

The Importance of DNA Replication Termination and the MHF Complex to Genome Stability



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DECLARATION

I hereby declare that this dissertation entitled “The Importance of DNA Replication Termination and the MHF Complex to Genome Stability” has been originally carried out by myself under the supervision of Professor Matthew Whitby, Department of Biochemistry, University of Oxford. This work has not formed the basis for award of any degree or diploma previously. The particulars given in the dissertation are true to the best of my knowledge.

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ABSTRACT

The final stages of replication fork termination requires the timely and orderly orchestration of catalytic and enzymatic activities. Given the complexity of this process, it is conceivable that the final stages of fork termination is susceptible to problems that could trigger recombination, which could lead to deleterious genomic rearrangements if ectopic homologous sequences are recombined. Using the site-specific *RTS1* barrier in fission yeast, I demonstrated that fork termination is generally not a recombinogenic process, and that hyper-recombination-induced by fork blockage at *RTS1* is largely a result of replication fork restart.

To investigate the actual mechanisms and proteins, which drive and influence recombination at a replication barrier, I studied the MHF proteins, which assist Fml1 in limiting crossovers during double-strand break (DSB) repair and promoting Rad51-mediated recombination at impeded replication forks, and are also components of the constitutive centromere-associated network (CCAN). Intriguingly, structural studies revealed that the MHF can exist as an octamer *in vitro*. I examined the biological significance of octameric MHF by employing three mutations that disrupt the octamer configuration *in vitro*. In fission yeast, these mutations cause hypersensitivity to methyl methanesulfonate (MMS), suggesting that the MHF octamer may have a role in DNA repair. One of the “octamerisation” mutants, exhibits greater hypersensitivity to MMS than the other two, and biochemical experiments indicated that this is because it confers an additional defect in MHF’s interaction with Fml1. Further genetic experiments on this mutant suggest that the ability of Fml1 to unwind D-loops depends more critically on its interaction with MHF than fork reversal. Additionally, I showed a synergistic interaction between Dcr1 and MHF, and demonstrated that in the absence of Dcr1, there is a greater need for recombination to tolerate/repair DNA damage. Lastly, I uncovered a novel function for the MHF in controlling the initiation of septation.

ABBREVIATIONS

ATPase	adenosine triphosphatase
BIR	break-induced replication
BND	benzoylated naphtholyated DEAE
bp	base pair
BSA	bovine serum albumin
CCAN	centromere-associated network
CDEI	centromere DNA element I
<i>Cen</i>	centromere
CENP-S	centromere-associated protein S
ChrI	chromosome I
ChrII	chromosome II
ChrIII	chromosome III
cM	CentiMorgan
CMG	Cdc45, Mcm2-7, GINS complex
cnt1	central core region 1
CPD	cyclobutane pyrimidine dimer
CPT	camptothecin
D-loop	displacement loop
DAPI	4',6-diamidino-2-phenylindole
DEAE	diethylaminoethanol
DEB	diepoxybutane
dHJ	double Holliday junction
diRNA	DSB-induced RNA
DMSO	dimethylsulfoxide
DNA	deoxyribonucleic acid
dNTP	deoxynucleotide triphosphate
DSB	double-strand break
DSBR	DNA double-strand break repair
dsDNA	double-stranded DNA
DTT	dithiothreitol
EDTA	ethylenediaminetetraacetic acid
EMMG	Edinburgh minimal media glutamate
EMSA	electrophoretic mobility shift assay
FA	Fanconi anaemia
FAAP250	Fanconi anaemia A-associated polypeptide with a mass of 250 kDa
FANCM	Fanconi's anaemia complementation group M
FOA	5' fluoroarotic acid
GFP	green fluorescent protein
<i>Gg</i>	<i>Gallus gallus</i>
GINS	go, ichi, nii, and san; five, one, two, and three in Japanese
Hef	helicase-associated endonuclease for fork structured DNA
HhH	helix-hairpin-helix
HJ	Holliday junction
HJURP	Holliday junction recognition protein

HR	homologous recombination
<i>Hs</i>	<i>Homo sapiens</i>
HU	hydroxyurea
ICL	interstrand crosslink
ID	FANCI-FANCD2 complex
imrL	inverted repeat sequences L
IR	ionizing radiation
KASH	Klarsicht, ANC-1, Syne Homology
kb	kilobases
KMN	KNL1, Mis12 complex and Ndc80 complex
LB	Luria-Bertani
LOH	loss of heterozygosity
M	minus
Mb	mega bases
MBP	maltose binding protein
MEA	malt extract agar
MHF	FANCM-associated histone-fold protein
MMC	mitomycin C
MMS	methyl methanesulfonate
Mph1	mutator phenotype
MPS1	mating-type pause site
MRN	Mre11-Rad50-Nbs1 complex
MRX	Mre11-Rad50-Xrs2 complex
NER	nucleotide excision repair
NHEJ	non-homologous end joining
ORC	origin recognition complex
otrL	outer repeat L
N3-MeA	3-methyladenine
P	plus
PCNA	proliferating cell nuclear antigen
PCR	polymerase chain reaction
<i>Pfu</i>	<i>Pyrococcus furiosus</i>
PMSF	phenylmethylsulphonylfluoride
rDNA	ribosomal DNA
RDR	recombination-dependent replication
RFB	replication fork barrier
RFC	replication factor C
RNAi	RNA interference
RPA	replication protein A
Rtf1	replication termination factor 1
<i>RTS1</i>	replication termination sequence 1
<i>RTS1-AO</i>	<i>RTS1</i> active orientation
<i>RTS1-IO</i>	<i>RTS1</i> inactive orientation
SAXS	small angle X-ray scattering
<i>Sc</i>	<i>Saccharomyces cerevisiae</i>
SDS	sodium dodecyl sulphate
SDS-PAGE	SDS-polyacrylamide gel electrophoresis
SDSA	synthesis-dependent strand annealing

