rfc5-S1*. The diagram of *S.cerevisiae* RFC is copied from Bowman *et al.*, and the position of pentapeptide insertions in *rfc1-44* and *rfc5-S1* are indicated.

5.2.3 Suppression of the temperature sensitive phenotype of *rfc1-44*

The suppression of the mutant phenotype of *rfc1-44* was examined by the spotting assay. Exponentially growing wild-type, *rfc1-44* and the double mutant cells (one of the five identical mutants, named as *rfc1-44 rfc5-S1*) were spotted on YE or YE plate with DNA damaging agents. The plates are incubated at 25°C or 35°C until the colonies form. Wild-type, *rfc1-44* and *rfc1-44 rfc5-S1* cells grew well at 25°C. *rfc1-44* showed temperature sensitive phenotype that was suppressed by the second mutation in $rfc5^{+}$ (Figure 5.3). However, the suppression of mutant phenotype by Rfc5 mutation was restricted to the temperature sensitivity. The sensitivity to DNA damaging agents was not suppressed by the mutation in Rfc5. Both *rfc1-44* and *rfc1-44 rfc5-S1* showed growth defect in the presence of 5mM HU or 0.005% MMS while wild-type cells grew well (Figure 5.3).

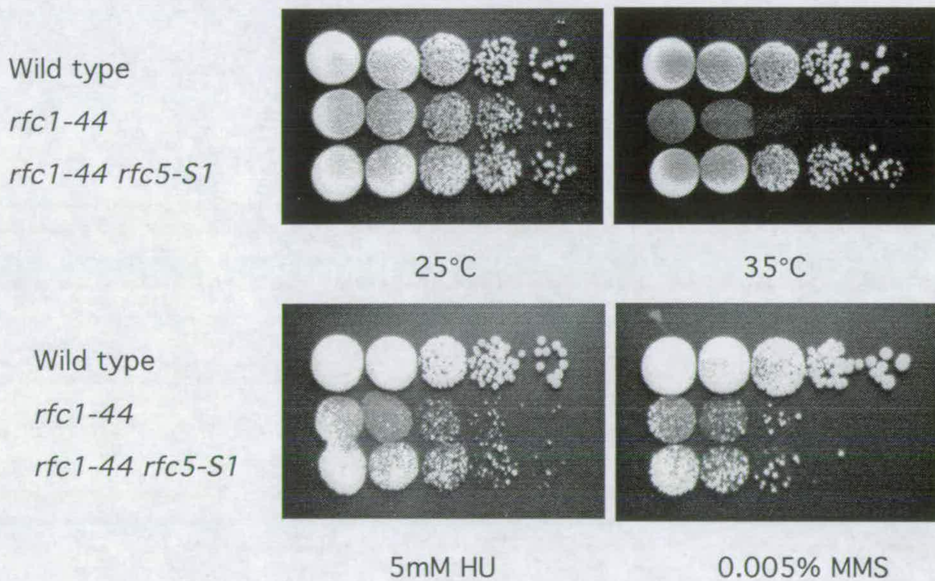


Figure 5.3 The suppression of the temperature-sensitive phenotype of *rfc1-44* by the mutation in Rfc5. Exponentially-growing wild-type, *rfc1-44* or *rfc1-44 rfc5-S1* cells were plated in YE or YE plate containing 5mM HU or 0.005% MMS. Cells were serially diluted 10-fold from 10^7 to 10^3/ml. 10μl of diluted cells were dropped on each spot. Cells were incubated at 25°C or 35°C. The cells spotted on the plate containing HU or MMS were incubated at 25°C.

5. 3 Screening of plasmid suppressors of *rfc1-44*

In an effort to identify genes which over-expression suppressed the *rfc1-44* phenotype, *S.pombe* genomic library was screened. Screening of proteins that suppress the temperature-sensitive phenotype of the mutant will provide a clue about the function of Rfc1 C-terminal domain. *S.pombe* genomic DNA library screening was performed to find out the protein that suppresses the mutant phenotype.

S.pombe genomic DNA libraries were kindly provided by Dr. Hiroyuki Tanaka (University of Edinburgh). Three restriction enzymes (*BamHI*, *SpeI* and *HindIII*) were used for the construction of genomic DNA libraries with the pSL backbone vector. All three libraries were transformed into *rfc1-44* (*ade6-M210 leu1-32 ura4-D18 h^-*) by electroporation and transformants were incubated overnight at 28°C before being transferred to 35°C and incubated until colonies formed. 26 colonies were formed at high temperature in *SpeI* library among 4.6 x 10^4 transfomants. In the *BamHI* library, 27 colonies were formed among 4.56 x 10^4 cells and 29 colonies were formed among 3.12 x 10^4 transformants in *HindIII* library (Table 5.1). However, many candidate cells did not grow when the cells were streaked on a YE plate and incubated at 35°C. Total of 13 transformants grew at high temperature.

Table 5.1 Screening of the plasmid suppressors of *rfc1-44*. To count the number of transformants, the transformants were diluted 100-fold and plated on EMM plate containing appropriate supplements and incubated at 25°C for 7 days. The number of colonies formed were counted and multiplied 100 times.

	BamHI	*HindIII*	*SpeI*
Transformants	4.56 x10^4	3.12 x 10^4	4.6 x 10^4
Candidates	27	26	29
Suppressors	3	7	3
rfc1	1	2	0
sks2$^+$	2	1	0

The plasmid DNA was recovered from *S.pombe* cells and transformed into *E.coli*. Plasmid DNA was purified and re-transformed into *rfc1-44* to check its suppression. 3 plasmids from *BamHI* library, 7 plasmids from *HindIII* library and 3 plasmids from *SpeI* library were able to suppress the temperature sensitive phenotype of *rfc1-44*. The plasmids were sequenced with pBS1 or pBS 2 primer (from Dr. Hiroyuki Tanaka). Three plasmids contained *rfc1* gene in their sequences (one from *BamHI* library and two from *HindIII* library) From the *SpeI* library no plasmid containing *rfc1* gene was isolated. The plasmid recovered from *HindIII* library lacked 20 nucleotides from the 5' end of $rfc1^+$. $rfc1^+$ contains *Hind III* restriction enzyme site about 20 nucleotide downstream from the 5' end of open reading frame indicating that the 20 nucleotides were deleted during the construction of library.

Most of other suppressors were encoding the genes involved in the metabolism of *S.pombe* cells or some mitochondrial genes that identified only once from all three libraries. Two plasmids from *BamHI* library and one plasmid from *HindIII* library contained identical protein called Sks2. The plasmids encoding *sks2+* suppressed the temperature-sensitive phenotype of *rfc1-44* (Figure 5.4).

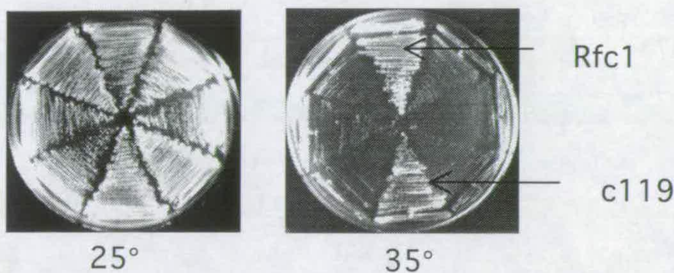


Figure 5.4 Suppression of the temperature sensitive phenotype of *rfc1-44*. The cells carrying either Rfc1 or Sks2 (c119) suppress the temperature-sensitive phenotype of *rfc1-44*. The candidates isolated from the screening with *S.pombe* genomic DNA library were patched on YE plate and incubated at 25°C or 35°C and incubated for 2 days.

Sks2 is a member of Heat Shock Protein 70 (HSP70) family playing a role as a chaperon. In *S.pombe sks2+* is in chromosome II encoding 62.7kDa protein (Figure 5.5). Sks2 was first identified as a suppressor for K-252α, a protein kinase inhibitor sensitive mutant (Usui *et. al.* 1997). $sks2^+$ disruption mutant cells did not show

increased sensitivity to K-252α but showed slow growth and elongated cell phenotype suggesting its involvement in cell cycle progression.

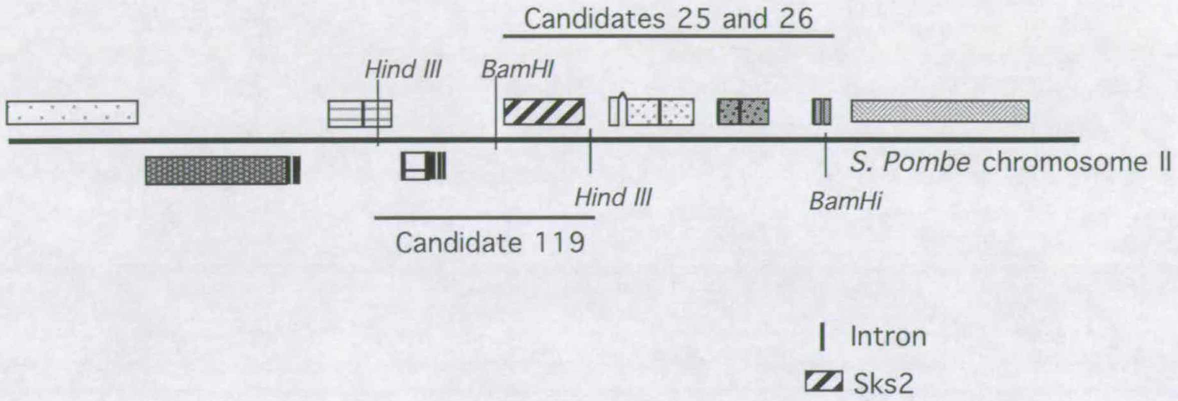


Figure 5.5 Chromosomal locus of *sks2*$^{+}$. *sks2*$^{+}$ is in *S.pombe* chromosome II between dihydrourine synthease and cyclophillin. The intron sequences within the gene are indicated. The DNA fragment isolated from the screening is indicated by the bar.

5.4 Discussion

In order to understand the role of the C-terminal domain of Rfc1, two genetic screens were performed. First, based on the result of crystal structure, the *rfc5* mutants were screened to know whether the Rfc1 C-terminal domain interacts with Rfc5 N-terminal domain. From the screening of 2,400 transformants, six mutant cells showed suppression of the temperature-sensitive phenotype of *rfc1-44*. Five mutant cells carried DNA with same mutation in the same position and one of the mutants was containing mutation in the four nucleotides downstream of the other five mutations. The sequence alignment results showed that the mutation in Rfc5 N-terminal domain is within the sequences known to be involved in the Rfc1 interaction. The density of the crystal structure in *rfc1-44* mutation region was too low to be interpreted, but the mutation was in proximity of the sequences that involved in the direct interaction with Rfc5. In the spotting assay, the isolated mutant strain (*rfc1-44 rfc5-S1*) suppressed the temperature-sensitive phenotype of *rfc1-44* but it did not suppress the sensitivity to DNA damaging agents. In the screening with *S.pombe* genomic DNA library, *sks2*$^+$ was isolated as a suppressor for the temperature-sensitive phenotype of *rfc1-44*.

The isolation of *rfc5* mutant suppressors for *rfc1-44* strongly suggests that the C-terminal domain of Rfc1 interacts with the N-terminal domain of Rfc5. The fact that position of Rfc5 and Rfc1 mutations are within or near the Rfc1-Rfc5 interaction region suggest that the temperature-sensitive mutant phenotype of *rfc1-44* may results from the failure of interaction with Rfc5. According to the RFC-DNA interaction model, the template strand of primer DNA positions within the gap between Rfc5 and Rfc1 C-terminal domain (Bowman *et al.*, 2004). If it is correct, it is possible that the C-terminal domain of Rfc1 plays important role for the DNA interaction. However, several previous biochemical results showed that the domain around the BRCT motif is responsible for the interaction with DNA (Fotedar *et al.*, 1996, Uhlmann *et al.* 1997). To understand whether the Rfc1 C-terminal domain interacts with the N-terminal domain of Rfc5 and whether the interaction is important for the binding to DNA, it will be necessary to purify RFC with the mutant large subunit and to examine the DNA binding activity of the mutant RFC *in vitro*.

It is reasonable that a heat shock protein was isolated as a suppressor of *rfc1-44*. The mutation in *rfc1-44* was generated by integration of five amino acids within the normal amino acid sequences of Rfc1. The integration of five amino acids can force conformational change that inactivates Rfc1. As a heat shock protein, Sks2 can unfold the distorted structure that resulted in the suppression of the temperature-sensitive phenotype of *rfc1-44*. This result also suggests that Rfc1 is a substrate for Sks2. However, the isolation of Sks2 as a suppressor for *rfc1-44* does not provide a clue about the function of Rfc1 C-terminal domain.

5.5 Summary

The suppressors of *rfc1-44* were isolated from the *rfc5* mutation library or *S.pombe* genomic DNA library. From *rfc5* mutation library, six suppressors were isolated that contained mutation within the amino acid sequences responsible for Rfc1 interaction. From *S.pombe* genomic DNA library, three suppressors were isolated that encoding the Sks2 gene. The interaction between Rfc5 and the C-terminal domain of Rfc1 suggests that these two subunits of RFC are responsible for the interaction with DNA.

Chapter 6 Discussion

6.1 Summary of results

This section summarises the results that have been presented in this work.

The role of Rfc1 C-terminal domain was examined in *S.pombe*. Insertion of a stop codon at the starting point of the C-terminal domain of Rfc1 resulted in a lethal phenotype. The lethal phenotype of *rfc1-ΔC* cells was rescued by expression of wild-type Rfc1.

Mutational analysis of C-terminal domain with point mutation of highly conserved amino acids or with random penta-peptide insertion mutation resulted in one lethal, two temperature-sensitive, one cold-sensitive and five DNA damaging agents sensitive mutants. The characteristics of *rfc1-44* and *rfc1-54*, the temperature-sensitive mutants, were examined. The mutant cells showed temperature-sensitive phenotype at 35°C and sensitivity to HU, MMS, and UV. *rfc1-44* did not show sensitivity to CPT while *rfc1-54* showed sensitivity to CPT.

rfc1-44 cells showed slow but steady growth and no obvious accumulation of 1C DNA at restrictive temperature. However, the chromosomes of mutant cells showed aberrant mobility in PFGE. The double mutant cells showed synthetic lethal phenotype when the *rfc1-44* was crossed with the mutant cells that have defect in Pol3, Cdc1, Cdc27 and Dna2. Over-expression of replication proteins such as DNA polymerase δ subunits, RFC small subunits or PCNA in *rfc1-44* did not rescue the temperature-sensitive phenotype of *rfc1-44*.

In a suppressor screening with three different *S.pombe* genomic libraries, three plasmids containing Sks2 protein were isolated as a suppressor from two different genomic libraries. In a screening with Rfc5 N-terminal domain mutation library, six suppressors were isolated that containing mutation in the Rfc1-Rfc5 interacting domains.

The role of three clamp loaders was examined. *rfc1-44 ctf18Δ*, *rfc1-44 ctf8Δ*, *rfc1-44 dcc1Δ* cells showed synthetic lethal phenotype while *rfc1-44 elg1Δ* and *rfc1-44 ctf18Δ elg1Δ* were viable at permissive temperature. The triple mutant cells showed accumulation of 1C DNA at restrictive temperature. Chromosomes of *rfc1-44 elg1Δ* showed aberrant mobility in the gel but moderate integrity was maintained for four

hours while the chromosomes of *rfc1-44* showed increasing abnormality over time. *elg1Δ*, or *elg1Δ ctf18Δ* showed better growth in the presence of high concentration of HU. Different from *S.cerevisiae ctf18Δ*, *S.pombe ctf18Δ* cells showed moderate growth defect at restrictive temperature. Triple mutant cells showed sensitivity to CPT while both *rfc1-44* and *rfc1-44 elg1Δ* were not sensitive to CPT. Over-expression of Elg1 in *rfc1-44* resulted in lethal phenotype and over-expression of Ctf18 in *rfc1-44* resulted in highly elongated cells at restrictive temperature. Over-expression of Elg1 in *ctf18Δ* increased the sensitivity to MMS and HU of the mutant cells.

6.2 Role of Rfc1 C-terminal domain

6.2.1 C-terminal domain of Rfc1 is essential

The lethal phenotype of *rfc1-ΔC* mutant cells indicated that the C-terminal domain of Rfc1 is essential for cell viability. The requirement of C-terminal domain for cell viability was supported by the transformation of wild-type Rfc1 in the C-terminal domain deletion mutant cells that rescued the viability of the mutant cells. The evolutionary conservation of C-terminal domain across species supports the idea of essential role of C-terminal domain in the RFC function. Interestingly, Ctf18 and Elg1, the large subunits of alternative RLC, also contain long C-terminal domains (Mayer *et al.*, 2001, Kanellis *et al.*, 2003, Bon-Aroya *et al.*, 2003, Bellaoui *et al.*, 2003). However, amino acid sequences of the C-terminal domain of three PCNA loaders are not conserved. It will be intriguing to examine whether the C-terminal domain of three clamp loaders are interchangeable.

6.2.2 rfc1-44 shows DNA replication defect

Incomplete growth arrest and no obvious accumulation of 1C DNA in *rfc1-44* cell at the restrictive temperature suggests that the temperature-sensitive mutant phenotype of the mutant cells did not result from a gross defect in DNA replication. However, the highly elongated phenotype at the restrictive temperature, aberrant mobility of

rfc1-44 chromosomes and the synthetic lethal phenotype of double mutant cells indicate that the *rfc1-44* has replication defect at the restrictive temperature.

Many chromosome integrity defects result from replication defects. The chromosomes from *mcm5* temperature-sensitive mutants migrated with significantly decreased intensity after the incubation at restrictive temperature (Takahashi *et al.*, 1994) suggesting that replication defect inhibits the normal mobility of chromosomes. The *cdc24* mutant chromosomes showed abnormal mobility that the chromosomes migrated faster than chromosome III, the smallest *S.pombe* chromosome, in addition to the chromosomes that did not enter the gel (Gould *et al.*, 1997). Cdc24 is a protein involved in S phase completion and Okazaki fragment maturation (Gould *et al.*, 1998 Tanaka *et al.*, 1999). The chromosomes from *rfc1-44* did not enter the gel or migrated faster than chromosome III similar to the chromosomes from *cdc24* mutant cells. The chromosome migration pattern of the mutant cells suggests that *rfc1-44* have defect in DNA replication.

The synthetic lethal phenotype of double mutants suggests that the two proteins are functionally linked (Bellaoui *et al.*, 2003, Smolikov *et al.*, 2004). The functions of many proteins are elucidated from the synthetic lethal phenotype of double mutant cells (Smolikov *et al.*, 2004, Smith *et al.*, 2004). The synthetic lethal phenotype of double mutants between *rfc1-44* and the replication mutants suggests that the temperature-sensitive mutant phenotype of *rfc1-44* resulted from the defect in DNA replication.

Moreover, the highly elongated phenotype seen with *rfc1-44* is commonly found in the DNA replication mutants including *pol3* temperature-sensitive mutant (Francesconi *et al.*, 1993), *mcm* mutants (Miyake *et al.*, 1993), DNA polymerase ε mutant (Feng *et al.*, 2003) and *pcn1* mutant (Waseem *et al.*, 1992). *rfc1-44* shows highly elongated phenotype at restrictive temperature.

Together, these results indicate that the *rfc1-44* has defect in DNA replication at restrictive temperature. The replication defect in *rfc1-44* suggests that the C-terminal domain plays a role in DNA replication.

6.2.3 Role of Rfc1 C-terminal domain

The characteristics of *rfc1-44* mutant cells suggest that the C-terminal domain of Rfc1 functions in DNA replication. The question to be answered, is what does the C-terminal domain of Rfc1 do in DNA replication?

The results from the screening of Rfc5 mutant suppressor suggest that the temperature-sensitive mutant phenotype of *rfc1-44* was caused from the failure of interaction between Rfc1 and Rfc5. The crystal structure of *S.cerevisiae* RFC has suggested that the 5' terminus of template strand of the primer is positioned near the opening between Rfc1 and Rfc5 (Bowman *et al.*, 2004). This model and the *rfc5* mutant suppressors suggest that the Rfc1 C-terminal domain is involved in the primer binding. Similar results were acquired from the structural analysis of archaeal RFC. The electron microscope analysis of *Pyrococcus furiosus* RFC-PCNA complex has revealed that the large and small subunit of *P.furiosus* RFC forms a horseshoe shaped structure. In structure analysis with the primed DNA, the 5' end of the primer was passing through the horseshoe (Miyata *et al.*, 2004). These results suggest that the Rfc1 C-terminal domain interacts with the N-terminal domain of Rfc5 to form a complex. There is a gap between Rfc1 and Rfc5 interaction that can bind and hold primed DNA. The temperature-sensitive mutant phenotype of *rfc1-44* might is caused from the distortion of three-dimensional structure by penta-peptide insertion resulting in failure of DNA binding.

However, the large subunit of *P.furiosus* RFC does not contain the C-terminal domain that binds and holds the primed DNA (Cann *et al.*, 2001). In addition, many previous data suggested that the DNA binding activity exist in the BRCT-domain containing N-terminal region of Rfc1 (Fotedar *et al.*, 1996, Burbelo *et al.*, 1993, Allen *et al.*, 1998). Reconstitution assay of human RFC with truncated Rfc1 suggested that the middle domain containing RFC box IV to VII is important for DNA binding (Uhlmann *et al.*, 1997) that is upstream sequences of the C-terminal domain. These results are the opposite of the results from structural analysis. It is required to purify recombinant RFC complex with the Rfc1 is replaced with *Rfc1-44* mutant and examine the DNA binding activity of mutant protein complex *in vitro*.

The crystal structure of *S.cerevisiae* RFC was examined with the mutant Rfc1 protein. 76 amino acids from the C-terminus and 294 amino acids from the N-terminus were truncated for the crystallization. The sequence alignment of Rfc1 C-terminal domain between *S.cerevsiae* and *S.pombe* indicates that the mutant Rfc1 only contains two third of the C-terminal domain. Moreover, the amino acids sequences involved in the Rfc5 interaction exist upstream of the *rfc1-44* mutation site which positions approximately one third from the N-terminus of C-terminal domain. This suggests that the primed-DNA binding activity by Rfc1 takes place in the upstream sequences of Rfc1 C-terminal domain. It still leaves a question about the role of C-terminal domain

The clue about the function of Rfc1 C-terminal domain can be found in the analysis of the τ subunit, a subunit of *E.coli* γ complex. τ and γ subunits of γ complex shares same amino acids sequences but the τ subunits contains additional amino acids at the C-terminal domain (Kodaira *et al.*, 1983). The τ subunit dimerises the DNA polymerase III core enzyme in *E.coli* (McHenry 1982) facilitating the concurrent synthesis of leading and lagging strand. τ subunit also interacts with DnaB helicase and the interaction of τ subunit with DNA polymerase and helicase is required to mediate the high rate of replication fork movement (Kim *et al.*, 1996). Recent biochemical data suggested that the C-terminal domain of τ subunit plays crucial role in the processivity switching in the lagging strand. In the assay, the τ subunit released the DNA polymerase from the double-strand DNA after the completion of Okazaki fragment synthesis facilitating the recycling of DNA polymerase in the lagging strand (Leu *et al.*, 2003). Interestingly, the τ subunit showed DNA binding activity in addition to the binding activity to the C-terminal domain of DNA polymerase (Lopez *et al.*, 2003).

Rfc1 and τ show similarity in that both proteins contain additional C-terminal domains. The C-terminal domain does not exist in the small subunits or in the δ, γ or δ' subunit suggesting the specific role of this region. In addition to that, the C-terminal domains of both proteins display DNA binding activity. The structural similarity and the DNA binding activity in the C-terminal domain suggest that both proteins play similar role. It is interesting that only the C-terminal domain of τ subunit is involved in the processivity switching (Leu *et al.*, 2003).

From the role of τ subunit, there is a possibility that the C-terminal domain is involved in the dimerization of leading and lagging strand DNA polymerases at the replication fork. Dimerization of DNA polymerases is important for concurrent leading and lagging strand DNA synthesis. Another possible role is that the C-terminal domain of Rfc1 may link DNA polymerase with DNA helicase facilitating the rapid DNA synthesis. However, no interaction between Rfc1 and any of DNA polymerase δ or ε subunit is known yet. There is no evidence neither about Rfc1 and MCM subunits interaction.

Another possible role for the Rfc1 C-terminal domain is in the recycling of DNA polymerase in the lagging strand synthesis like the C-terminal domain of τ subunit. Although RFC is thought to be released from the DNA after the loading of PCNA at the replication fork (Jeruzalmi *et al.*, 2001) it seems true that it is also required for the Okazaki fragment maturation. For example, RFC interacts with the DNA ligase I (Levin *et al.*, 2004) suggesting the novel function of RFC in the Okazaki fragment maturation. Therefore, it is possible that the RFC complex moves with DNA polymerase δ at the replication fork and plays roles in the junction of two Okazaki fragment by releasing the DNA polymerase δ for re-cycling and by helping the DNA ligase I for the ligation of two DNA strands.

6.3 Role of three PCNA loaders

6.3.1 Eukaryotic cells contain three PCNA loaders

Failure to get *rfc1-44 ctf18Δ*, *rfc1-44 dcc1Δ*, and *rfc1-44 ctf8Δ* suggests that the *rfc1-44* requires the function of Ctf18-RLC for viability. The recovery of viability of *rfc1-44 ctf18Δ* double mutant by further deletion of Elg1 suggests that the Elg1 plays a negative role related to RFC function. Ctf18-RLC loads PCNA onto DNA (Bermudez *et al.*, 2003). *In vivo* and *in vitro* interaction between Elg1 and PCNA suggests that Elg1-RLC is another PCNA loader (Kanellis *et al.*, 2003). All these facts and results acquired in this study indicate that eukaryotic cells contain three PCNA loaders. The results from over-expression of proteins in mutant cells and sensitivity test of mutant cells for DNA damaging agents suggest that RFC play a

key role for PCNA loading while Ctf18-RLC plays supporting role and Elg1-RLC plays negative role.

Ctf18 is a protein involved in the sister chromatid cohesion in *S.cerevisiae* (Hanna *et al.*, 2001). Although Ctf18 is not essential for cell viability, it is essential for sister chromatid cohesion. The deletion of *ctf18* resulted in failure of sister chromatid cohesion and pre-anaphase accumulation of cells (Hanna *et al.*, 2001). *In vitro* reconstitution assay of Ctf18-RLC indicates that Ctf18-RLC load PCNA on DNA (Bermudez *et al.*, 2003). However, Ctf18-RLC could not substitute for RFC in the promotion of SV40 replication (Shiomi *et al.*, 2004). In addition to PCNA, Ctf18 also interacts genetically with Ctf7, an essential protein for sister chromatid cohesion play a role in the establishment of cohesion (Skibbens *et al.*, 1999). From the interaction of Ctf18 with Ctf7 and PCNA, it was suggested that Ctf18 links DNA replication and sister chromatid cohesion (Skibbens *et al.*, 1999).

However, it is not clear that the function of Ctf18 as a sister chromatid cohesion protein and as a PCNA loader is the same. More study is required to know whether Ctf18 and Ctf18-RLC have different function.

The less severe abnormality in the chromosome structure observed by PFGE in *rfc1-44 elg1Δ* double mutant than *rfc1-44* suggest that Elg1-RLC plays a role in maintenance of chromosome integrity. The role of Elg1 in the maintenance of chromosome integrity is one of the best known functions of Elg1 (Bellaoui *et al.*, 2003, Kanellis *et al.*, 2003, Ben-Aroya *et al.*, 2003). Therefore, the chromosome integrity in *rfc1-44 elg1Δ* double mutant cells is the consequence of either Elg1-RLC function or Elg1 function. Elg1 seems to play many roles in the progression of cell cycle. It is suggested that Elg1 is involved in the maintenance of chromosome integrity (Bellaoui *et al.*, 2003, Kanellis *et al.*, 2003, Ben-Aroya *et al.*, 2003), S phase progression (Kanellis *et al.*, 2003,Bellaoui *et al.*, 2003), Okazaki fragment maturation (Kanellis *et al.*, 2003), DNA checkpoint (Kanellis *et al.*, 2003, Ben-Aroya *et al.*, 2003, Bellaoui *et al.*, 2003) homologous recombination (Bellaoui *et al.*, 2003 Kanellis *et al.*, 2003) and telomere length maintenance (Kanellis *et al.*, 2003, Smolikov *et al.*, 2004). However, it is not known whether Elg1 plays various role or specific function of Elg1 results in various phenotypes.

Many data including the results described in the previous section (Chapter 4) suggest that there are three PCNA loaders in eukaryotic cells; one essential and two non-essential clamp loaders for cell viability. The question is why does the eukaryotic cell require three PCNA loaders? Three explanations are possible.

6.3.2 Different DNA substrate

Many biochemical data suggest that the large subunit of RFC possesses DNA binding activity (Fotedar *et al.*, 1996, Uhlmann *et al.*, 1997, Allen *et al.*, 1998). Although the small subunits (p40, p37 and p36 of human RFC) showed DNA binding activity, they showed DNA biding activity only in the low salt concentration suggesting that the DNA binding activity of small subunit is not a major factor influencing the binding of RFC to primed DNA (Cai *et al.*, 1997). As a result it seems likely that the large subunit of RFC contains DNA binding activity. If this is true then it is possible that the large subunit of Ctf18-RLC and Elg1-RLC contains DNA binding activity too. In addition to DNA replication, PCNA is also involved in various DNA metabolism such as DNA repair and sister chromatid cohesion. For the recognition of damaged DNA or DNA with a specific structure, eukaryotic cells may require different DNA recognition protein from RFC. If this is true, Ctf18-RLC may recognize the DNA structure in the sister chromatid cohesion. The interaction of Ctf7 with Ctf18 and PCNA suggest that the PCNA plays a role in sister chromatid cohesion (Skibbens *et al.*, 1999). In sister chromatid cohesion, Ctf18-RLC rather than RFC may load PCNA by recognition of sister chromatid-specific DNA structure. The fact that $elg1^+$ deletion mutant strain showed a defect in the recovery from replication fork stalling (Bellaoui *et al.*, 2003, Kanellis *et al.*, 2003) suggest that the Elg1-RLC plays a role in stalled replication fork. In addition, both Ctf18 and Elg1 play a redundant role in DNA damage checkpoint with Rad24 (Naiki *et al.*, 2001, Kanellis *et al.*, 2003) suggesting that the Ctf18-RLC and Elg1-RLC may recognize aberrant DNA substrate and load PCNA onto the damaged DNA.

6.3.3 Modified PCNA

The second possible role of three PCNA loaders is that they can load normal or modified PCNA. The idea of Elg1-RLC and Ctf18-RLC as modified PCNA loaders came from the report that the PCNA is modified by SUMO or ubiquitin (Hoege *et al.*, 2002). (See section 1.1.3.5) Ubiquitin-modified PCNA is involved in Rad6-dependent DNA repair pathway while the SUMO-modified PCNA is involved in replication (Hoege *et al.*, 2002). Mono-ubiquitinated or SUMO-modified PCNA is involved in the switch of DNA polymerases after the UV irradiation by interaction with DNA polymerase η or ζ (Stelter and Ulrich 2003, Kannouche *et al.*, 2004) facilitating the DNA repair by trans-lesion DNA polymerases.

A question needs to be answered about the presence of modified PCNA. How is modified PCNA loaded onto damaged DNA? One possibility is that Elg1-RLC and Ctf18-RLC load modified PCNA. This seems reasonable because Elg1 and Ctf18 play a role in the DNA damage checkpoint (Naiki *et al.*, 2001, Kanellis *et al.*, 2003, Bellaoui *et al.*, 2003, Ben-Aroya *et al.*, 2003). Another possibility is that, like PCNA at the replication fork, modified PCNA is loaded onto the damaged DNA by RFC. However, accessory proteins or another mechanism is required to distinguish PCNA and modified PCNAs at replication fork or at the damaged DNA in this case. The selection of PCNA must be accurate because the recruitment of trans-lesion DNA polymerase at replication fork can cause multiple replication error and inhibit the processive DNA synthesis. Therefore it seems more efficient to have two different clamp loaders that recognize the modified PCNA rather than have RFC and accessory proteins.

Preliminary Western blotting data with tagged Elg1 supports the function of Elg1 as a loader of modified PCNA in response to DNA damage. The endogenous protein level of Elg1 was lower than that of Rfc1 or Ctf18. When the cells were treated with MMS for 3 hours, the level of Elg1 has increased about three times (Stuart MacNeill personal communication). This result suggests that the Elg1 is involved in DNA damage response pathway. The requirement of Ctf18 for the viability of *rfc1-44* indicates that Ctf18 plays a positive role for cell viability (Chapter 4). Taken together, these results suggest that Ctf18-RLC loads SUMO-modified PCNA that involved in the DNA replication and Elg1-RLC loads ubiquitinated PCNA.

6.3.4 PCNA unloader

The third possible role of Elg1-RLC and Ctf18-RLC is as PCNA unloaders. The photo bleaching analysis with GFP-labeled PCNA has suggested that PCNA is not synthesized *de novo* but recycled in the lagging strand synthesis (Sporbert *et al.*, 2002). In contrast to leading strand synthesis where the DNA synthesis is continuous and requires one PCNA trimer complex to tether DNA polymerase δ, lagging strand synthesis requires many PCNAs for the synthesis of short DNA fragments because PCNA is required for each Okazaki fragments. For the synthesis of new Okazaki fragment, the eukaryotic cells can either recycle PCNA that was used for the synthesis previous Okazaki fragment or synthesize new PCNA molecule. In the case of RPA, the protein complex is assembled *de novo* for the new Okazaki fragment synthesis (Sporbert *et al.*, 2002). In the photobeaching assay of GFP-PCNA, the protein showed little turnover at replication site suggesting that it remains associated with the replication machinery through the multiple rounds of Okazaki fragment synthesis. It also showed that the transition from earlier to later replicons occur by dis-assembly into a nucleoplasmic pool of rapidly diffusing subcomponents and reassembly at newly activated sites.