SF2	superfamily 2
SIN	septation initiation network
siRNA	short interfering RNA
SLX-MUS	MUS81-EME1 and SLX1-SLX4
<i>Sp</i>	<i>Schizosaccharomyces pombe</i>
SPR	surface plasmon resonance
sRNA	small RNA
SSA	single-strand annealing
SSC	saline sodium citrate
ssDNA	single-stranded DNA
SUN	Sad1p, UNC-84
TBZ	thiabendazole
tDNA	transfer DNA
<i>Tel</i>	telomere
TLS	translesion synthesis
Top1	topoisomerase I
tRNA	transfer RNA
TS	temperature sensitive
UV	ultraviolet light
YE	yeast extract
YES	yeast extract with supplements
2D	two-dimensional

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Chapter 1: Introduction

Fidelity in the replication of deoxyribonucleic acid (DNA) is crucial for inheriting a complete and accurate genome, which encodes information essential for life. In eukaryotes, replication is achieved through the firing of replication forks from origins along the length of each chromosome. Each origin fires to produce two bi-directionally moving replication forks which eventually will merge with opposing forks or reach the end of chromosome arms (i.e. the telomeres). With each successive cell division, a human diploid cell has to accurately replicate the sequence of 6 billion base pairs (bps) and segregate sister chromatids equally at mitosis to ensure genome stability (McGlynn and Lloyd 2002a).

DNA lesions, DNA secondary structures and protein-DNA barriers pose as accidental or programmed barriers to replication fork progression (Lambert and Carr 2013). Such barriers impede the progression of the fork and can prevent the completion of replication if not dealt with appropriately. Incomplete replication can lead to DNA breakage during chromosome segregation in anaphase. This can ultimately result in cell death. Therefore, cells have evolved mechanisms like homologous recombination (HR) to restore DNA integrity and restart replication at blocked or broken replication forks (Hoeijmakers 2001; Aplan 2006; Jefford and Irminger-Finger 2006; Halazonetis et al. 2008; Chen et al. 2010).

In this chapter, I review the process of HR and its related proteins. As the molecular mechanisms involved in HR are best understood in the context of double-strand break (DSB) repair, I will first describe the various models of HR in DSB repair. Building on this, I will provide an overview of the key factors

that drive HR and discuss the involvement of HR in DNA replication. Finally, I will introduce the following HR proteins, FANCM (Fanconi's anaemia complementation group M), Mhf1 (FANCM-associated histone-fold protein 1) and Mhf2, in which much of my research focuses on.

1.1 Homologous recombination in double-strand break repair

HR is a conserved, high fidelity DNA repair mechanism that cells employ to mediate the transfer of genetic information between homologous or near homologous (homeologous) DNA sequences (Heyer et al. 2006). Besides being necessary for the repair of DNA DSBs, HR is used to repair single-stranded DNA (ssDNA) gaps and interstrand crosslinks (ICLs), recover stalled and broken replication forks (Li and Heyer 2008), and maintain the telomeres. Apart from that, cells exploit the properties of HR to carry out various biological processes. These include: 1) antigenic variation in pathogens (e.g. *Neisseria gonorrhoeae*) to escape their host's immune system (Robertson and Meyer 1992), 2) mating-type switching in budding yeast and fission yeast to enable self-fertilisation (Haber 2012), 3) creating new combinations of parental alleles for genetic diversity during meiosis I (Champion and Hawley 2002), and 4) producing chiasmata, which direct the correct monopolar attachment of the spindle so that the homologues are segregated properly during meiosis I (Whitby 2005; Phadnis et al. 2011).

Other than HR, the other main pathway cells use in the repair of DSBs is non-homologous end joining (NHEJ) (Pfeiffer et al. 2000). These two processes are fundamentally distinct and function in competition with each other (Haber 2000). HR involves the use of an intact homologous DNA molecule as a

template to retrieve genetic information lost due to the DSB (allelic recombination). As such, HR is considered to be an error-free pathway, however it can also result in deleterious genomic change if recombination occurs between ectopic homologous sequences (e.g. DNA repeats in different parts of the genome). Thus, the employment of HR has to be well regulated. On the other hand, NHEJ involves the re-ligation of two DNA ends with little or no base pairing at the junction. NHEJ is therefore considered to be an error-prone pathway. In the G1 phase of the cell cycle, when the DNA has not been replicated and there is only the homologous chromosome available as a template for repair, NHEJ is predominantly used to re-ligate the broken DNA ends. In the late S and G2/M phases of the cell cycle, when a sister chromatid is available as a template for repair, HR is preferentially used to repair the DSB (Heyer et al. 2010).

1.1.1 Models for homologous recombination in double-strand break repair

The DNA double-strand break repair (DSBR) model emerged in 1983 (Szostak et al. 1983), following the discovery by Szostak and colleagues that DSBs were strong initiators of recombination (Orr-Weaver et al. 1981; Szostak et al. 1983). Not only has this model been able to explain meiotic segregation patterns, some of its key features have also been confirmed by physical analyses of meiotic recombination intermediates, primarily in *Saccharomyces cerevisiae* (Paques and Haber 1999; Krogh and Symington 2004). In spite of that, the DSBR model cannot explain all aspects of mitotic and meiotic recombination and therefore additional models have been

proposed. These include synthesis-dependent strand annealing (SDSA), break-induced replication (BIR) and single-strand annealing (SSA).

All HR pathways generally consist of three stages. In the first stage, pre-synapsis, broken DNA is resected by exonucleases to generate 3' ssDNA tails onto which the central recombinase, Rad51, loads to form the Rad51 nucleoprotein filament. In the second stage, synapsis, the Rad51 nucleoprotein filament searches for a homologous DNA template through homologous pairing before strand invasion occurs. DNA polymerases subsequently extend the invaded tail to restore any DNA that was lost at the DNA break site. In the third stage, post-synapsis, the recombination intermediates are variously resolved by DNA structure-specific nucleases and helicases/translocases.

1.1.1.1 The double-strand break repair model

In the DSBR model, the Rad51 nucleoprotein filament invades a double-stranded DNA (dsDNA) homologue, generating a displacement loop (D-loop). DNA synthesis extends the D-loop, enabling the other resected DSB end to anneal to it – a process that is termed second end capture (Figure 1.1). Once captured, the D-loop serves as a template to prime DNA synthesis to fill in the gap. This leads to the formation of a double Holliday junction (dHJ). Branch point migration of each Holliday junction (HJ) can extend or limit the region of DNA heteroduplex formed by strand exchange. Cleavage of the dHJ resolves the two conjoined DNA molecules, resulting in the formation of either a crossover or a non-crossover recombinant depending on how each HJ is cleaved (Figure 1.1). Alternatively, branch migration of the HJs towards each