These results suggest that the PCNA in one Okazaki fragment needed to be unloaded to be used in the subsequent Okazaki fragment synthesis. For this purpose it is possible that eukaryotic cells have evolved to have PCNA loader and unloader to facilitate the loading and unloading of PCNA at each Okazaki fragment for efficient and rapid synthesis of the lagging strand DNA.

However, both Ctf18 and Elg1 are not essential proteins suggesting that the PCNA unloading activity of these complexes must be shared with RFC. The unloading activity of RFC was reported before (Yao *et al.*, 1996). In addition to that the small subunit of RFC contains PCNA unloading activity too suggesting that Ctf18-RLC and Elg1-RLC can have PCNA unloading activity (Cai *et al.*, 1997).

For the identification of the role of three clamp loaders in eukaryotic cells, more studies are required.

6.4 Conclusion

The C-terminal domain of Rfc1 is essential for cell viability. *rfc1-44*, a temperature-sensitive mutant containing a mutation in the C-terminal domain of Rfc1 shows abnormal chromosome mobility, highly elongated phenotype and synthetic lethal phenotype when combined with other replication mutant strains, suggesting that the C-terminal domain of Rfc1 plays a role in DNA replication. The temperature-sensitive mutant phenotype of the *rfc1-44* cells were suppressed by further mutation of the N-terminal domain of Rfc5 suggesting that the C-terminal domain is important for the interaction with Rfc5.

Ctf18, Dcc1 and Ctf8, components of Ctf18-RLC, were required for the viability of *rfc1-44* whilst Elg1 was not. Restoration of viability of *rfc1-44 ctf18Δ* double mutant cells by further deletion of Elg1 suggests that Elg1 plays a negative role in eukaryotic cells. The positive and negative role of Ctf18 and Elg1 was further supported by the lethal phenotype of *rfc1-44* in the presence of high-level of Elg1 and growth defect of *ctf18* deletion strain at high temperature. These results suggest that RFC plays a key role in DNA replication and that Elg1-RLC and Ctf18-RLC can play negative and positive roles respectively, when RFC function is impaired.

Chapter 7. Materials and methods

7.1 General

7.1.1 Chemicals

Chemicals used in the experiments were purchased from Sigma, Fluka, ICN or Bio-Rad if not mentioned.

7.1.2 Enzymes

Restriction enzymes, DNA polymerases, DNA ligase or any other specialized enzymes were purchased from New England Biolabs or Promega. Enzymes were used in accordance with the manufacturers' instructions.

7.1.3 Commonly used buffers

Buffer	Formula
6X DNA loading buffer	0.25% bromophenol blue, 0.25% xylene cyanol FF, 30% glycerol
TE pH7.5	10mM Tris-Cl pH7.5, 1mM EDTA pH8.0
TE pH 8.0	10mM Tris-Cl pH8.0, 1mM EDTA pH8.0
TSB	10% Polyethylene glycol 3000, 10mM $MgCl_2$, 10mM $MgSO_4$, 5% DMSO, L-broth up to final volume of 20ml
TSBG	TSB+20mM glucose

7.2 Oligonucleotides

Oligonucleotides were routinely ordered from Amersham Pharmacia or Sigma-Genosys. Stock solutions were 100μM in H_2O and working concentrations were 10μM for PCR amplification and 2μM for sequencing reactions. The oligonucleotides used for subcloning, PCR and sequencing are listed below.

F: forward primer
R: reverse primer
Underline: restriction enzyme site

Name	Description	Sequences
RFC1cm-1 (F)	Subcloning of Rfc1-CTD	5'-AACTGCAGAAGGATCCTAGCTTCTTTCGTGGCTGGTTC-3'
RFC1cm-2 (R)	Subcloning of Rfc1-CTD	5'-AACTGCAGAAGGATCCGTTAACACAGCAATGTAGCG-3'
RFC1cm-3 (F)	PCR for the integration check; Sequencing	5'-TCTATAGCGTATCGTGAAGG-3'
RFC1cm-4 (F)	Stop codon insertion at the starting point of CTD	5'-TATGTCTTGCGTGCGACCTTAGATTAATTGAGCTTCTTTCGTGGCTGGTT-3'
RFC1cm-5 (R)	Stop codon insertion at the starting point of CTD	5'-AACCAGCCACGAAAGAAGCTCAATTAATCTAAGGTCGCACGCAAGACATA-3'
RFC1cm-6 (R)	PCR for the integration check; Sequencing	5'-TAACTCATATACAATCCGTA-3'
RFC1cm-7 (F)	Subcloning of chromosome integration construct	5'-ACTGCAGAGGATCCGCAGTCCGATATGCGCCAAA-3'
RFC1cm-8 (F)	PCR-based point mutation of highly-conserved amino acids	5'-AACTGCAGAAGGATCCTATACAAGTTAAGCTGTTCAGAAAT-3'
RFC1cm-11 (R)	Sequencing to confirm integration	5'-AACTGCAGAAGGATCCGCACTTCGCGCTGAGCAG-3'
RFC1cm-13 (F)	Point mutation of Asp739Ala	5'-TTACTAATTGGTTGGGTGCTAACTCGAAGACAAATA-3'
RFC1cm-14 (R)	Point mutation of Asp739Ala	5'-TATTTGCTTCGATACGACCCAACCAATTAGTAA-3'
RFC1cm-15 (F)	Point mutation of Lys745Ala	5'-ATAACTCAAGACAAATGCATTATATCGTATGCTTC-3'

RFC1cm-16 (R)	Point mutation of Lys745Ala	5'-GAAGCATACGATATAATCGATTTGTCTTCGA GTTAT-3'
RFC1cm-17 (F)	Point mutation of Arg748Ala	5'-AGACAAATAAATTATATGCTATGCTTCGTGA ATAC-3'
RFC1cm-18 (R)	Point mutation of Arg748Ala	5'-GTATCTCACGAAGCATACGATATAATTTATT TGTCT-3'
RFC1cm-19 (F)	Point mutation of Glu807Ala	5'-ATATTATCTTAATCGTGCAGATTTTGATTCA ATAA-3'
RFC1cm-20 (R)	Point mutation of Glu807Ala	5'-TTATTGAATCAAAATCTGCACGATTTAGATA ATAT-3'
RFC1cm-21 (F)	Point mutation of Asp808Ala	5'-CCGTTATTGAATCAAAAGCTTCACGATTTAG ATAA-3'
RFC1cm-22 (R)	Point mutation of Asp808Ala	5'-CCGTTATTGAATCAAAAGCTTCACGATTTTA GAAA-3'
RFC1cm-23 (F)	Point mutation of Glu814Ala	5'-TTTTGATTCAATAACGGCATTAGTTCTTCCT GCAG-3'
RFC1cm-24 (R)	Point mutation of Glu814Ala	5'-CTGCAGGAAGAACTAATGCCGTTATTGAAT CAAAA-3'
RFC1cm-25 (F)	Point mutation of Lys834Ala	5'-CCATACCGACTGCTGCAGCATCTGCATTCAC AAGAA-3'
RFC1cm-26 (R)	Point mutation of Lys834Ala	5'-TTCTTGTGAATGCAGATCGTGCAGCAGTCGG TATGG-3'
RFC1cm-27 (F)	Point mutation of Arg839Ala	5'-CAAAATCTGCATTCACAGCAAAATACAATTC TTCGA-3'
RFC1cm-28 (R)	Point mutation of Arg839Ala	5'-TCGAAGAATTGTATTTTCGTGTGAATGCAGA TTTTG-3'
RFC1cm-29 (F)	Point mutation of Asn842Ala	5'-CATTCACAAGAAATACGCTTCTTCGACTCAC CCCA-3'
RFC1cm-30 (R)	Point mutation of Asn842Ala	5'-TGGGGTGACTCGAAGAACGGTATTTTCTTGT GAATG-3'
RFC1cm-31 (F)	Removal of *KpnI* site in nt 2891(GTA-GTT)	5'-GACAAATTTATTTCGGTTCAAAGAAACCTA AAAAA-3'
RFC1cm-32 (R)	Removal of *KpnI* site in nt 2891(GTA-GTT)	5'-TTTTTTAGGTTTCTTTGGAACCGAAATAAAT TTGTC-3'
pUR19-1	Subcloning of constructs, PCR for chromosome integration	5'-ATGACCATGATTACGCCAAG-3'

pUR18N-1	The PCR for integration check	5'-GTAAAACGACGGCCAGTGC-3'
RFC5-1	Sequencing of chromosomally-integrated DNA	5'-AACTGCAGAAGGATCCAGGCCCCTACGCCAGAGGAA-3'
RFC5-2	Sequencing of chromosomally-integrated DNA	5'-AACTGCAGAAGGATCCAGTCTCCAGGCAACAAGCAACT-3'
RFC5-3	PCR for chromosome integration, sequencing	5'-AACTGCAGAAGGATCCCTAATAAGAAGGTAACTCCATAC-3'
RFC5-4	Subcloning of Rfc5 N-terminus construct	5'-AACTCAGAAGGATCCATGTGTAAACTAGCGAGAACG-3'
RFC5-5	Subcloning of Rfc5 N-terminus construct	5'-AAAACTGCAGAATGCAGTGAGAAAGAAGATCA-3'

7.3 Plasmids

For the genetic analysis of the Rfc1 C-terminal domain, pUR19 or its derivatives were used. For the integration of most of the mutation construct (pJKE1), a derivative of pUR19 in which the *ars1*$^+$ sequence has been deleted was used. DNA encoding the 130 amino acids upstream of C-terminal domain plus the Rfc1 C-terminal domain itself was constructed from pUR19-Rfc1 (pJKE11) by PCR and other modifications were performed with pJKE11.

7.3.1 Plasmids used for mutation analysis

CTD: C-terminal domain
a.a : amino acid

Name	Description	Construction
pJKE1	Deletion of ars1 sequence from pUR19	This study
pJKE11	The integration construct of Rfc1-CTD (130a.a upstream of CTD+ Rfc1-CTD)	This study
pJKE31	Deletion of *Kpn I* site from pJKE1	This study
pJKE32	The integration construct in pJKE31	This study
pJKE33	The integration construct with Asp739Ala	This study
pJKE34	The integration construct with Lys745Ala	This study
pJKE35	The integration construct with Arg748Ala	This study
pJKE36	The integration construct with Glu807Ala	This study
pJKE37	The integration construct with Asp808Ala	This study
pJKE38	The integration construct with Glu814Ala	This study
pJKE39	The integration construct with Lys834Ala	This study
pJKE40	The integration construct with Arg839Ala	This study
pJKE41	The integration construct with Asn842Ala	This study
pJKE42	The integration construct with *Kpn I* site mutation	This study
pJKE43	The integration construct with QGVPL insertion after Ile753	This study
pJKE44	The integration construct with IRGTP insertion after Ser811	This study

pJKE45	The integration construct with VRGTP insertion after Ala801	This study
pJKE46	The integration construct with SRGTP insertion after Gly852	This study
pJKE47	The integration construct with HLGAP insertion after Lys677	This study
pJKE49	The integration construct with LGVPQ insertion after Lys638	This study
pJKE50	The integration construct with VGVPH insertion after Asp870	This study
pJKE51	The integration construct with MKGYP insertion after Pro858	This study
pJKE52	The integration construct with LGVPL insertion after Met880	This study
pJKE53	The integration construct with LMGVP insertion after Tyr650	This study
pJKE54	The integration construct with PVGVP insertion after Leu780	This study
pJKE59	The integration construct with the stop codons inserted at the starting point of Rfc1-CTD	This study
pUR19-Rfc1	Template for the PCR amplification of CTD and the chromosomal integration construct.	Stuart MacNeill

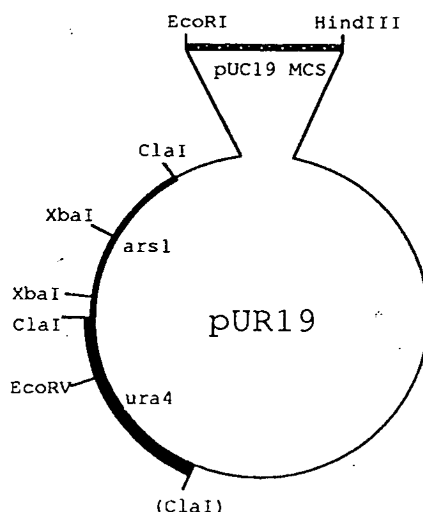


Figure 7.1 Restriction map of pUR19. The pUR19 vector contains the *ura4*$^{+}$ gene allowing for selection of the plasmid in media lacking uracil. *S.pombe ars1* sequence allows amplification of the plasmid in the *S.pombe* cells. For modification and molecular cloning, pUR19 contains the ampicillin resistant gene and ColE1 compatibility group of origin of replication of *E.coli*. The vector was modified from the pUC19 vector by Barbet et al., 1992.

The pJKE1 was plasmid constructed by removal of the *ars1* sequence from pUR19. The vector was digested with *Cla I* restriction enzyme and the digest was run on 0.8% agarose. The 5.5kb vector was purified from the agarose gel slice and re-ligated.

7.3.2 Plasmids used for the expression of proteins

Generally, pREP vectors were used for the expression of proteins. pREP vectors contains nmt1 promoter and nmt1 terminator sequences that over-express proteins. The level of protein expression is regulated by the kind of nmt1 promoter that was generated by the mutation. The level of protein expression can be inhibited by thiamine that repress the transcription. pREP vectors contains either *LEU2* or *ura4*$^{+}$ nutritional marker allowing selection of plasmid-containing cells in media lacking either leucine or uracil.

Plasmid	Function	Reference
pUR19-Rfc1	The large subunit of *S. pombe* RFC	Stuart MacNeill

pcL-Rfc1C30	The large subunit of *S. pombe* RFC	H. Tanaka
pCL-Rfc1C60	The large subunit of *S. pombe* RFC	H. Tanaka
pCL-Rfc1C109	The large subunit of *S. pombe* RFC	Tanaka *et al.*, 1999
pREP3X-Rfc2	RFC small subunit	Reynolds *et al.*, 1999
pUR19-Rfc3	RFC small subunit	Gray and MacNeill 2000
pUR19-Rfc4	RFC small subunit	Stuart MacNeill
pUR19-Rfc5	RFC small subunit	Stuart MacNeill
pREP41X-Pol3	The large subunit of *S. pombe* DNA polymerase δ	Stuart MacNeill
pREP3X-Cdc1	DNA polymerase δ subunit	MacNeill *et al.*, 1996
pREP3X-Cdc27	DNA polymerase δ subunit	MacNeill *et al.*, 1996
pREP3X-Cdm1	DNA polymerase δ subunit	Reynolds *et al.* 1998
pREP3X-Cdc17	DNA ligase I	Martin and MacNeill 2004
pIRT2-Pcn1	PCNA	
pREP1-Dna2	Exo/endo nuclease	Kang *et al.*, 2000
pAL-Cdc24	Rfc1 binding protein	Tanaka *et al.*, 1999
pcL-Pfh1	DNA helicase	Tanaka *et al.*, 2002
pcL-Rad17	Rad24 homologue	H. Tanaka
pREP3XH6		Stuart MacNeill
pREP1-Rfc1	The large subunit of RFC	Stuart MacNeill
pREP3X-Elg1	Chromosome integrity protein	Stuart MacNeill
pREP3X-Ctf18	Chromatid cohesion protein	Stuart MacNeill

7.4 General bacterial methods

7.4.1 *E.coli* strains

Strain	Purpose	Genotype
DH5α	General subcloning	F'*l endA1 hsdR17* ($r_k^- m_k^+$)*glnV44 thi-1 recA1 gyrA (Nal^r) relA1Δ (lacIZYA-argF) U169deoR (φ80dacΔ(lacZ)M15)*
GM2163	Dam-, Dcm- strain	F- *ara-14 leuB6 fhuA31 lacY1 tsx78 glnV44 galK2 galT22 McrA dcm-6 hisG4 rfbD1 rsoK136* (Str^R)*dam13::Tn9*(Cam^R) *xylA5 ntk-1 thi-1 mcrB1 hsdR2*
DS941	Transposon recipient	*AB1157 recF* $lacI^q$ *lacZdel.M15*
FH1046	Tn4430Ω containing strain	

7.4.2 Bacterial media

LB (Luria broth) 10g/l Bacto-tryptone
5g/l Bacto-yeast extract
5g/l NaCl
adjust the pH to 7.0 with NaOH
autoclave and store at room temperature

For LB plate, agar was added to a final concentration of 2%

SOB 20g/l tryptone
5g/l yeast extract
0.5g/l NaCl

Shake until the solutions have dissolved. Add 10ml of a 250mM solution of KCl. Adjust the pH of the medium to 7.0 with NaOH. Sterilize by autoclaving and add 5ml of 2M $MgCl_2$ just before use.