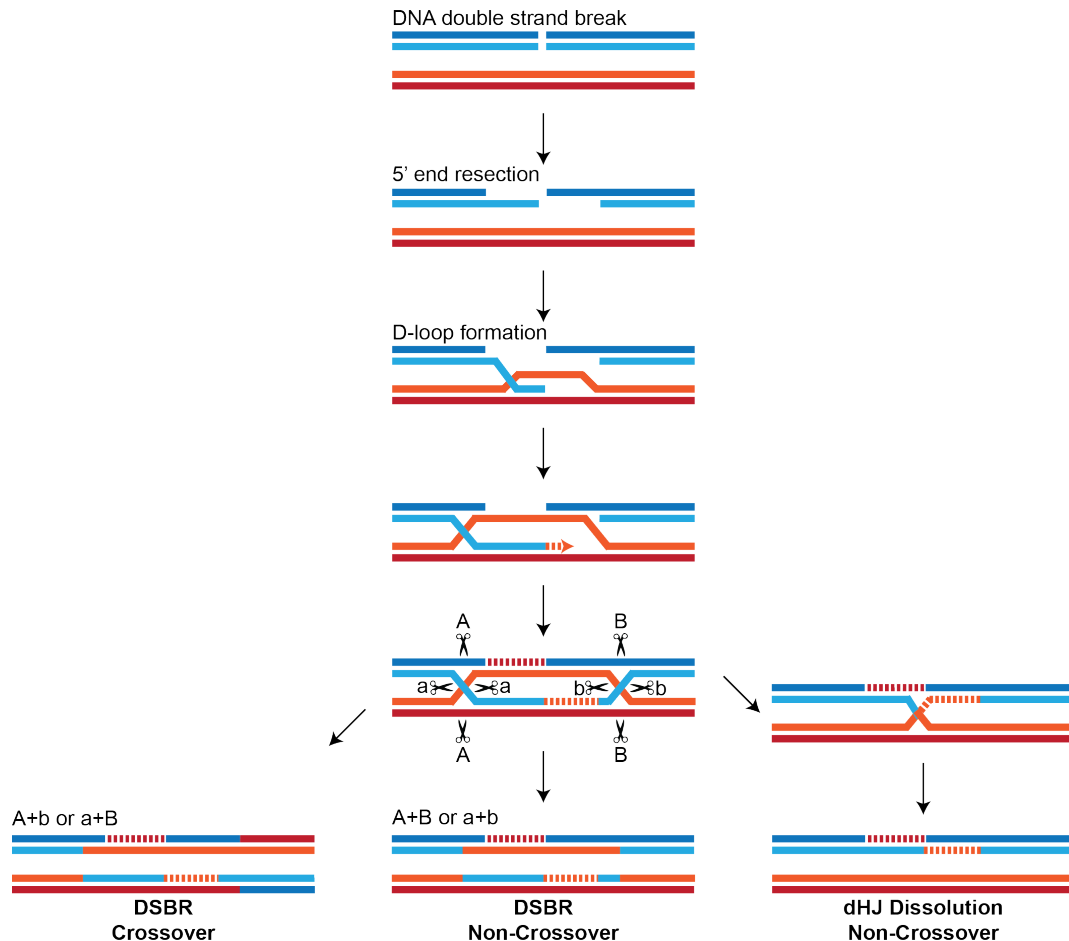


Figure 1.1 The double-strand break repair model

In DSBR, the 5' ends of the DSB are resected to generate a substrate with 3' ssDNA tails followed by Rad51 mediated strand invasion into a homologous DNA molecule (sister chromatid or homologous chromosome), which forms a D-loop. New DNA synthesis is primed from the 3' end of the invading strand. The D-loop then anneals to the other resected DSB end – a process termed second end capture. Filling of the gaps and ligation forms a dHJ. The dHJ is resolved by endonucleatic cleavage of a pair of strands at each HJ to form crossover ($A+b$ or $a+B$) or non-crossover ($A+B$ or $a+b$) recombinants. Alternatively, dHJ dissolution can occur to form non-crossover recombinants.

other causes them to convert into a hemicatenane that is eliminated by the action of a type I topoisomerase. This method of dHJ processing is termed dissolution and is catalysed by the concerted action of a RecQ-type helicase and topoisomerase III, generating non-crossovers.

A crossover occurs when the two HJs are resolved in the opposite sense, giving rise to reciprocal exchange of DNA that flanks the dHJ. A non-crossover occurs when the two HJs are resolved in the same sense, giving rise to a product where there is no exchange between the DNA flanking the dHJ. In support of this model, physical analysis of meiotic and mitotic recombination intermediates in *S. cerevisiae* has shown the presence of single-end DNA invasion intermediates and dHJs (Schwacha and Kleckner 1995; Hunter and Kleckner 2001; Bzymek et al. 2010).

1.1.1.2 Synthesis-dependent strand annealing model

Subsequent studies of some DSB-induced gene conversion events showed lower levels of associated crossing over than would be expected from the DSBR model (Nassif et al. 1994; Ferguson and Holloman 1996). This led to the proposal of the SDSA model (Nassif, Penney et al. 1994). As with the DSBR model, the recombination by SDSA initiates at DSBs, and the 3' end of the invading strand functions as a primer for DNA synthesis. However, as an alternative to dHJ formation, the newly synthesised 3' end of the invaded strand becomes displaced from the template and anneals to the resected strand of the second DSB end (Figure 1.2). This can occur by helicases that actively disrupt the annealing of the newly synthesised 3' end. The gaps in the DNA that remain from when the two ends of the DSBs anneal are then filled in

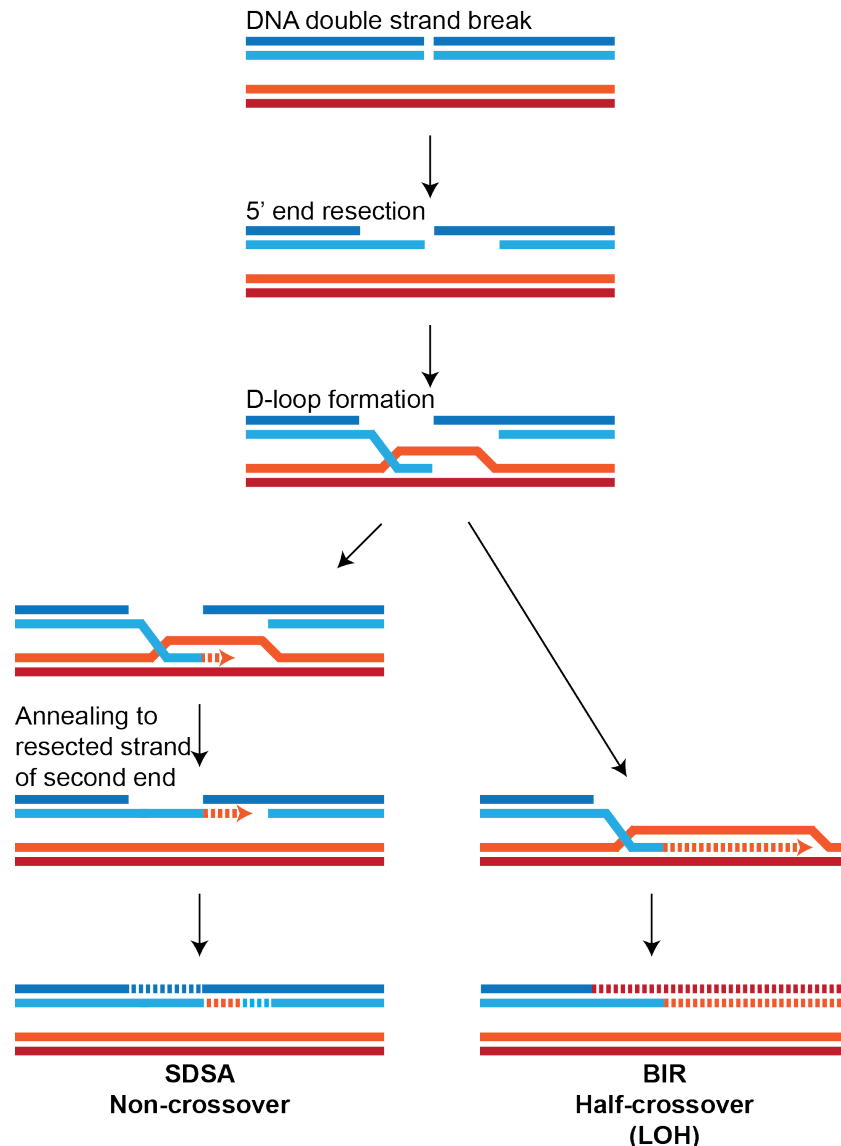


Figure 1.2 A schematic for double-strand break repair by synthesis-dependent strand annealing and break-induced replication pathways