SOC SOB + 20mM glucose

7.4.3 Antibiotics

For the subcloning of a plasmid that containing antibiotic-resistant gene, or for the selection and maintenance of *E.coli* strain which contained an antibiotic-resistant gene, antibiotics were added to the media.

Antibiotic	Stock solution	Working concentration
Ampicillin	100mg/ml in H_2O	100μg/ml
Chloramphenicol	34mg/ml in ethanol	34μg/ml
Kanamycin	10mg/ml in H_2O	10μg/ml
Streptomycin	10mg/ml in H_2O	10μg/ml

7.4.4 Transformation

For general subcloning, the Inoue method (Inoue *et al.*, 1990) was used to prepare the competent cells and to transform the plasmid. For mutagenesis of *rfc1*$^+$ (chapter 2) or *rfc5*$^+$ (chapter 5) gene, the DMSO (Chung and Miller 1988) method was used.

7.4.4.1 Inoue method

1) Competent cell preparation

Competent cells were prepared according to the Inoue method.

A single colony that had been incubated for 16-20 hours at 37°C was picked and inoculated into 25ml of LB medium in 250ml flask. After incubation at 37°C for 8 hours with vigorous shaking, 8ml of starter culture were transferred into 250ml of SOB and incubated overnight at 18°C. Cells were transferred to ice-water for 10min when the OD_{600} of culture reached 0.55. Cells were harvested by centrifugation at 2,500g for 10min at 4°C and washed with 80ml of ice-cold Inoue transformation buffer. Pellets from the wash were resuspended in 20ml of ice-cold Inoue transformation buffer and 1.5ml of DMSO were added. After 10min incubation on ice, the cells were aliquoted and stored at –70°C.

2) Transformation

Competent cells were thawed on ice and aliquoted into pre-chilled 1.5ml eppendorf tubes. 100-500ng of DNA of interest was added to the tube and then was swirled gently using a pipette. The tubes were incubated on ice for 30min and transferred to a 42°C water bath. Cells were heat shocked for 1 min, transferred to ice and incubated for 2 min. 900μl of SOC were added and the cells were incubated at 37°C for 1 hour with vigorous shaking. Cells were plated on LB containing appropriate antibiotics.

Inoue transformation buffer (1L)

$MnCl_2$-4H_2O	10.88g
$CaCl_2$-2H_2O	2.2g
KCl	18.65g
PIPES (0.5M pH6.7)	20ml
H_2O	to 1 liter

Filter-sterilize Inoue transformation buffer and store at -20°C.

7.4.4.2 DMSO method

E.coli cells with OD_{600} of 0.5 were spined at RT for 10min at 3,000rpm. The pellets were resuspended in 0.1 volume of TSB and incubated on ice for 10 minutes. 100μl of the competent cells were added to the tube containing 100-500ng of DNA and incubated 30 minutes on ice. 0.9ml of TSBG were added to the tube and incubated for 1 hour at 37°C with shaking. Cells were plated on LB plate containing appropriate antibiotics.

7.5 Fission Yeast methods

7.5.1 Fission yeast media

Edinburgh Minimal Media (EMM) –used for vegetative growth

3g/l potassium hydrogen phthalate	(14.7mM)
2.2g/l Na_2HPO_4	(15.5mM)
5g/l NH_4Cl	(93.5mM)
2% (w/v) glucose	(111mM)
20ml/l salts	(50X stock)
1ml/l vitamins	(1000X stock)
0.1ml/l minerals	(10,000X stock)

50X Salts

52.5g/l $MgCl_2.6H_2O$	(0.26M)
0.735g/l $CaCl_2.2H_2O$	(4.99mM)
50g/l KCl	(0.67M)
2g/l Na_2SO_4	(14.1mM)

1000X vitamins

1g/l pantothenic acid	(4.20mM)
10g/l nicotinic acid	(81.2mM)
10g/l inositol	(55.5mM)
10mg/l biotin	(40.8uM)

10,000X minerals

5g/l boric acid	(80.9mM)
4g/l $MnSO_4$	(23.7mM)
4g/l $ZnSO_4.7H_2O$	(13.9mM)
2g/l $FeCl_2.6H_2O$	(7.40mM)
0.4g/l molybdic acid	(2.47mM)
1g/l KI	(6.02mM)

0.4g/l $CuSO_4.5H_2O$ (1.60mM)

10g/l citric acid (47.6mM)

ME (Malt Extract) –used for conjugation and sporulation

30g/l malt extract

20g/l Bacto agar

0.25g/l leucine

0.25g/l uracil

0.25g/l histidine

0.25g/l adenine

YE (Yeast Extract)-used for vegetative growth-inhibits conjugation and sporulation

0.5% (w/v) Oxoid yeast extract

3.0% (w/v) glucose

7.5.2 Strain used

strain	genotype	reference
Sp292	*leu1-32 ura4-D18 ade6-M210 h$^-$*	
Sp295	*leu1-32 ura4-D18 ade6-M216 h$^+$*	
rfc1-D1	*rfc1+/rfc1-ΔC leu1-32 ura4-D18 ade6-M210/ade6-M216*	This study
pol3-ts3	*pol3-ts3 leu1-32 ura4-D18 ade-M210 h$^+$*	
cdc1-P13	*cdc1-P13 h$^-$*	MacNeill *et al.*, 1996
cdc27-P11	*cdc27-P11 h$^-$*	MacNeill *et al.*, 1996
cdc27-D1	*cdc27-D1 leu1-32 h$^-$*	H.Tanaka
dna2-20	*dna2-20 h$^-$*	Kang *et al.*, 2000
cdc24-M38	*cdc24-M38 leu1-32 h$^+$*	

rfc1-33	*rfc1⁺/rfc1-33 leu1-32 ura4-D18 ade6-M210 / ade6-M206*	This study
rfc1-34	*rfc1⁺/rfc1-34 leu1-32 ura4-D18 ade6-M210/ ade6-M206*	This study
rfc1-35	*rfc1⁺/rfc1-35 leu1-32 ura4-D18 ade6-M210 /ade6-M206*	This study
rfc1-36	*rfc1⁺/rfc1-36 leu1-32 ura4-D18 ade6-M210/ade6-M206*	This study
rfc1-37	*rfc1⁺/rfc1-37 leu1-32 ura4-D18 ade6-M210/ade6-M206*	This study
rfc1-38	*rfc1⁺/rfc1-38 leu1-32 ura4-D18 ade6-M210/ade6-M206*	This study
rfc1-39	*rfc1⁺/rfc1-39 leu1-32 ura4-D18 ade6-M210/ade6-M206*	This study
rfc1-40	*rfc1⁺/rfc1-40 leu1-32 ura4-D18 ade6-M210/ade6-M206*	This study
rfc1-41	*rfc1⁺/rfc1-41 leu1-32 ura4-D18 ade6M210/ade6M206*	This study
rfc1-43	*rfc1⁺/rfc1-43 leu1-32 ura4-D18 ade6-M210/ade6-M206*	This study
rfc1-45	*rfc1⁺/rfc1-45 leu1-32 ura4-D18 ade6-M210/ade6-M206*	This study
rfc1-46	*rfc1⁺/rfc1-46 leu1-32 ura4-D18 ade6-M210/ade6-M206*	This study
rfc1-47	*rfc1⁺/rfc1-47 leu1-32 ura4-D18 ade6-M210/ade6-M206*	This study
rfc1-48	*rfc1⁺/rfc1-48 leu1-32 ura4-D18 ade6-M210/ade6-M206*	This study
rfc1-49	*rfc1⁺/rfc1-49 leu1-32 ura4-D18 ade6-M210/ade6-M206*	This study
rfc1-50	*rfc1⁺/rfc1-50 leu1-32 ura4-D18 ade6-*	This study

	M210/ade6-M206	
rfc1-51	*rfc1*$^{+}$*/rfc1-51 leu1-32 ura4-D18 ade6-M210/ade6-M206*	This study
rfc1-52	*rfc1*$^{+}$*/rfc1-52 leu1-32 ura4-D18 ade6-M210/ade6-M206*	This study
rfc1-53	*rfc1*$^{+}$*/rfc1-53 leu1-32 ura4-D18 ade6-M210/ade6-M206*	This study
rfc1-54	*rfc1-54 leu1-32 ura4-D18 ade6-M210 h*$^{-}$	This study
rfc1-44	*rfc1-44h*$^{+}$	This study
	rfc1-44h^{-+}	This study
	rfc1-44 leu1-32 ura4-D18	This study
elg1Δ	*elg1::natMX6 leu1-32 ura4-D18 h*$^{-}$	Stuart MacNeill
ct18Δ	*ctf18::natMX6 leu1-32 ura4-D18*	Stuart MacNeill
elg1Δctf18Δ	*elg1::natMX6 ctf18::kanMX6 h*$^{-}$	Stuart MacNeill
rfc1-44 elg1Δ	*rfc1-44 elg1::natMX6h*$^{+}$	This study
rfc1-44 elg1Δ ctf18Δ	*rfc1-44 elg1::natMX6 ctf18::kanMX6 h*$^{+}$	This study

7.5.3 Drugs used

Various chemicals were used to select for the deletion strain or to test sensitivity. Typically chemicals were added to the media immediately before use. For the preparation of plates YE-agar was melted and cooled to 55°C before the addition of chemicals. In the case of camptothecin, 10mM stock solution was prepared by dissolving the powder in DMSO and immediately adding to the media. The clonNAT (Nourseothricin, a complex produced by *Streptomyces noursei*) containing plate was prepared from 200mg/ml clonNat stock solution.

Drug /treatment	Abbreviation	Supplier/ Cat.No	Final concentration	Application
G418-sulphate	G418	Gibco-BRL/ 11811-031	0.1mg/ml	Selection for G418^{res} clones
5-fluoroorotic acid	5-FOA	Melford Laboratories Ltd/F5001	1mg/ml	Selection for ura^{-} clones
Hydroxyurea	HU	Sigma H-8627	6-12mM	Test sensitivity
Camptothecin	CPT	Sigma C-9911	6-10μM	Test sensitivity
Methylmethane sulphonate	MMS	Aldrich 12,992-5	0.0025%-0.01%	Test sensitivity
Ultraviolet light	UV	Stratalinker 2400 (Stratagne)	100-300 J/Cm^2	Test sensitivity
clonNAT	NAT	WERNER BioAgents 1.0000	100μg/ml	Construction of deletion strain

7.5.4 *S. pombe* methods

7.5.4.1 Maintenance

For the general maintenance of the *S.pombe* cells, YE media was used. A single colony was selected by streaking the cells on a plate and then patching on agar medium. For specific purposes such as selection of transformants or checking of a yeast genotype, EMM + supplements was used.

In most case, yeast cells were incubated at 32°C, while temperature sensitive strains were incubated at 25°C, as a permissive temperature, or 36.5°C as a restrictive temperature.

For the liquid cultures, 10ml of YE was inoculated using a fresh colony of the strain and incubated for 1 day until cells were in early stationary phase. These pre-culture were used to inoculate typically 200ml of YE or EMM + supplements and grown at permissive temperature with shaking.

7.5.4.2 Yeast transformation

Yeast cells reaching the absorbance of OD_{600} 0.2-0.4 in YE or EMM + supplements were harvested by centrifugation at 3,000rpm for 5min at RT. Cells were washed three times with 15ml of ice-cold 1.2M sorbitol and spun as before. Cells were resuspended in ice-cold 1.2M sorbitol at a final concentration of 1x 10^9cells/ml. 200μl of cell suspension were mixed with 1μg of DNA of interest and transferred into sterile ice-cold cuvette. Electroporation was performed at 200Ω, 2.25V, 50μF with a Gene Pulser II (Bio-Rad). 0.5ml of ice-cold 1.2M sorbitol was added immediately after the electroporation and 0.2ml of transformants were plated on EMM + supplements. Cells were incubated for 3 days at 32°C or 7 days at 25°C in the case of temperature sensitive mutants.

7.5.4.3 Genetic crosses

For the crossing of cells, haploid strains with opposite mating types were used.
A loopful of each of the two haploid cells of interest was resuspended in 20μl of DW and spotted on ME medium. After air-drying of the ME plate, the cells were incubated for 2-3 days at 28°C and the formation of asci were examined under the microscope.

7.5.4.4 Tetrad analysis

A loopful of cells was patched on one side of a YE plate. Single asci were separated from other cells by micromanipulator and incubated for several hours at 32°C or 28°C as appropriate until the asci wall had broken down. Each ascus was separated and placed into a line by micromanipulator (MSM system, Singer Instruments) and incubated 2-3days at 32°C or 3-4 days at 28°C in the case of the temperature sensitive mutant.

7.5.4.5 Random spore analysis

A loopful of cells was dissolved in 500μl of DW. The 10μl of 10-fold diluted helicase (*Helix pomatia* Juice) were added and incubated overnight at 28°C.

Disruption of cell wall and membrane was checked under the microscope and the spores were washed three times with DW. The number of spores was counted using a hemacytometer and similar or equal numbers of spores were plated on selective medium.

7.5.4.6 Spotting assay

Exponentially growing *S. pombe* cells were taken and the cell numbers determined using a Beckman Coulter Counter Z2. 1 x 10^7 /ml of cells were serially diluted ten fold to 1x 10^3 cells/ml with sterile H_2O. 10μl of each dilution was spotted on selective media. Spotted cells were incubated at 32°C or 25°C until the colony formation. Sometimes cells were incubated at 36.5°C to check the temperature-sensitive phenotype.

7.5.4.7 Measurement of cell number

Accurate cell number was determined using a Coulter Counter Z2 (Beckman). 100μl of cell suspension was added to 10ml of Isoton II (Beckman Cat. No. 8448011). The cells were sonicated for 10 sec at 15% amplitude with a high-density ultrasonic processor (Sonics and Materials) and counted with the Coulter Counter according to the manufacturer's instructions.

7.5.4.8 Genomic DNA preparation from *S.pombe*

10ml of *S.pombe* of interest was cultured at 32°C or 25°C (temperature-sensitive mutant) in EMM media. Cells were harvested by centrifugation at 3,000 rpm for 5 min and washed with 5ml of dH_2O. The cell pellet was resuspended in 0.2ml of Extraction buffer and transferred to a 2ml Eppendorf tube containing 500mg of glass beads (0.4mm BDH 150294N). 0.2ml of phenol:chloroform:isoamylalcohol= 25:24:1 were added and vortexed briefly. The tube was transferred to VIBREX (IKA type VX2E) and vibrated for 10min. Cell extract was centrifuged for 10min at 13,000rpm at RT and the upper phase solution transferred into a new tube. The DNA-containing solution was diluted 10-fold before use. Typically 2μl of the 1/10 diluted DNA were used for PCR reactions.

7.5.4.9 Generation of mutations in the *S.pombe* genome

A plasmid carrying a mutation within the gene of interest and the *ura4*$^+$ gene was constructed by conventional subcloning procedures. The plasmid was cut with appropriate restriction enzyme and purified using the QIAquick Gel Extraction kit (QIAGEN Cat. No. 28704). The linear DNA was then transformed into *S.pombe* cells by electroporation and cells were plated on EMM + supplements. Single colonies were isolated by streaking a colony on EMM + supplements and subsequently replica-plated on YE and EMM + supplements. The integration of the plasmid into the *S.pombe* chromosome was checked by PCR with primers that only yield a product in the integrated mutant genome. The PCR product was purified and sequenced to check the mutation where required. If required the *ura4*$^+$ gene was removed by plating the cells on YE + 5-FOA (1mg/ml). The mutation on the *S.pombe* chromosome was checked by PCR and DNA sequencing after the 5-FOA selection.

7.5.4.10 5-FOA selection

YE-agar-5-FOA plates were made by dissolving 1mg/ml 5-FOA in YE agar solution. YE liquid was inoculated with the yeast cells containing the *ura4+* gene and incubated overnight before being spread on a YE-agar-5-FOA plate. The cells were incubated at 32°C or 25°C (in the case of temperature-sensitive mutant cells) until colonies were visible.