In SDSA, new DNA synthesis is primed from the 3' end of the invading strand. The invading strand is displaced and annealed to the resected second end of the DSB. Gap filling and ligation of nicks then complete repair. This pathway exclusively generates non-crossover recombinants. In BIR, a one-ended DSB invades a homologous DNA template to form a D-loop. New DNA synthesis is primed from the 3' end of the invading strand and extended, leading to a migrating bubble that persists for potentially hundreds of kilobases, down the length of the chromosome and to the end of the telomere. DNA synthesis is conservative resulting in the formation of a half-crossover recombinant. If a homologue instead of the sister chromatid was invaded, it could result in LOH.

through DNA synthesis, followed by DNA ligation. This type of HR would exclusively give rise to non-crossovers and was proposed as a model to account for the paucity of crossovers in mitotic cells (Nassif et al. 1994). Additionally, the observation that newly synthesized DNA associated with recombinants is found on the same heteroduplex (i.e. conservative DNA synthesis) corroborated this model (Ira et al. 2006). The existence of such recombinants is not explained by the DSBR model, which predicts semi-conservative DNA synthesis (Gilbertson and Stahl 1996). In contrast to commonly held assumptions that crossovers and non-crossovers arise from alternative resolution of the same dHJ intermediate, studies of meiotic recombination in *S. cerevisiae* suggest that non-crossovers predominantly arise from the SDSA pathway, while crossovers predominantly arise from the DSBR pathway involving the biased resolution of a dHJ (Allers and Lichten 2001). If such were true, the decision to repair a DSB via a crossover or non-crossover would have had to be made prior to the formation of the dHJ.

1.1.1.3 Break-induced replication model

BIR involves the repair of a one-ended DSB, or a two-ended DSB that only shares homology with an intact DNA sequence on one end, or two free DSB ends finding homology in different areas of the genome. A one-sided DSB can arise from the breakage of a replication fork, or the uncapping and erosion of telomeres. In the absence of second end capture, extension of the 3' invading strand accompanied by branch migration of the D-loop, establishes a replication fork. This can continue for hundreds of kilobases (kb) till the end of the chromosome is reached, or convergence with an opposing replication fork

occurs. DNA synthesis is conservative resulting in the formation of a half-crossover recombinant. If the homolog instead of the sister chromatid is invaded, it can result in an extensive loss of heterozygosity (LOH) (Morrow et al. 1997; Bosco and Haber 1998) (Figure 1.2).

1.1.1.4 Single-strand annealing model

HR can also proceed in a Rad51 independent pathway through SSA. SSA occurs typically at DSBs flanked by homologous sequences, for example, DNA repeats (Lin et al. 1984). Here, DNA ends are resected to expose the complementary 3' ssDNA tails that can simply anneal to each other, facilitated by Rad52's strand annealing properties. The non-homologous DNA between the annealed repeats is flipped out, forming 3' flaps. These 3' flaps are subsequently cleaved by nucleotide excision repair (NER) proteins, Rad1 and Rad10, and a mismatch recognition complex, Msh2-Msh3 (Fishman-Lobell and Haber 1992). Final processing involves gap filling and the ligation of nicks (Paques and Haber 1999) (Figure 1.3). SSA generates deletions between flanking direct repeats and it is thus regarded as a mutagenic sub-pathway of HR.

1.1.2 Mechanism of homologous recombination

As introduced above, HR has a few sub-pathways that can be differentially employed in various contexts. A large number of recombination factors exist to accommodate this versatility. In this section, I provide an overview of the key factors that drive HR. The focus is on the mechanistic phases of recombination and the principal proteins that execute them, identifying key

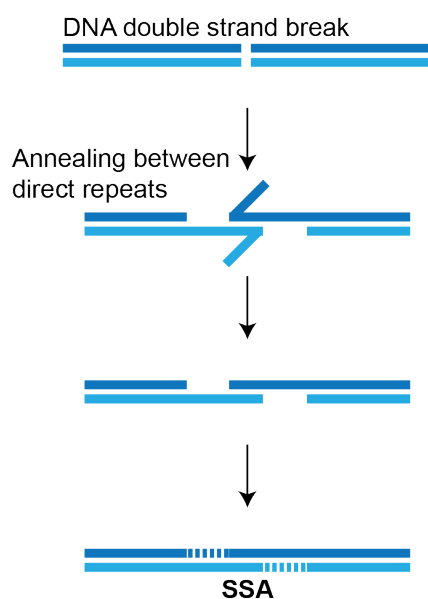


Figure 1.3 A schematic for double-strand break repair by single-strand annealing

SSA can happen when DSBs occur within direct DNA repeats. Rad52 can anneal complementary sequences in the direct repeats in a Rad51-independent manner. The 3' flaps between the direct repeats can then be processed by DNA endonucleases, the gaps can be filled and the resulting DNA nicks can be ligated. SSA leads to a deletion of the intervening sequence and of one of the repeats.

regulatory transitions, structures and mechanisms. Meiotic HR factors are included in this section if they are also applicable to vegetative cells.

I will focus on what we know about HR in *Schizosaccharomyces pombe*, since it is the model organism I use in my research. Alongside that, I will allude to key insights derived from other widely used model organisms, including the bacterium *Escherichia coli* and the budding yeast *S. cerevisiae*. In Table 1.1 there is a list of the key HR proteins from these organisms as well as their equivalents/homologues from mammalian cells.

1.1.2.1 Pre-synapsis: resection

In fission yeast, resection involves a surprising complexity of four nucleases Mre11-Rad50-Nbs1 (MRN), Exo1, Dna2, Ctp1, and a helicase Rqh1. Resection is thought to begin the moment the heterotrimeric DNA binding complex, MRN, is recruited to the DSB site. Both Mre11 and Rad50 proteins are well conserved in all organisms, while Nbs1 is weakly conserved. A functional analogue of the MRN complex exists in budding yeast (Mre11-Rad50-Xrs2 [MRX]) (Alani et al. 1990; Nairz and Klein 1997; Tsubouchi and Ogawa 1998; Nicolette et al. 2010) and in human (MRE11-RAD50-NBS1 [MRN]) (Yuan and Chen 2010). The MRN/MRX complexes have three important functions: 1) DSB recognition, 2) initiation of DSB sensing and cell cycle checkpoint signalling, and 3) regulation of chromatin remodelling in the vicinity of the DSB (Williams et al. 2007). Four main observations have implicated the MRN/MRX complex in catalysing end resection to produce 3' ssDNA tails. Firstly, sub-nuclear foci containing both Rad50 and Mre11 have been observed in mammalian and yeast cells following γ -irradiation

Table 1.1 Homologous Recombination Proteins

	<i>E. coli</i>	<i>S. cerevisiae</i>	<i>S. pombe</i>	Mammals
Pre-synapsis Processing of broken DNA ends	RecBCD			
	SbcD-SbcC	Mre11-Rad50-Xrs2	Mre11-Rad50-Nbs1	MRE11-RAD50-NBS1
		Sae2	Ctp1	CtIP
	RecQ	Sgs1	Rqh1	BLM
		Dna2	Dna2	DNA2
	RecE, RecJ	Exo1	Exo1	EXO1
Synapsis Homologous pairing and DNA strand exchange	SSB	RPA	RPA	RPA
	RecA	Rad51	Rad51	RAD51
	RecO	Rad52	Rad52	RAD52
		Rad59		
	RecF-RecR	Rad55-Rad57	Rad55-Rad57	RAD51B, RAD51C-XRCC3
		Sae3-Mei5	Swi5-Sfr1	SWI5-SFR1
		Shu1-Shu2-Psy3-Csm2	Rlp1-Sws1-Rdl1	XRCC2-SWS1-RAD51D-SWSAP1
		Rad54	Rad54	RAD54
Post-synapsis Resolution of recombination intermediates	RecQ	Sgs1	Rqh1	BLM, WRN, RECQ1, RECQ4, RECQ5
	Top3	Top3	Top3	TOPOIII α
		Mph1	Fml1	FANCM
			Fbh1	FBH1
		Srs2	Srs2	PARI
	RecG			
	RuvABC			
	RusA			
		Mus81-Mms4	Mus81-Eme1	MUS81-EME1
		Yen1		GEN1
		Slx1-Slx4	Slx1-Slx4	SLX1-SLX4

(Nelms et al. 1998); secondly, Mre11 has been observed to localise to DSB sites before end resection occurs in budding yeast (Lisby et al. 2004); thirdly, null mutants of *mre11*, *rad50* and *xrs2* having retarded resection of HO-induced DSBs at the budding yeast mating-type locus (Ivanov et al. 1994; Tsubouchi and Ogawa 1998); and fourthly, an accumulation of un-resected meiotic DSBs in *mre11* nuclease defective and *rad50S* mutant budding yeast cells (Alani et al. 1990; Tsubouchi and Ogawa 1998).

Biochemical studies have revealed that Mre11 has a 3' → 5' exonuclease activity and a DNA structure-specific endonuclease activity that acts on 5' overhangs, 3' flaps and 3' branches (Trujillo and Sung 2001). The Mre11 exonuclease activity has the opposite polarity needed to produce 3' ssDNA tails, and thus, Mre11 needs to work in conjunction with other factors to produce the overhang. Rad50 greatly stimulates the nuclease activities of Mre11, and provides a means to hold the broken DNA ends together (Trujillo and Sung 2001). The budding yeast Xrs2 orthologue of Nbs1, on the other hand has DNA binding activity that helps target the complex to DNA ends (Trujillo et al. 2003). Notably, the MRN/MRX complexes can displace Ku, a NHEJ initiation protein, suggesting that MRN/MRX has a role in preventing NHEJ (Wu et al. 2008).