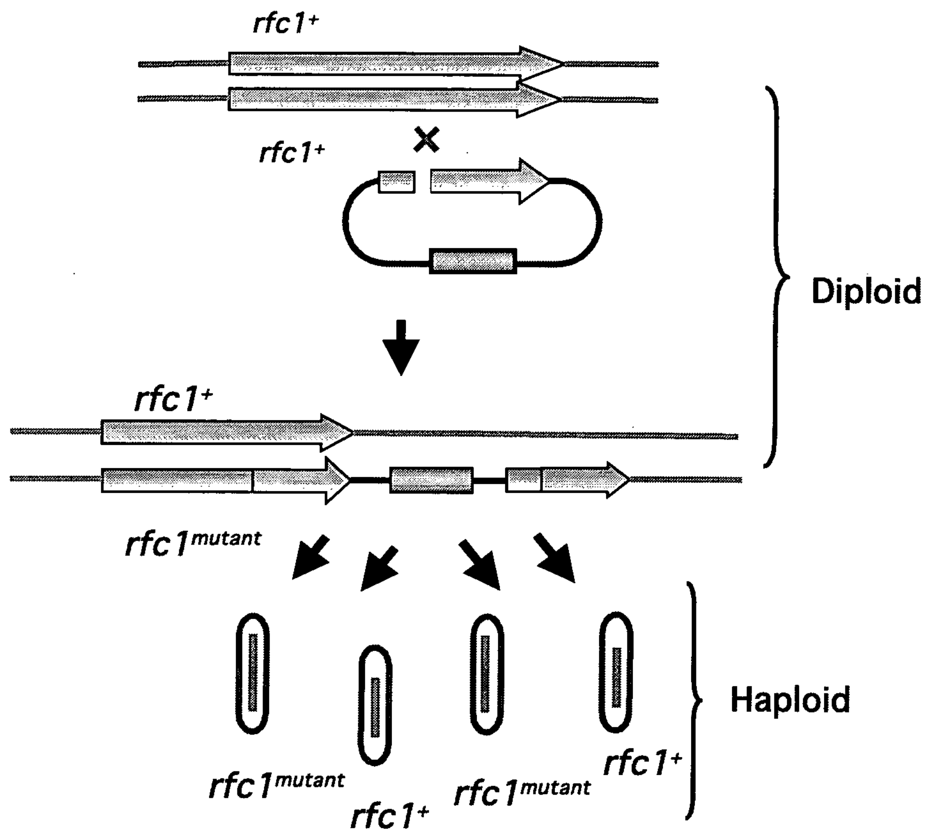


Figure 7.2 Integration of mutation into the *S.pombe* chromosome. The plasmid was linearised by digestion by a restriction enzyme before transformation into *S.pombe* cells. Homologous integration takes place via the site of single cut and the whole plasmid containing the mutation is integrated into the chromosome.

7.5.5 Isolation of *rfc1-44* suppressors

Either *S.pombe* genomic DNA or DNA from *rfc5* mutation library was transformed into the *rfc1-44* strain by electroporation. The resulting transformants were plated on EMM plate contaning appropriate nutritional markers and incubated overnight at 28°C. Except for some control plates which continued to be incubated at 28°C to check the transformation efficiency, the plates were transferred to 35°C and incubated further 5-7 days. Single colonies were acquired by streaking the colonies that grew at the high temperature on EMM plate containing the appropriate nutritional markers. For the *rfc5* mutant integration, single colonies were replicated subsequently on YE plate and EMM plates containing appropriate nutritional markers to check the stable integration of the plasmid into the *S.pombe* genome. Plasmid DNA was recovered from *S.pombe* by phenol-chloroform extraction of cell extracts and transformed into *E.coli* DH5α. The suppressor gene was examined by sequencing with a primer which could amplify either the N- or C-terminus of the integrated gene. For the *rfc5* mutation library, integrated *rfc5* DNA was amplified from the *S.pombe* genomic DNA and the PCR product was sequenced to examine the mutation site

7.6 General DNA methods

7.6.1 Preparation of plasmid DNA

Small scale preparations of plasmid DNA were usually carried out using a QIAprep Spin miniprep kit (QIAGEN Cat. No. 27104). The DNA was resuspended in 100μl of 10mM Tris-Cl pH 8.0. Typically, 10μg of plasmid DNA were acquired from miniprep. Large scale preparation of DNA was carried out using a QIAfilter Midi kit (QIAGEN Cat. No. 12243). In all cases plasmid DNA was prepared according to the manufacturers' instructions. Typically 50μg of plasmid DNA were acquired using the Midi kit.

7.6.2 Restriction enzyme digestion

Typically enzyme reactions were carried out in a volume of 20μl using various amounts of DNA according to the purpose. For analysis of the DNA, 300ng of mini-prepped DNA was used while 1μg was used for a preparative digest. Supplied reaction buffer was used and BSA was added where recommended. 1μl of enzyme (between 2 and 10 units) was added and incubated for 1 hour at 37°C. The digested DNA was then either analyzed by agarose gel electrophoresis or purified using the QIAquick Gel Extraction Kit (QIAGEN Cat. No. 28704) or the MinElute Gel Extraction Kit (QIAGEN Cat. No. 28604)

7.6.3 Purification of DNA from agarose gel slices

DNA fragments of interest were separated by agarose gel electrophoresis and visualized using a UV transilluminator. The target DNA was cut from the gel with a scalpel and transferred to a 1.5ml tube. It was then purified using either a MinElute Gel Extraction Kit (QIAGEN Cat. No. 28604) or a QIAquick Gel Extraction Kit (QIAGEN Cat. No. 28704) according to the manufacturers' instructions. In the case of MinElute Gel Extraction Kit, the DNA was eluted in 10μl of 10mM Tris-Cl pH8.0. In the case of QIAquick Gel Extraction Kit, it was eluted in 50μl.

7.6.4 DNA ligation

0.1-0.5μg of DNA was used in a 10μl reaction. 1 unit of T4 DNA ligase (Promega Cat. No. M180A) was used with supplied reaction buffer. Approximately 1:5 molar ratio of vector and insert DNA was used for the reaction which was incubated overnight at 16°C. The ligated DNA was then transformed into *E.coli* cells

7.6.5 Agarose gel electrophoresis

Agarose gel electrophoresis was performed to analyze the DNA, to purify a DNA fragment or to check a PCR product. 0.8-1%(w/v) agrose gels were used depending on the size of the DNA. The gel was prepared by melting an appropriate amount of agarose powder in 1X TAE buffer. Hot agarose was cooled and a final concentration of 0.1μg/ml of ethidium bromide was added prior to pouring the gel. The DNA sample was mixed with 6X sample buffer and 1X TAE to a final concentration of 1X and loaded on to the gel. The gel was run for 40 min at 120V with running time varying depending on the size of the DNA. 1X TAE was used as running buffer. 2-4μl of 100μg/ml 1kb$^+$ ladder (Invitrogen Cat.No. 10787-018) was run alongside the sample as a DNA molecular weight marker. The DNA was visualized using a UV-transilluminator and photographed using the GelDoc system.

7.6.7 DNA amplification by PCR

7.6.7.1 General PCR

Polymerase chain reaction (PCR) was used to amplify DNA fragments from either plasmid DNA or *S.pombe* genomic DNA. Generally 20μl reactions were carried out for analytical and 100μl for the preparative PCR reaction. Taq DNA polymerase (Roche Cat. No. 1146165) was used for the analytical PCR reaction and Pfu polymerase (Promega Cat. No. M774B) was used for the preparative PCR. For the amplification of DNA using Pfu, 2.5mM of $MgSO_4$ was added to each reaction because the supplied Pfu buffer from the manufacturer does not contain magnesium.

20μl reactions were set up as follow

DNA	20-200ng
10X polymerase buffer	2μl
25mM $MgSO_4$	2μl
2mM dNTP	2ul
5' oligonucleotide (10pmol/μl)	1ul
3' oligonucleotide (10pmol/μl)	1ul
Taq	0.5μl
dH_2O	up to 20μl

0.2ml tubes (Anexis Cat. No. 321-10-051) were used for the PCR reaction. The 'Genius' PCR machine (Techne) was used with the following standard program

step1 : Denaturation	94°C	1min	30 cycles
step2 : Primer annealing	55°C	45sec	
step3 : Extension	72°C	2min	

7.6.7.2 Colony PCR

Colony PCR was performed to check the efficiency of the subcloning. From the LB plate containing appropriate antibiotics, transformant *E.coli* colonies were picked and resuspended in 30μl of DW. 2μl of the cell suspension was used as a template. PCR reaction was performed by general PCR method.

7.6.8 DNA Sequencing

Template DNA was prepared by either the purification of plasmid DNA using the QIAprep Spin miniprep kit (QIAGEN Cat. No. 27104) or by purification of the PCR product using the MinElute Gel Extraction Kit (QIAGEN Cat. No. 28604).

Reactions were performed with the BigDye terminator cycle sequencing kit (Amersham Bioscience). A typical reaction mix was set up as follows.

Template DNA (100-250μg)	1μl
BigDye	4μl
Primer (2pmol)	1μl
DW	up to 10μl

Step 1 : 96°C for 30 seconds	
Step 2 : 50°C for 15 seconds	25 cycles
Step 3 : 60°C for 4 minutes	

Reaction mixtures then transferred into 1.5ml tube and 10μl of DW was added. The DNA was run by the ICMB sequencing service (University of Edinburgh) on an ABI PRISM 377 DNA sequencer and the sequences analyzed using the Sequencher and Gene Jockey II programmes.

7.7 Mutagenesis

7.7.1 PCR-based point mutation

pJKE11 was used as a template for the PCR reaction. RFC1cm-8 was used as a 5' end primer and RFC1cm-6 was used as a 3' end primer. The primers RFC1cm-13 through to RFC1cm-32 primers were both 36 bases long. The central DNA sequences of each contained the designed mutation flanked on either side by appropriate RFC1 sequence. First, two independent PCR reactions were performed with the RFC1cm-8 and each of RFC1cm-14, 16, 18, 20, 22, 24, 26,28, 30 or 32 and each of RFC1cm-13, 15, 17, 19, 21, 23, 25, 27, 29 or 31 and RFC1cm-6. PFU was used as the DNA polymerase and the general PCR method was used. The PCR products were run on a 1% agarose gel and the DNA products were removed. For each mutation, both gel fragments were put in an 1.5ml tube and 100μl of DW were added. The gel slices were then incubated at 37°C for 15minutes. 20μl of the DNA solution was taken from each tube and used as a template for a second PCR. In the second PCR, RFC1cm-8 and RFC1cm-6 were used as a primer set. The second PCR products were run on a 0.9% agarose gel and the DNA purified using the MinElute Gel Extraction Kit (QIAGEN Cat. No. 28604). Purified DNA was cut with restriction enzyme *EcoRI* and *KpnI* that is endogenous in Rfc1 gene and ligated to the pJKE32 that is cut by *EcoRI* and *KpnI.* The ligated plasmid was transformed into DH5α and the plasmid was purified by using QIAprep Spin miniprep kit (QIAGEN Cat. No. 27104).

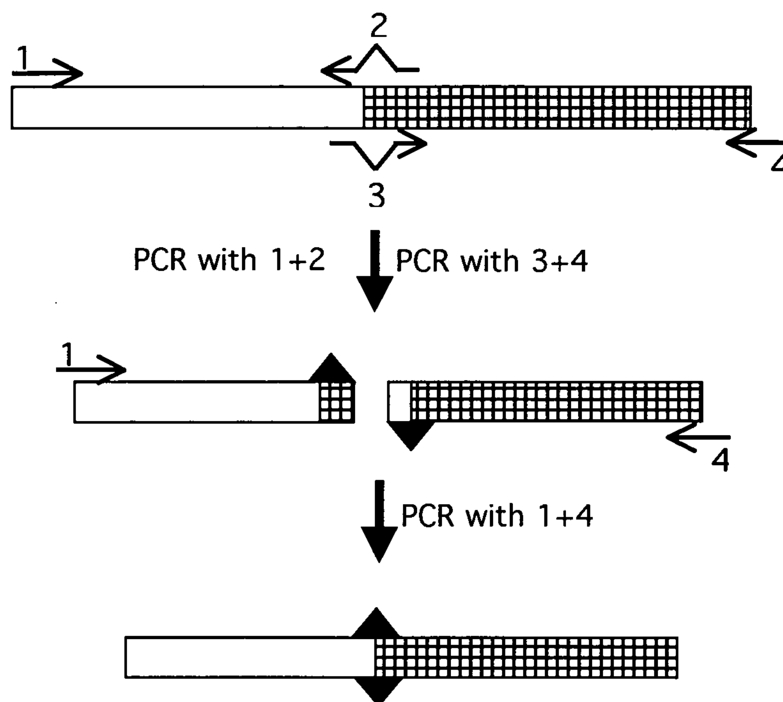


Figure 7.3 PCR-based point mutation. The two sets of oligos were used for the amplification of two DNA fragment both containing the same mutation. Primers 2 and 3 were designed to generate overlapping mutation containing PCR products. The two DNA fragments were then used as a template for the second PCR reaction with the primers that bind 3' or 5' end of the final PCR product.

7. 7.2 Transposon based penta-peptide insertion

For the Rfc1 C-terminal domain study, the chromosome integration construct (See chapter 2) was used as a target gene. For analysis of the Rfc5 N-terminal domain, the N-terminal 1070 nucleotides were used as a template for the mutagenesis. For the insertion and cleavage of transposon in Rfc1 C-terminal domain, the endogenous KpnI restriction enzyme site was removed by substituting adenine at 2891 to thymidine without changing the corresponding amino acid sequence by PCR-based point mutation.

Plasmid containing the gene of interest (pJKE32) was transformed into the FH1046

E.coli strain which contains Tn4430 transposon and cells were plated on LB+ 50μg/ml kanamycin + 100μg/ml ampicillin plate. 20 colonies were isolated and suspended in 40μl of LB. DS941 recipient cells were picked from an LB plate containing 50μg/ml streptomycin and were suspended in 100μl of LB. 2.5μl of FH1046 cells from each transformation were mixed with 2.5μl of DS941 cells and the mixtures were spotted on LB plates. The spots were air-dried and incubated for 3 hours at 37°C. A loopful of cells were resuspended in 300μl of LB. 100μl of cells were plated an LB plate containing 100μg/ml ampicillin, 50μg/ml kanamycin and 50μg/ml streptomycin and incubated overnight at 37°C. The integration of the transposon into the target gene was checked by colony PCR with the pUR19-1 and RFC1cm-7 primers which will only yield a product if integration has not occurred. DS941 cells containing the transposon were amplified by inoculating the cells in 5ml of liquid LB containing 100μg/ml ampicillin, 50μg/ml kanamycin and 50μg/ml streptomycin solution and incubating the cells overnight at 37°. The plasmids were prepared by QIAprep Spin miniprep kit (QIAGEN Cat. No. 27104). The tansposon was removed from the plasmid by *Kpn I* restriction enzyme digestion and re-ligation of the DNA. The insertion of transposon into the target gene and the position of insertion were checked by sequencing with RFC1cm-6 primer and RFC1cm-7 primer.

For the construction of Rfc5 N-terminus mutation library, some of the protocols were altered. After the transformation of target plasmid into the FH1046 *E.coli* strain all of the colony forming cells were collected and diluted 1/10 before mating with the DS941 recipient strain. All the DS941 cells forming colonies on LB+ Ampicillin + Kanamycin + Streptomycin were collected and diluted 1/10 before amplification in liquid media. Plasmid DNAs were purified by QIAGEN Midi-prep kit (Cat. No. 12145).

7.8 Biochemical methods

7.8.1 Flow cytometry

Wild-type or mutant cells were inoculated in 10ml of YE solution and incubated for 24 hours. The cells were transferred into 200ml of EMM + supplements and incubated overnight. 5ml of exponentially-growing cells were taken from the culture and collected through filtering apparatus containing a filter paper (25mm 0.45um Whatman Cat. No. 7184-002). At the same time, 100µl of the cells were taken and used to count the number of cells by Coulter counter. The cells were washed with 5ml of ice–cold STOP buffer through filtering apparatus. The filter paper containing the cells was removed from the filtering apparatus and moved into 15ml centrifuge tube. Cells were washed off from the filter paper with 10ml of 70% ethanol and stored at 4°C. Approximately 1 x 10^7 cells were taken and centrifuged at 3,000rpm for 5min at RT. Cells were washed with 4ml of SCB (50mM sodium citrate buffer pH 7.0) and resuspended in 0.5ml of SCB containing 100µg/ml Rnase A (SIGMA R4875). Cells were incubated overnight at 37°C and 0.5ml of SCB containing 4µg/ml propidium iodide was added. After 2 hours incubation at 4°C in the dark, the cells were diluted 1:20 with SCB containing 4µg/ml propidium iodide to a final volume of 2ml in a 75mm test tube (Elkay Laboratory Cat. No. 0002032/53). The cells were briefly sonicated (10sec in 15% amplitude) before analysis in the FACSCailbur Flow Cytometer (Becton Dickinson). The results were analyzed by Cellquest software.