Current models based on studies performed on budding yeast, have suggested that DNA end resection is a two-step process that can be divided into resection initiation and resection extension. It is proposed that in resection initiation, MRX recruits another endonuclease Sae2 (the budding yeast homologue of the fission yeast Ctp1). MRX-Sae2 catalyses the initial

elimination of 50 to 100 nucleotides from the 5' end of the break, thus generating short 3' ssDNA overhangs (Mimitou and Symington 2008). Extensive resection is then facilitated by two parallel pathways which depend on: 1) the 5' → 3' exonuclease Exo1, and 2) the concerted action of the 3' → 5' DNA helicase Sgs1 (the budding yeast homologue of the fission yeast Rqh1), with the exonuclease Dna2 (Mimitou and Symington 2008; Zhu et al. 2008).

1.1.2.2 Pre-synapsis to synapsis: Rad51 nucleoprotein filament formation and strand invasion

After 3' ssDNA tails have been generated by resection, replication protein A (RPA) binds to the ssDNA to eliminate secondary structures, to facilitate Rad51 loading. RPA is a highly conserved heterotrimeric ssDNA binding protein important for DNA replication, repair and recombination. Besides removing secondary structures, RPA also protects the 3' ssDNA tail from nucleolytic attack, and aids Rad51 nucleoprotein filament invasion by binding to the displaced strand during D-loop formation (Eggler et al. 2002).

During synapsis, the Rad51 nucleoprotein filament searches for a homologous DNA template through strand invasion and homologous pairing. Rad51 is a highly conserved orthologue of the principal recombination enzyme in *E. coli*, RecA. Rad51 is non-essential in budding and fission yeasts, and is even dispensable in some recombination pathways (i.e. SSA). However, RAD51 is essential for viability in mammals and the deletion of RAD51 in mice is embryonic lethal (Tsuzuki et al. 1996; Sonoda et al. 1998). Biochemical studies have shown that like RecA, Rad51 oligomerises on

resected 3' ssDNA tails to form extensive right-handed helical nucleoprotein filaments that catalyse strand exchange between a circular ssDNA molecule and a homologous linear dsDNA molecule (Ogawa et al. 1993; Sung 1994; Namsaraev and Berg 1997). Nucleoprotein filament formation stretches DNA from 12.5 bps per turn to 18.6 bps per turn (Ogawa et al. 1993). This stretch increases the accessibility of the bps within the filament for efficient strand invasion and base pairing. Some aspects of nucleoprotein filament dynamics are dependent on ATP hydrolysis by Rad51 (Baumann et al. 1996). Although the formation of Rad51 nucleoprotein filaments is facilitated by RPA, paradoxically, RPA has a higher affinity for ssDNA than Rad51. This forms a kinetic barrier against Rad51 filament assembly, necessitating mediator proteins to allow timely Rad51 filament formation on RPA-coated ssDNA.

Rad52 is a mediator protein, which is thought to displace RPA from ssDNA to aid Rad51 loading (Benson et al. 1998; Song and Sung 2000; Kim et al. 2002; Sugiyama and Kowalczykowski 2002). Besides being a mediator protein, Rad52 can also promote strand annealing of RPA-bound complementary ssDNA (Kagawa et al. 2002). Rad52 is required for all recombination whether they are Rad51-dependent or not (Doe et al. 2004).

Apart from Rad52, several other mediator proteins have been identified. Rad55 and Rad57 are Rad51 paralogues that mediate Rad51-dependent strand exchange by stabilising Rad51 nucleoprotein filaments (Sung 1997). They are classified as Rad51 paralogues because they share sequence similarity to Rad51. The mammalian Rad51 paralogues RAD51B, RAD51C, RAD51D, XRCC2 and XRCC3 variously interact with each other to stabilise

Rad51 nucleoprotein filaments (Bishop et al. 1998). Rad55 and Rad57 form a stable heterodimer and *in vitro*, are able to overcome RPA inhibition of Rad51-mediated strand exchange (Sung 1997).

Additionally, fission yeast and mammals have Swi5 and Sfr1 mediator proteins, which mediate Rad51 nucleation and stimulate Rad51-mediated strand exchange (Akamatsu et al. 2003; Akamatsu et al. 2007; Haruta et al. 2008).

Further, fission yeast has two highly divergent Rad51 paralogs, Rlp1 and Rdl1, which function with Sws1 as HR mediator proteins (Khasanov et al. 2004; Martin et al. 2006). Shu1 and Psy3, and XRCC2 and RAD51D are homologues of Rlp1 and Rdl1 in budding yeast and human respectively. Also, Shu2 in budding yeast has been identified to be the homologue of Sws1 (Sasanuma et al. 2013)

Another protein that aids Rad51 nucleoprotein formation and function is Rad54, which is conserved from yeasts to human and is a member of the Swi2/Snf2 family of DNA-dependent adenosine triphosphatases (ATPases). It has been proposed that Rad54 promotes the homology search by causing the transient melting of sections of duplex DNA, thereby allowing recognition of homology via base pairing (Petukhova et al. 1999; Van Komen et al. 2000). Importantly, the dsDNA translocase activity of Rad54 dissociates Rad51 from dsDNA filaments after strand invasion. Not only does this allow Rad51 to be recycled for new rounds of recombination, it also gives a DNA polymerase access to the invading 3' strand for DNA extension to restore lost information (Solinger et al. 2002; Chi et al. 2006; Heyer et al. 2006; Li and Heyer 2009).

1.1.2.3 Post-synapsis: resolution of recombination intermediates

In the last stage, post-synapsis, one of the three sub-pathways of HR (DSBR, SDSA, BIR) (Figure 1.1 and Figure 1.2) ensues (West 2003; Krogh and Symington 2004; Heyer 2007; San Filippo et al. 2008). Each of these sub-pathways has specific enzymatic requirements that are still only partially defined.