STOP buffer	150mM NaCl
	50mM NaF
	10mM EDTA
	1mM NaN_3

7.8.2 Pulse Field Gel Electrophoresis

Wild-type or mutant cells were inoculated in 10ml of YE solution and incubated at 25°C for 24 hours. The cells were transferred into EMM + appropriate amino acid solution and incubated overnight at 25°C. Exponentially growing cells were taken and harvested by centrifugation at 3,000rpm at RT for 5min. 100µl of the cells were aliquoted and used for the counting of cell number by the Coulter counter. The cells were washed twice by resuspending the cells in 40ml CSE (20mM citrate-phosphate pH5.6, 1.2 M sorbitol, 40mM EDTA) and spinning down as before. The supernatant was discarded and the pellet cells were resuspended in 10ml CSE containing 1.5mg/ml Zymolyase-20T (ICN Cat. No. 32092) and incubated for 1 hour at 37°C. Cells were gently spun down by centrifugation at 1,500 rpm for 5 min at RT. The supernatant was removed and the cell pellets were resuspended in TSE (20mM citrate-phosphate pH5.6, 1.2 M sorbitol, 40mM EDTA) to a final concentration of 6 X 10^8/ml. The cells were warmed by incubating the cell suspension at 37°C water bath for 20 min. The same volume of 1% low melting point agarose (Bio-Rad Cat. No. 162-0017) was added and mixed well. 100µl of the cell suspension-agarose was taken and transferred into a Plug mold (Bio-Rad Cat, No. 170-3713) and cooled. The gelled plugs were incubated at 55°C for 90 min in 0.25M EDTA, 50mM TrisCl pH7.5, 1% SDS and further incubated for 48 hours in % lauryl sarcosine, 0.5M EDTA (pH 9.5), 1mg/ml proteinase K. The plugs were washed three times with Tris-EDTA (pH 8.0) before loading.

A 0.6% chromosomal grade agarose (Bio-Rad Cat. No. 162-0135) gel was prepared by melting 0.6g of the agarose in 100ml of 0.5X TAE. The plugs were cut to the well size and inserted into the well of 0.6% agarose gel and the wells were sealed with 0.6% low melting point agarose. The gel was run in Bio-Rad CHEF DRII system (Cat. No. 170-3612~170 3729) under the following conditions.

switch time : 1,800 seconds
voltage : 2V/Cm
buffer : 0.5 X TAE (replaced with the fresh buffer after 36 hours)
run time : 72 hours
temperature : 14°C

The gel was stained with 0.1μg/ml EtBr in DW for 1 hour and destained with DW for 3 hours.

References

Aladjem, M.I., Rodewald, L.W., Kolman, J.L. and Wahl, G.M. (1998) Genetic dissection of a mammalian replicator in the human β-globin locus. Science, 281, 1005-1009.

Allen, B.L., Uhlmann, F., Gaur, L.K., Mulder, B.A., Posey, K.L., Jones, L.B. and Hardin, S.H. (1998) DNA recognition properties of the N-terminal DNA binding domain within the large subunit of replication factor C. Nucleic Acids Res, 26, 3877-3882.

Altman, A.L. and Fanning, E. (2001) The Chinese hamster dihydrofolate reductase replication origin β is active at multiple ectopic chromosomal locations and requires specific DNA sequence elements for activity. Mol Cell Biol, 21, 1098-1110.

Amin, N.S., Tuffo, K.M. and Holm, C. (1999) Dominant mutations in three different subunits of replication factor C suppress replication defects in yeast PCNA mutants. Genetics, 153, 1617-1628.

Anderson, L.A. and Perkins, N.D. (2002) The large subunit of replication factor C interacts with the histone deacetylase, HDAC1. J Biol Chem, 277, 29550-29554.

Aparicio, O.M., Stout, A.M. and Bell, S.P. (1999) Differential assembly of Cdc45p and DNA polymerases at early and late origins of DNA replication. Proc Natl Acad Sci U S A, 96, 9130-9135.

Aparicio, O.M., Weinstein, D.M. and Bell, S.P. (1997) Components and dynamics of DNA replication complexes in *S. cerevisiae*: redistribution of MCM proteins and Cdc45p during S phase. Cell, 91, 59-69.

Arroyo, M.P., Downey, K.M., So, A.G. and Wang, T.S. (1996) *Schizosaccharomyces pombe* proliferating cell nuclear antigen mutations affect DNA polymerase δ processivity. J Biol Chem, 271, 15971-15980.

Austin, R.J., Orr-Weaver, T.L. and Bell, S.P. (1999) Drosophila ORC specifically binds to ACE3, an origin of DNA replication control element. Genes Dev, 13, 2639-2649.

Ayyagari, R., Impellizzeri, K.J., Yoder, B.L., Gary, S.L. and Burgers, P.M. (1995) A mutational analysis of the yeast proliferating cell nuclear antigen indicates distinct roles in DNA replication and DNA repair. Mol Cell Biol, 15, 4420-4429.

Bauer, G.A. and Burgers, P.M. (1988) The yeast analog of mammalian cyclin/proliferating-cell nuclear antigen interacts with mammalian DNA polymerase δ. Proc Natl Acad Sci U S A, 85, 7506-7510.

Bauer, G.A. and Burgers, P.M. (1988) Protein-protein interactions of yeast DNA polymerase III with mammalian and yeast proliferating cell nuclear antigen (PCNA)/cyclin. Biochim Biophys Acta, 951, 274-279.

Beckwith, W.H., Sun, Q., Bosso, R., Gerik, K.J., Burgers, P.M. and McAlear, M.A. (1998) Destabilized PCNA trimers suppress defective Rfc1 proteins in vivo and in vitro. Biochemistry, 37, 3711-3722.

Bell, S.P. and Dutta, A. (2002) DNA replication in eukaryotic cells. Annu Rev Biochem, 71, 333-374.

Bellaoui, M., Chang, M., Ou, J., Xu, H., Boone, C. and Brown, G.W. (2003) Elg1 forms an alternative RFC complex important for DNA replication and genome integrity. EMBO J, 22, 4304-4313.

Ben-Aroya, S., Koren, A., Liefshitz, B., Steinlauf, R. and Kupiec, M. (2003) ELG1, a yeast gene required for genome stability, forms a complex related to replication factor C. Proc Natl Acad Sci U S A, 100, 9906-9911.

Bermudez, V.P., Maniwa, Y., Tappin, I., Ozato, K., Yokomori, K. and Hurwitz, J. (2003) The alternative Ctf18-Dcc1-Ctf8-replication factor C complex required for sister chromatid cohesion loads proliferating cell nuclear antigen onto DNA. Proc Natl Acad Sci U S A, 100, 10237-10242.

Biamonti, G., Paixao, S., Montecucco, A., Peverali, F.A., Riva, S. and Falaschi, A. (2003) Is DNA sequence sufficient to specify DNA replication origins in metazoan cells? Chromosome Res, 11, 403-412.

Boulet, A., Simon, M., Faye, G., Bauer, G.A. and Burgers, P.M. (1989) Structure and function of the *Saccharomyces cerevisiae* CDC2 gene encoding the large subunit of DNA polymerase III. EMBO J, 8, 1849-1854.

Bowman, G.D., O'Donnell, M. and Kuriyan, J. (2004) Structural analysis of a eukaryotic sliding DNA clamp-clamp loader complex. Nature, 429, 724-730.

Brown, G.W. and Kelly, T.J. (1998) Purification of Hsk1, a minichromosome maintenance protein kinase from fission yeast. J Biol Chem, 273, 22083-22090.

Bryant, J.A., Moore, K. and Aves, S.J. (2001) Origins and complexes: the initiation of DNA replication. J Exp Bot, 52, 193-202.

Bunz, F., Kobayashi, R. and Stillman, B. (1993) cDNAs encoding the large subunit of human replication factor C. Proc Natl Acad Sci U S A, 90, 11014-11018.

Cai, J., Gibbs, E., Uhlmann, F., Phillips, B., Yao, N., O'Donnell, M. and Hurwitz, J. (1997) A complex consisting of human replication factor C p40, p37, and p36 subunits is a DNA-dependent ATPase and an intermediate in the assembly of the holoenzyme. J Biol Chem, 272, 18974-18981.

Cann, I.K., Ishino, S., Yuasa, M., Daiyasu, H., Toh, H. and Ishino, Y. (2001) Biochemical analysis of replication factor C from the hyperthermophilic archaeon *Pyrococcus furiosus*. J Bacteriol, 183, 2614-2623.

Celniker, S.E., Sweder, K., Srienc, F., Bailey, J.E. and Campbell, J.L. (1984) Deletion mutations affecting autonomously replicating sequence ARS1 of *Saccharomyces cerevisiae.* Mol Cell Biol, 4, 2455-2466.

Chong, J.P., Hayashi, M.K., Simon, M.N., Xu, R.M. and Stillman, B. (2000) A double-hexamer archaeal minichromosome maintenance protein is an ATP-dependent DNA helicase. Proc Natl Acad Sci U S A, 97, 1530-1535.

Chuang, L.S., Ian, H.I., Koh, T.W., Ng, H.H., Xu, G. and Li, B.F. (1997) Human DNA-(cytosine-5) methyltransferase-PCNA complex as a target for $p21^{WAF1}$. Science, 277, 1996-2000.

Chung and Miller (1988) A rapid and convenient method for the preparation and storage of competent bacterial cells. Nucleic Acids Res. 16. 3580.

Coxon, A., Maundrell, K. and Kearsey, S.E. (1992) Fission yeast *cdc21*$^{+}$ belongs to a family of proteins involved in an early step of chromosome replication. Nucleic Acids Res, 20, 5571-5577.

Cullmann, G., Fien, K., Kobayashi, R. and Stillman, B. (1995) Characterization of the five replication factor C genes of *Saccharomyces cerevisiae.* Mol Cell Biol, 15, 4661-4671.

D'Urso, G., Grallert, B. and Nurse, P. (1995) DNA polymerase α, a component of the replication initiation complex, is essential for the checkpoint coupling S phase to mitosis in fission yeast. J Cell Sci, 108 (Pt 9), 3109-3118.

D'Urso, G. and Nurse, P. (1997) *Schizosaccharomyces pombe cdc20*$^{+}$ encodes DNA polymerase ε and is required for chromosomal replication but not for the S phase checkpoint. Proc Natl Acad Sci U S A, 94, 12491-12496.

Delidakis, C. and Kafatos, F.C. (1989) Amplification enhancers and replication origins in the autosomal chorion gene cluster of *Drosophila.* EMBO J, 8, 891-901.

Dijkwel, P.A. and Hamlin, J.L. (1995) The Chinese hamster dihydrofolate reductase origin consists of multiple potential nascent-strand start sites. Mol Cell Biol, 15, 3023-3031.

Ellison, V. and Stillman, B. (1998) Reconstitution of recombinant human replication factor C (RFC) and identification of an RFC subcomplex possessing DNA-dependent ATPase activity. J Biol Chem, 273, 5979-5987.

Egel R. Fission yeast in general genetics. p. 1-10. The molecular biology of *Schizosaccharomyces pombe.* Springer, Verlag Berlin Heidelberg New York

Feng, W., Rodriguez-Menocal, L., Tolun, G. and D'Urso, G. (2003) *Schizosacchromyces pombe* Dpb2 binds to origin DNA early in S phase and is required for chromosomal DNA replication. Mol Biol Cell, 14, 3427-3436.

Fien, K. and Stillman, B. (1992) Identification of replication factor C from *Saccharomyces cerevisiae*: a component of the leading-strand DNA replication complex. Mol Cell Biol, 12, 155-163.

Fotedar, R., Mossi, R., Fitzgerald, P., Rousselle, T., Maga, G., Brickner, H., Messier, H., Kasibhatla, S., Hubscher, U. and Fotedar, A. (1996) A conserved domain of the large subunit of replication factor C binds PCNA and acts like a dominant negative inhibitor of DNA replication in mammalian cells. EMBO J, 15, 4423-4433.

Francesconi, S., Park, H. and Wang, T.S. (1993) Fission yeast with DNA polymerase δ temperature-sensitive alleles exhibits cell division cycle phenotype. Nucleic Acids Res, 21, 3821-3828.

Fried, L.M., Koumenis, C., Peterson, S.R., Green, S.L., van Zijl, P., Allalunis-Turner, J., Chen, D.J., Fishel, R., Giaccia, A.J., Brown, J.M. and Kirchgessner, C.U. (1996) The DNA damage response in DNA-dependent protein kinase-deficient SCID mouse cells: replication protein A hyperphosphorylation and p53 induction. Proc Natl Acad Sci U S A, 93, 13825-13830.

Gary, R., Ludwig, D.L., Cornelius, H.L., MacInnes, M.A. and Park, M.S. (1997) The DNA repair endonuclease XPG binds to proliferating cell nuclear antigen (PCNA) and shares sequence elements with the PCNA-binding regions of FEN-1 and cyclin-dependent kinase inhibitor p21. J Biol Chem, 272, 24522-24529.

Gary, S.L. and Burgers, M.J. (1995) Identification of the fifth subunit of *Saccharomyces cerevisiae* replication factor C. Nucleic Acids Res, 23, 4986-4991.

Gerik, K.J., Li, X., Pautz, A. and Burgers, P.M. (1998) Characterization of the two small subunits of *Saccharomyces cerevisiae* DNA polymerase δ. J Biol Chem, 273, 19747-19755.

Gilbert, D.M. (2001) Making sense of eukaryotic DNA replication origins. Science, 294, 96-100.

Gomes, X.V. and Burgers, P.M. (2000) Two modes of FEN1 binding to PCNA regulated by DNA. EMBO J, 19, 3811-3821.

Gomes, X.V., Gary, S.L. and Burgers, P.M. (2000) Overproduction in *Escherichia coli* and characterization of yeast replication factor C lacking the ligase homology domain. J Biol Chem, 275, 14541-14549.

Gomes, X.V. and Burgers, P.M. (2001) ATP utilization by yeast replication factor C. I. ATP-mediated interaction with DNA and with proliferating cell nuclear antigen. J Biol Chem, 276, 34768-34775.

Gomes, X.V., Schmidt, S.L. and Burgers, P.M. (2001) ATP utilization by yeast replication factor C. II. Multiple stepwise ATP binding events are required to load proliferating cell nuclear antigen onto primed DNA. J Biol Chem, 276, 34776-34783.

Gould, K.L., Burns, C.G., Feoktistova, A., Hu, C.P., Pasion, S.G. and Forsburg, S.L. (1998) Fission yeast *cdc24(+)* encodes a novel replication factor required for chromosome integrity. Genetics, 149, 1221-1233.

Green, C.M., Erdjument-Bromage, H., Tempst, P. and Lowndes, N.F. (2000) A novel Rad24 checkpoint protein complex closely related to replication factor C. Curr Biol, 10, 39-42.

Guenther B., Onrust R., Sali A., O'Donnell M. and Kuriyan J. (1997) Crystal structure of the δ' subunit of the clmap-loader complex of E.coli DNA polymerase III. Cell, 91, 335-345

Hallet, B., Sheratt, D.J. and Hayes F. (1997) Pentapeptide scanning mutagenesis: random insertion of a variable five amino acid cassette in a target protein. Nucleic Acids Res, 25, 1866-1867.

Hamatake, R.K., Hasegawa, H., Clark, A.B., Bebenek, K., Kunkel, T.A. and Sugino, A. (1990) Purification and characterization of DNA polymerase II from the yeast *Saccharomyces cerevisiae*. Identification of the catalytic core and a possible holoenzyme form of the enzyme. J Biol Chem, 265, 4072-4083.

Hanna J.S., Kroll E.S., Lundblad V. and Spencer F.A. (2001) *Saccharomyces cerevisiae* CTF18 and CTF4 are required for sister chromatid cohesion. Mol Cell Biol, 21 3144-3158.

Haracska, L., Unk, I., Johnson, R.E., Johansson, E., Burgers, P.M., Prakash, S. and Prakash, L. (2001) Roles of yeast DNA polymerases δ and ζ and of Rev1 in the bypass of abasic sites. Genes Dev, 15, 945-954.

Hasan, S., Hassa, P.O., Imhof, R. and Hottiger, M.O. (2001) Transcription coactivator p300 binds PCNA and may have a role in DNA repair synthesis. Nature, 410, 387-391.

Hayes, F., Hallet, B. and Cao Y. (1997) Insertion mutagenesis as a tool in the modification of protein function : Extended substrate specificity conferred by pepta-peptide insertions in the Ω-loop of TEM-1 β-lactamase. J Biol Chem, 272 28833-28836

He, H., Tan, C.K., Downey, K.M. and So, A.G. (2001) A tumor necrosis factor α- and interleukin 6-inducible protein that interacts with the small subunit of DNA polymerase δ and proliferating cell nuclear antigen. Proc Natl Acad Sci U S A, 98, 11979-11984.

Henneke, G., Gueguen, Y., Flament, D., Azam, P., Querellou, J., Dietrich, J., Hubscher, U. and Raffin, J.P. (2002) Replication factor C from the hyperthermophilic archaeon *Pyrococcus abyssi* does not need ATP hydrolysis for clamp-loading and contains a functionally conserved RFC PCNA-binding domain. J Mol Biol, 323, 795-810.

Hingorani, M.M. and Coman, M.M. (2002) On the specificity of interaction between the *Saccharomyces cerevisiae* clamp loader replication factor C and primed DNA templates during DNA replication. J Biol Chem, 277, 47213-47224.

Hoege, C., Pfander, B., Moldovan, G.L., Pyrowolakis, G. and Jentsch, S. (2002) RAD6-dependent DNA repair is linked to modification of PCNA by ubiquitin and SUMO. Nature, 419, 135-141.

Hofmann, J.F. and Beach, D. (1994) Cdt1 is an essential target of the Cdc10/Sct1 transcription factor: requirement for DNA replication and inhibition of mitosis. EMBO J, 13, 425-434.

Holmes, A.M. and Haber, J.E. (1999) Double-strand break repair in yeast requires both leading and lagging strand DNA polymerases. Cell, 96, 415-424.

Hong, S., Park, S.J., Kong, H.J., Shuman, J.D. and Cheong, J. (2001) Functional interaction of bZIP proteins and the large subunit of replication factor C in liver and adipose cells. J Biol Chem, 276, 28098-28105.

Hughes, P., Tratner, I., Ducoux, M., Piard, K. and Baldacci, G. (1999) Isolation and identification of the third subunit of mammalian DNA polymerase δ by PCNA-affinity chromatography of mouse FM3A cell extracts. Nucleic Acids Res, 27, 2108-2114.

Inoue, H., Nojima, H. and Okayama H. (1990) High efficiency transformation of Escherichia coli with plasmid. Gene, 96, 23-28.

Jacquier, A., Legrain, P. and Dujon, B. (1992) Sequence of a 10.7 kb segment of yeast chromosome XI identifies the APN1 and the BAF1 loci and reveals one tRNA gene and several new open reading frames including homologs to RAD2 and kinases. Yeast, 8, 121-132.

Jallepalli, P.V., Brown, G.W., Muzi-Falconi, M., Tien, D. and Kelly, T.J. (1997) Regulation of the replication initiator protein p65cdc18 by CDK phosphorylation. Genes Dev, 11, 2767-2779.

Jeruzalmi, D., O'Donnell, M. and Kuriyan, J. (2001) Crystal structure of the processivity clamp loader γ (γ) complex of *E. coli* DNA polymerase III. Cell, 106, 429-441.

Jeruzalmi, D., Yurieva, O., Zhao, Y., Young, M., Stewart, J., Hingorani, M., O'Donnell, M. and Kuriyan, J. (2001) Mechanism of processivity clamp opening by the δ subunit wrench of the clamp loader complex of *E. coli* DNA polymerase III. Cell, 106, 417-428.

Jiang, W., McDonald, D., Hope, T.J. and Hunter, T. (1999) Mammalian Cdc7-Dbf4 protein kinase complex is essential for initiation of DNA replication. EMBO J, 18, 5703-5713.

Jiang, W., Wells, N.J. and Hunter, T. (1999) Multistep regulation of DNA replication by Cdk phosphorylation of HsCdc6. Proc Natl Acad Sci U S A, 96, 6193-6198.

Johansson, E., Garg, P. and Burgers, P.M. (2004) The Pol32 subunit of DNA polymerase δ contains separable domains for processive replication and proliferating cell nuclear antigen (PCNA) binding. J Biol Chem, 279, 1907-1915.

Johnson, R.E., Kovvali, G.K., Guzder, S.N., Amin, N.S., Holm, C., Habraken, Y., Sung, P., Prakash, L. and Prakash, S. (1996) Evidence for involvement of yeast proliferating cell nuclear antigen in DNA mismatch repair. J Biol Chem, 271, 27987-27990.

Johnston, L.H., Barker, D.G. and Nurse, P. (1986) Cloning and characterization of the *Schizosaccharomyces pombe* DNA ligase gene CDC17. Gene, 41, 321-325.

Kalejta, R.F., Li, X., Mesner, L.D., Dijkwel, P.A., Lin, H.B. and Hamlin, J.L. (1998) Distal sequences, but not ori-β/OBR-1, are essential for initiation of DNA replication in the Chinese hamster DHFR origin. Mol Cell, 2, 797-806.

Kamimura, Y., Tak, Y.S., Sugino, A. and Araki, H. (2001) Sld3, which interacts with Cdc45 (Sld4), functions for chromosomal DNA replication in *Saccharomyces cerevisiae.* EMBO J, 20, 2097-2107.

Kanellis, P., Agyei, R. and Durocher, D. (2003) Elg1 forms an alternative PCNA-interacting RFC complex required to maintain genome stability. Curr Biol, 13, 1583-1595.

Kannouche, P.L., Wing, J. and Lehmann, A.R. (2004) Interaction of human DNA polymerase η with monoubiquitinated PCNA: a possible mechanism for the polymerase switch in response to DNA damage. Mol Cell, 14, 491-500.

Kelly, T.J. and Brown, G.W. (2000) Regulation of chromosome replication. Annu Rev Biochem, 69, 829-880.

Kelman, Z. and Hurwitz, J. (2000) A unique organization of the protein subunits of the DNA polymerase clamp loader in the archaeon *Methanobacterium thermoautotrophicum ΔH.* J Biol Chem, 275, 7327-7336.

Kim, H.S. and Brill, S.J. (2001) Rfc4 interacts with Rpa1 and is required for both DNA replication and DNA damage checkpoints in *Saccharomyces cerevisiae*. Mol Cell Biol, 21, 3725-3737.

Kim, J. and MacNeill, S.A. (2003) Genome stability: a new member of the RFC family. Curr Biol, 13, R873-875.

Kim, S., Dallmann, H.G., McHenry, C.S. and Marians, K.J. (1996) Coupling of a replicative polymerase and helicase: a τ-DnaB interaction mediates rapid replication fork movement. Cell, 84, 643-650.

Kim, S.M. and Huberman, J.A. (1998) Multiple orientation-dependent, synergistically interacting, similar domains in the ribosomal DNA replication origin of the fission yeast, *Schizosaccharomyces pombe*. Mol Cell Biol, 18, 7294-7303.

Kleczkowska, H.E., Marra, G., Lettieri, T. and Jiricny, J. (2001) hMSH3 and hMSH6 interact with PCNA and colocalize with it to replication foci. Genes Dev, 15, 724-736.

Klemm, R.D., Austin, R.J. and Bell, S.P. (1997) Coordinate binding of ATP and origin DNA regulates the ATPase activity of the origin recognition complex. Cell, 88, 493-502.

Kodaira, M., Biswas, S.B. and Kornberg, A. (1983) The dnaX gene encodes the DNA polymerase III holoenzyme τ subunit, precursor of the gamma subunit, the dnaZ gene product. Mol Gen Genet, 192, 80-86.

Kodaira, M., Biswas, S.B. and Kornberg, A. (1983) The dnaX gene encodes the DNA polymerase III holoenzyme τ subunit, precursor of the gamma subunit, the dnaZ gene product. Mol Gen Genet, 192, 80-86.

Kong, X.P., Onrust, R., O'Donnell, M. and Kuriyan, J. (1992) Three-dimensional structure of the β subunit of *E. coli* DNA polymerase III holoenzyme: a sliding DNA clamp. Cell, 69, 425-437.

Krause, S.A., Loupart, M.L., Vass, S., Schoenfelder, S., Harrison, S. and Heck, M.M. (2001) Loss of cell cycle checkpoint control in *Drosophila* Rfc4 mutants. Mol Cell Biol, 21, 5156-5168.

Krishna, T.S., Fenyo, D., Kong, X.P., Gary, S., Chait, B.T., Burgers, P. and Kuriyan, J. (1994) Crystallization of proliferating cell nuclear antigen (PCNA) from *Saccharomyces cerevisiae.* J Mol Biol, 241, 265-268.

Labib, K., Diffley, J.F. and Kearsey, S.E. (1999) G1-phase and B-type cyclins exclude the DNA-replication factor Mcm4 from the nucleus. Nat Cell Biol, 1, 415-422.

Labib, K., Tercero, J.A. and Diffley, J.F. (2000) Uninterrupted MCM2-7 function required for DNA replication fork progression. Science, 288, 1643-1647.

Leatherwood, J., Lopez-Girona, A. and Russell, P. (1996) Interaction of Cdc2 and Cdc18 with a fission yeast ORC2-like protein. Nature, 379, 360-363.

Lee, S.H., Ishimi, Y., Kenny, M.K., Bullock, P., Dean, F.B. and Hurwitz, J. (1988) An inhibitor of the in vitro elongation reaction of simian virus 40 DNA replication is overcome by proliferating-cell nuclear antigen. Proc Natl Acad Sci U S A, 85, 9469-9473.

Lee, S.H., Kwong, A.D., Ishimi, Y. and Hurwitz, J. (1989) Studies on the DNA elongation inhibitor and its proliferating cell nuclear antigen-dependent control in simian virus 40 DNA replication *in vitro*. Proc Natl Acad Sci U S A, 86, 4877-4881.

Lee, S.H., Eki, T. and Hurwitz, J. (1989) Synthesis of DNA containing the simian virus 40 origin of replication by the combined action of DNA polymerases alpha and delta. Proc Natl Acad Sci U S A, 86, 7361-7365.

Lee, S.H. and Hurwitz, J. (1990) Mechanism of elongation of primed DNA by DNA polymerase δ, proliferating cell nuclear antigen, and activator 1. Proc Natl Acad Sci U S A, 87, 5672-5676.

Lee, S.H., Kwong, A.D., Pan, Z.Q. and Hurwitz, J. (1991) Studies on the activator 1 protein complex, an accessory factor for proliferating cell nuclear antigen-dependent DNA polymerase δ. J Biol Chem, 266, 594-602.

Leu, F.P., Georgescu, R. and O'Donnell, M. (2003) Mechanism of the *E. coli* τ processivity switch during lagging-strand synthesis. Mol Cell, 11, 315-327.

Levin, D.S., Bai, W., Yao, N., O'Donnell, M. and Tomkinson, A.E. (1997) An interaction between DNA ligase I and proliferating cell nuclear antigen: implications for Okazaki fragment synthesis and joining. Proc Natl Acad Sci U S A, 94, 12863-12868.

Levin, D.S., Vijayakumar, S., Liu, X., Bermudez, V.P., Hurwitz, J. and Tomkinson, A.E. (2004) A conserved interaction between the replicative clamp loader and DNA ligase in eukaryotes: implications for Okazaki fragment joining. J Biol Chem, 279, 55196-55201.

Lewin, B. (2002) DNA replication. p. 387-413 Genes VII. Oxford University press. Great Clarendan street, Oxford.

Li, X. and Burgers, P.M. (1994) Molecular cloning and expression of the *Saccharomyces cerevisiae* RFC3 gene, an essential component of replication factor C. Proc Natl Acad Sci U S A, 91, 868-872.

Liang, C. and Stillman, B. (1997) Persistent initiation of DNA replication and chromatin-bound MCM proteins during the cell cycle in cdc6 mutants. Genes Dev, 11, 3375-3386.

Liu, J., Smith, C.L., DeRyckere, D., DeAngelis, K., Martin, G.S. and Berger, J.M. (2000) Structure and function of Cdc6/Cdc18: implications for origin recognition and checkpoint control. Mol Cell, 6, 637-648.

Liu, L., Mo, J., Rodriguez-Belmonte, E.M. and Lee, M.Y. (2000) Identification of a fourth subunit of mammalian DNA polymerase δ. J Biol Chem, 275, 18739-18744.

Longley, M.J., Pierce, A.J. and Modrich, P. (1997) DNA polymerase δ is required for human mismatch repair in vitro. J Biol Chem, 272, 10917-10921.

Lopez de Saro, F.J., Georgescu, R.E. and O'Donnell, M. (2003) A peptide switch regulates DNA polymerase processivity. Proc Natl Acad Sci U S A, 100, 14689-14694.

Lu, L., Zhang, H. and Tower, J. (2001) Functionally distinct, sequence-specific replicator and origin elements are required for Drosophila chorion gene amplification. Genes Dev, 15, 134-146.

MacNeill, S.A., Moreno, S., Reynolds, N., Nurse, P. and Fantes, P.A. (1996) The fission yeast Cdc1 protein, a homologue of the small subunit of DNA polymerase δ, binds to Pol3 and Cdc27. EMBO J, 15, 4613-4628.

Majka, J. and Burgers, P.M. (2003) Yeast Rad17/Mec3/Ddc1: a sliding clamp for the DNA damage checkpoint. Proc Natl Acad Sci U S A, 100, 2249-2254.

Marahrens, Y. and Stillman, B. (1992) A yeast chromosomal origin of DNA replication defined by multiple functional elements. Science, 255, 817-823.

Masai, H., Miyake, T. and Arai, K. (1995) *hsk1*$^{+}$, a *Schizosaccharomyces pombe* gene related to *Saccharomyces cerevisiae* CDC7, is required for chromosomal replication. EMBO J, 14, 3094-3104.

Matsumiya, S., Ishino, S., Ishino, Y. and Morikawa, K. (2002) Physical interaction between proliferating cell nuclear antigen and replication factor C from *Pyrococcus furiosus*. Genes Cells, 7, 911-922.

Matsumoto, Y., Kim, K., Hurwitz, J., Gary, R., Levin, D.S., Tomkinson, A.E. and Park, M.S. (1999) Reconstitution of proliferating cell nuclear antigen-dependent repair of apurinic/apyrimidinic sites with purified human proteins. J Biol Chem, 274, 33703-33708.

Mayer, M.L., Gygi, S.P., Aebersold, R. and Hieter, P. (2001) Identification of RFC(Ctf18p, Ctf8p, Dcc1p): an alternative RFC complex required for sister chromatid cohesion in *S. cerevisiae.* Mol Cell, 7, 959-970.

McAlear, M.A., Tuffo, K.M. and Holm, C. (1996) The large subunit of replication factor C (Rfc1p/Cdc44p) is required for DNA replication and DNA repair in *Saccharomyces cerevisiae*. Genetics, 142, 65-78.

McHenry, C.S. (1982) Purification and characterization of DNA polymerase III'. Identification of τ as a subunit of the DNA polymerase III holoenzyme. J Biol Chem, 257, 2657-2663.

Melendy, T. and Stillman, B. (1991) Purification of DNA polymerase δ as an essential simian virus 40 DNA replication factor. J Biol Chem, 266, 1942-1949.

Miyake, S., Okishio, N., Samejima, I., Hiraoka, Y., Toda, T., Saitoh, I. and Yanagida, M. (1993) Fission yeast genes *nda1*$^{+}$ and *nda4*$^{+}$, mutations of which lead to S-phase block, chromatin alteration and Ca2+ suppression, are members of the CDC46/MCM2 family. Mol Biol Cell, 4, 1003-1015.

Miyake, S. and Yamashita, S. (1998) Identification of *sna41* gene, which is the suppressor of *nda4* mutation and is involved in DNA replication in *Schizosaccharomyces pombe*. Genes Cells, 3, 157-166.

Montecucco, A., Rossi, R., Levin, D.S., Gary, R., Park, M.S., Motycka, T.A., Ciarrocchi, G., Villa, A., Biamonti, G. and Tomkinson, A.E. (1998) DNA ligase I is recruited to sites of DNA replication by an interaction with proliferating cell nuclear antigen: identification of a common targeting mechanism for the assembly of replication factories. EMBO J, 17, 3786-3795.

Moon, K.Y., Kong, D., Lee, J.K., Raychaudhuri, S. and Hurwitz, J. (1999) Identification and reconstitution of the origin recognition complex from *Schizosaccharomyces pombe*. Proc Natl Acad Sci U S A, 96, 12367-12372.

Muzi Falconi, M., Brown, G.W. and Kelly, T.J. (1996) *cdc18*$^{+}$ regulates initiation of DNA replication in *Schizosaccharomyces pombe*. Proc Natl Acad Sci U S A, 93, 1566-1570.

Naiki, T., Shimomura, T., Kondo, T., Matsumoto, K. and Sugimoto, K. (2000) Rfc5, in cooperation with rad24, controls DNA damage checkpoints throughout the cell cycle in *Saccharomyces cerevisiae.* Mol Cell Biol, 20, 5888-5896.

Nishitani, H., Lygerou, Z., Nishimoto, T. and Nurse, P. (2000) The Cdtl protein is required to license DNA for replication in fission yeast. Nature, 404, 625-628.

Ogawa, Y., Takahashi, T. and Masukata, H. (1999) Association of fission yeast Orpl and Mcm6 proteins with chromosomal replication origins. Mol Cell Biol, 19, 7228-7236.

Ohta S., Shiomi Y., Sugimoto K., Obuse C. and Tsurimoto T. (2002) A proteomics approach to identify proliferating cell nuclear antigen (PCNA)-binding proteins in human cell lysates. Identification of the human CHL12/RFCs2-5 complex as a novel PCNA-binding protein. J. Biol. Chem. 277, 40362-40367.

Okuno, Y., Okazaki, T. and Masukata, H. (1997) Identification of a predominant replication origin in fission yeast. Nucleic Acids Res, 25, 530-537.

Okuno, Y., Satoh, H., Sekiguchi, M. and Masukata, H. (1999) Clustered adenine/thymine stretches are essential for function of a fission yeast replication origin. Mol Cell Biol, 19, 6699-6709.

Otterlei, M., Warbrick, E., Nagelhus, T.A., Haug, T., Slupphaug, G., Akbari, M., Aas, P.A., Steinsbekk, K., Bakke, O. and Krokan, H.E. (1999) Post-replicative base excision repair in replication foci. EMBO J, 18, 3834-3844.

Pak, D.T., Pflumm, M., Chesnokov, I., Huang, D.W., Kellum, R., Marr, J., Romanowski, P. and Botchan, M.R. (1997) Association of the origin recognition complex with heterochromatin and HP1 in higher eukaryotes. Cell, 91, 311-323.

Pape, T., Meka, H., Chen, S., Vicentini, G., van Heel, M. and Onesti, S. (2003) Hexameric ring structure of the full-length archaeal MCM protein complex. EMBO Rep, 4, 1079-1083.

Parker, A.E., Clyne, R.K., Carr, A.M. and Kelly, T.J. (1997) The *Schizosaccharomyces pombe rad11*$^{+}$ gene encodes the large subunit of replication protein A. Mol Cell Biol, 17, 2381-2390.

Pasion, S.G. and Forsburg, S.L. (1999) Nuclear localization of *Schizosaccharomyces pombe* Mcm2/Cdc19p requires MCM complex assembly. Mol Biol Cell, 10, 4043-4057.

Pearson, W.R. and Lipman, D.S. (1988) Improved tools or biological sequence comparison. Proc Natl Acad Sci U S A, 85, 2444-2448.

Pelizon, C., Diviacco, S., Falaschi, A. and Giacca, M. (1996) High-resolution mapping of the origin of DNA replication in the hamster dihydrofolate reductase gene domain by competitive PCR. Mol Cell Biol, 16, 5358-5364.

Pennaneach, V., Salles-Passador, I., Munshi, A., Brickner, H., Regazzoni, K., Dick, F., Dyson, N., Chen, T.T., Wang, J.Y., Fotedar, R. and Fotedar, A. (2001) The large subunit of replication factor C promotes cell survival after DNA damage in an LxCxE motif- and Rb-dependent manner. Mol Cell, 7, 715-727.

Perkins, G. and Diffley, J.F. (1998) Nucleotide-dependent prereplicative complex assembly by Cdc6p, a homolog of eukaryotic and prokaryotic clamp-loaders. Mol Cell, 2, 23-32.

Petersen, B.O., Lukas, J., Sorensen, C.S., Bartek, J. and Helin, K. (1999) Phosphorylation of mammalian CDC6 by cyclin A/CDK2 regulates its subcellular localization. EMBO J, 18, 396-410.

Pignede, G., Bouvier, D., de Recondo, A.M. and Baldacci, G. (1991) Characterization of the POL3 gene product from *Schizosaccharomyces pombe* indicates inter-species conservation of the catalytic subunit of DNA polymerase δ. J Mol Biol, 222, 209-218.

Podust, V.N., Chang, L.S., Ott, R., Dianov, G.L. and Fanning, E. (2002) Reconstitution of human DNA polymerase δ using recombinant baculoviruses: the p12 subunit potentiates DNA polymerizing activity of the four-subunit enzyme. J Biol Chem, 277, 3894-3901.

Prelich, G., Kostura, M., Marshak, D.R., Mathews, M.B. and Stillman, B. (1987a) The cell-cycle regulated proliferating cell nuclear antigen is required for SV40 DNA replication *in vitro*. Nature, 326, 471-475.

Prelich, G., Tan, C.K., Kostura, M., Mathews, M.B., So, A.G., Downey, K.M. and Stillman, B. (1987b) Functional identity of proliferating cell nuclear antigen and a DNA polymerase-δ auxiliary protein. Nature, 326, 517-520.

Prelich, G. and Stillman, B. (1988) Coordinated leading and lagging strand synthesis during SV40 DNA replication in vitro requires PCNA. Cell, 53, 117-126.

Rauen, M., Burtelow, M.A., Dufault, V.M. and Karnitz, L.M. (2000) The human checkpoint protein hRad17 interacts with the PCNA-like proteins hRad1, hHus1, and hRad9. J Biol Chem, 275, 29767-29771.

Reynolds, N., Fantes, P.A. and MacNeill, S.A. (1999) A key role for replication factor C in DNA replication checkpoint function in fission yeast. Nucleic Acids Res, 27, 462-469.

Reynolds, N., Warbrick, E., Fantes, P.A. and MacNeill, S.A. (2000) Essential interaction between the fission yeast DNA polymerase δ subunit Cdc27 and Pcn1 (PCNA) mediated through a C-terminal p21(Cip1)-like PCNA binding motif. EMBO J, 19, 1108-1118.

Romanowski, P., Madine, M.A., Rowles, A., Blow, J.J. and Laskey, R.A. (1996) The *Xenopus* origin recognition complex is essential for DNA replication and MCM binding to chromatin. Curr Biol, 6, 1416-1425.

Royzman, I., Austin, R.J., Bosco, G., Bell, S.P. and Orr-Weaver, T.L. (1999) ORC localization in *Drosophila* follicle cells and the effects of mutations in dE2F and dDP. Genes Dev, 13, 827-840.

Saha, S., Shan, Y., Mesner, L.D. and Hamlin, J.L. (2004) The promoter of the Chinese hamster ovary dihydrofolate reductase gene regulates the activity of the local origin and helps define its boundaries. Genes Dev, 18, 397-410.

Schmidt, S.L., Gomes, X.V. and Burgers, P.M. (2001) ATP utilization by yeast replication factor C. III. The ATP-binding domains of Rfc2, Rfc3, and Rfc4 are essential for DNA recognition and clamp loading. J Biol Chem, 276, 34784-34791.

Schmidt, S.L., Pautz, A.L. and Burgers, P.M. (2001) ATP utilization by yeast replication factor C. IV. RFC ATP-binding mutants show defects in DNA replication, DNA repair, and checkpoint regulation. J Biol Chem, 276, 34792-34800.

Schramke, V., Luciano, P., Brevet, V., Guillot, S., Corda, Y., Longhese, M.P., Gilson, E. and Geli, V. (2004) RPA regulates telomerase action by providing Est1p access to chromosome ends. Nat Genet, 36, 46-54.

Schwacha, A. and Bell, S.P. (2001) Interactions between two catalytically distinct MCM subgroups are essential for coordinated ATP hydrolysis and DNA replication. Mol Cell, 8, 1093-1104.

Seybert, A., Scott, D.J., Scaife, S., Singleton, M.R. and Wigley, D.B. (2002) Biochemical characterisation of the clamp/clamp loader proteins from the euryarchaeon *Archaeoglobus fulgidus*. Nucleic Acids Res, 30, 4329-4338.

Shareef, M.M., King, C., Damaj, M., Badagu, R., Huang, D.W. and Kellum, R. (2001) *Drosophila* heterochromatin protein 1 (HP1)/origin recognition complex (ORC) protein is associated with HP1 and ORC and functions in heterochromatin-induced silencing. Mol Biol Cell, 12, 1671-1685.

Sherman, D.A. and Forsburg, S.L. (1998) *Schizosaccharomyces pombe* Mcm3p, an essential nuclear protein, associates tightly with Nda4p (Mcm5p). Nucleic Acids Res, 26, 3955-3960.

Sherman, D.A., Pasion, S.G. and Forsburg, S.L. (1998) Multiple domains of fission yeast Cdc19p (MCM2) are required for its association with the core MCM complex. Mol Biol Cell, 9, 1833-1845.

Shimomura, T., Ando, S., Matsumoto, K. and Sugimoto, K. (1998) Functional and physical interaction between Rad24 and Rfc5 in the yeast checkpoint pathways. Mol Cell Biol, 18, 5485-5491.

Shiomi, Y., Usukura, J., Masamura, Y., Takeyasu, K., Nakayama, Y., Obuse, C., Yoshikawa, H. and Tsurimoto, T. (2000) ATP-dependent structural change of the eukaryotic clamp-loader protein, replication factor C. Proc Natl Acad Sci U S A, 97, 14127-14132.

Simon, M., Giot, L. and Faye, G. (1991) The 3' to 5' exonuclease activity located in the DNA polymerase δ subunit of *Saccharomyces cerevisiae* is required for accurate replication. EMBO J, 10, 2165-2170.

Skibbens, R.V., Corson, L.B., Koshland, D. and Hieter, P. (1999) Ctf7p is essential for sister chromatid cohesion and links mitotic chromosome structure to the DNA replication machinery. Genes Dev, 13, 307-319.

Smith, S., Hwang, J.Y., Banerjee, S., Majeed, A., Gupta, A. and Myung, K. (2004) Mutator genes for suppression of gross chromosomal rearrangements identified by a genome-wide screening in *Saccharomyces cerevisiae.* Proc Natl Acad Sci U S A, 101, 9039-9044.

Smolikov, S., Mazor, Y. and Krauskopf, A. (2004) ELG1, a regulator of genome stability, has a role in telomere length regulation and in silencing. Proc Natl Acad Sci U S A, 101, 1656-1661.

Spiga, M.G. and D'Urso, G. (2004) Identification and cloning of two putative subunits of DNA polymerase ε in fission yeast. Nucleic Acids Res, 32, 4945-4953.

Sporbert, A., Gahl, A., Ankerhold, R., Leonhardt, H. and Cardoso, M.C. (2002) DNA polymerase clamp shows little turnover at established replication sites but sequential *de novo* assembly at adjacent origin clusters. Mol Cell, 10, 1355-1365.

Stelter, P. and Ulrich, H.D. (2003) Control of spontaneous and damage-induced mutagenesis by SUMO and ubiquitin conjugation. Nature, 425, 188-191.

Sugimoto, K., Ando, S., Shimomura, T. and Matsumoto, K. (1997) Rfc5, a replication factor C component, is required for regulation of Rad53 protein kinase in the yeast checkpoint pathway. Mol Cell Biol, 17, 5905-5914.

Takahashi, K., Yamada, H. and Yanagida, M. (1994) Fission yeast minichromosome loss mutants mis cause lethal aneuploidy and replication abnormality. Mol Biol Cell, 5, 1145-1158.

Tan, S. and Wang, T.S. (2000) Analysis of fission yeast primase defines the checkpoint responses to aberrant S phase initiation. Mol Cell Biol, 20, 7853-7866.

Tanaka, H., Tanaka, K., Murakami, H. and Okayama, H. (1999) Fission yeast cdc24 is a replication factor C- and proliferating cell nuclear antigen-interacting factor essential for S-phase completion. Mol Cell Biol, 19, 1038-1048.

Tanaka, T., Knapp, D. and Nasmyth, K. (1997) Loading of an Mcm protein onto DNA replication origins is regulated by Cdc6p and CDKs. Cell, 90, 649-660.

Tercero, J.A., Labib, K. and Diffley, J.F. (2000) DNA synthesis at individual replication forks requires the essential initiation factor Cdc45p. EMBO J, 19, 2082-2093.

Tsurimoto, T. and Stillman, B. (1989) Purification of a cellular replication factor, RF-C, that is required for coordinated synthesis of leading and lagging strands during simian virus 40 DNA replication *in vitro*. Mol Cell Biol, 9, 609-619.

Tsurimoto, T. and Stillman, B. (1990) Functions of replication factor C and proliferating-cell nuclear antigen: functional similarity of DNA polymerase accessory proteins from human cells and bacteriophage T4. Proc Natl Acad Sci U S A, 87, 1023-1027.

Tsurimoto, T. and Stillman, B. (1991a) Replication factors required for SV40 DNA replication *in vitro*. I. DNA structure-specific recognition of a primer-template junction by eukaryotic DNA polymerases and their accessory proteins. J Biol Chem, 266, 1950-1960.

Tsurimoto, T. and Stillman, B. (1991b) Replication factors required for SV40 DNA replication *in vitro*. II. Switching of DNA polymerase α and δ during initiation of leading and lagging strand synthesis. J Biol Chem, 266, 1961-1968.

Uchiumi, F., Ohta, T. and Tanuma, S. (1996) Replication factor C recognizes 5'-phosphate ends of telomeres. Biochem Biophys Res Commun, 229, 310-315.

Uchiumi, F., Watanabe, M. and Tanuma, S. (1999) Characterization of telomere-binding activity of replication factor C large subunit p140. Biochem Biophys Res Commun, 258, 482-489.

Uhlmann, F., Cai, J., Gibbs, E., O'Donnell, M. and Hurwitz, J. (1997) Deletion analysis of the large subunit p140 in human replication factor C reveals regions required for complex formation and replication activities. J Biol Chem, 272, 10058-10064.

Uhlmann, F., Gibbs, E., Cai, J., O'Donnell, M. and Hurwitz, J. (1997) Identification of regions within the four small subunits of human replication factor C required for complex formation and DNA replication. J Biol Chem, 272, 10065-10071.

Umar, A., Buermeyer, A.B., Simon, J.A., Thomas, D.C., Clark, A.B., Liskay, R.M. and Kunkel, T.A. (1996) Requirement for PCNA in DNA mismatch repair at a step preceding DNA resynthesis. Cell, 87, 65-73.

Usui, T., Yoshida, M., Kasahara, K., Honda, A., Beppu, T. and Horinouchi, S. (1997) A novel HSP70 gene of *Schizosaccharomyces pombe* that confers K-252α resistance. Gene, 189, 43-47.

Waga, S., Masuda, T., Takisawa, H. and Sugino, A. (2001) DNA polymerase ε is required for coordinated and efficient chromosomal DNA replication in *Xenopus* egg extracts. Proc Natl Acad Sci U S A, 98, 4978-4983.

Wang, Y., Cortez, D., Yazdi, P., Neff, N., Elledge, S.J. and Qin, J. (2000) BASC, a super complex of BRCA1-associated proteins involved in the recognition and repair of aberrant DNA structures. Genes Dev, 14, 927-939.

Warbrick, E., Lane, D.P., Glover, D.M. and Cox, L.S. (1995) A small peptide inhibitor of DNA replication defines the site of interaction between the cyclin-dependent kinase inhibitor $p21^{WAF1}$ and proliferating cell nuclear antigen. Curr Biol, 5, 275-282.

Waseem, N.H., Labib, K., Nurse, P. and Lane, D.P. (1992) Isolation and analysis of the fission yeast gene encoding polymerase δ accessory protein PCNA. EMBO J, 11, 5111-5120.

Weinreich, M., Palacios DeBeer, M.A. and Fox, C.A. (2004) The activities of eukaryotic replication origins in chromatin. Biochim Biophys Acta, 1677, 142-157.

Wohlschlegel, J.A., Dwyer, B.T., Dhar, S.K., Cvetic, C., Walter, J.C. and Dutta, A. (2000) Inhibition of eukaryotic DNA replication by geminin binding to Cdt1. Science, 290, 2309-2312.

Yao, N., Turner, J., Kelman, Z., Stukenberg, P.T., Dean, F., Shechter, D., Pan, Z.Q., Hurwitz, J. and O'Donnell, M. (1996) Clamp loading, unloading and intrinsic stability of the PCNA, beta and gp45 sliding clamps of human, *E. coli* and T4 replicases. Genes Cells, 1, 101-113.

Yuzhakov, A., Kelman, Z., Hurwitz, J. and O'Donnell, M. (1999) Multiple competition reactions for RPA order the assembly of the DNA polymerase δ holoenzyme. EMBO J, 18, 6189-6199.

Zou, L., Mitchell, J. and Stillman, B. (1997) CDC45, a novel yeast gene that functions with the origin recognition complex and Mcm proteins in initiation of DNA replication. Mol Cell Biol, 17, 553-563.

Zuo, S., Gibbs, E., Kelman, Z., Wang, T.S., O'Donnell, M., MacNeill, S.A. and Hurwitz, J. (1997) DNA polymerase δ isolated from *Schizosaccharomyces pombe* contains five subunits. Proc Natl Acad Sci U S A, 94, 11244-11249.

Zou, L. and Stillman, B. (1998) Formation of a preinitiation complex by S-phase cyclin CDK-dependent loading of Cdc45p onto chromatin. Science, 280, 593-596.

Zou, L. and Stillman, B. (2000) Assembly of a complex containing Cdc45p, replication protein A, and Mcm2p at replication origins controlled by S-phase cyclin-dependent kinases and Cdc7p-Dbf4p kinase. Mol Cell Biol, 20, 3086-3096.

Zou, L., Cortez, D. and Elledge, S.J. (2002) Regulation of ATR substrate selection by Rad17-dependent loading of Rad9 complexes onto chromatin. Genes Dev, 16, 198-208.

Zou, L. and Elledge, S.J. (2003) Sensing DNA damage through ATRIP recognition of RPA-ssDNA complexes. Science, 300, 1542-1548.