RecQ-type DNA helicases (Rqh1 in fission yeast; Sgs1 in budding yeast; and BLM, WRN, RECQ1 and RECQ4 in mammals) and Fml1 (Mph1 in budding yeast; and FANCM in mammals) have been shown to unwind D-loops, and may all have a role in promoting the SDSA sub-pathway which leads exclusively to non-crossovers (Watt et al. 1996; Hanada et al. 1997; Stewart et al. 1997; Ira et al. 2003; Sharma et al. 2005; Bachrati et al. 2006; Dupaigne et al. 2008; Sun et al. 2008; Lorenz et al. 2009; Prakash et al. 2009; Lorenz et al. 2012).

Additionally, fission yeast Fbh1, budding yeast Srs2, mouse FBH1 and human RECQ5 have been shown to disrupt Rad51 nucleoprotein filaments, further driving the SDSA pathway (Ira et al. 2003; Krejci et al. 2003; Veaute et al. 2003; Hu et al. 2007; Dupaigne et al. 2008; Lorenz et al. 2009; Simandlova et al. 2013).

Besides, in yeast, Rqh1 and Sgs1 helicases interact with Rmi1 and Top3 in dHJ dissolution (Gangloff et al. 1994; Bennett et al. 2000; Mullen et al. 2005; Mankouri and Hickson 2007). The same is true in humans with BLM

interacting with Rmi1, Rmi2 and TopoIII α (Wu and Hickson 2003; Ralf et al. 2006; Raynard et al. 2006; Singh et al. 2008).

Mutations in the human FANCM and RecQ homologues, BLM, WRN and RECQ4, are genetically linked to the autosomal recessive diseases, Fanconi anaemia (FA), Bloom's, Werner's, and Rothmund-Thomson syndromes respectively, all of which are characterised by heightened cancer predisposition, due to genome instability arising from inappropriate recombination, highlighting the importance of these helicases (Ellis et al. 1995; Yu et al. 1996; Gray et al. 1997; Kitao et al. 1999; Kennedy and D'Andrea 2005).

In *E. coli*, the main HJ resolvase is RuvC (Bennett et al. 1993), which acts in a complex with RuvA and RuvB, and makes symmetrical nicks at HJs. RuvC is able to direct the orientation of resolution towards the generation of crossover or non-crossover recombinants, depending on the way the RuvABC complex assembles on the junction (van Gool et al. 1999). In *S. pombe*, Mus81-Eme1 (Mus81-Mms4 in *S. cerevisiae*; and MUS81-EME1 in mammals) has been proposed to be the main HJ resolvase. (Boddy et al. 2001). In meiotic fission yeast cells, Mus81-Eme1 plays a central role in cleaving recombination intermediates to form crossovers (Osman et al. 2003; Smith et al. 2003). Although Mus81-Eme1 has some activity on intact HJs, its preferred substrates are branched DNA structures like 3' flaps, nicked HJs, D-loops and replication forks (Whitby et al. 2003; Osman and Whitby 2007). Also, budding yeast Yen1 and mammalian GEN1 have been identified as HJ resolvases (Ip et al. 2008; Blanco et al. 2010). Both cleave HJs symmetrically to produce a

nicked duplex, which can be readily ligated (West 2009; Rass et al. 2010). Additionally, the Slx1-Slx4 endonuclease complex has been identified in yeast and humans (Fricke and Brill 2003; Coulon et al. 2004). Its preferred substrates are Y junction, 5' flap, and replication fork structures (Fricke and Brill 2003). In human cells, there are two defined pathways of HJ resolution. MUS81-EME1 and SLX1-SLX4 (SLX-MUS) mediate the first pathway, while the second pathway is mediated by GEN1. Biochemical analysis has demonstrated that SLX-MUS coordinates the catalytic sites of the two endonucleases during HJ resolution, cleaving substrates more efficiently and orderly than when SLX1-SLX4 is alone (Garner et al. 2013; Wyatt et al. 2013). It is interesting to note that in budding yeast, fission yeast and human cells, Mus81-Mms4, Mus81-Eme1, Yen1, MUS81-EME1 and SLX1-SLX4 are not activated until late G2/M, suggesting that recombination intermediates are mainly processed by mechanisms such as dHJ dissolution that avoid the formation of crossovers, and HJ resolution acts only as a backup mechanism should the dHJ persist till the end of mitosis (Matos et al. 2011; Dehe et al. 2013; Sarbajna et al. 2014).

1.2 DNA replication and homologous recombination

DNA replication is a crucial process that demands exceptional fidelity to ensure accurate transmission of the genetic code from one generation to the next. The replication machinery often encounters impediments that are likely to stop it in its tracks. If not dealt with appropriately, these impediments have the potential to give rise to genetic defects. Thus, although inherently accurate

and highly processive, DNA replication relies on a close interplay with HR to overcome such impediments.

1.2.1 Fundamentals of DNA replication

When a replication origin fires, the DNA double helix is unwound to form a replication bubble with two opposing replication forks that will move bi-directionally. A highly ordered multi-subunit enzyme complex known as the replisome assembles at each replication fork. Structural and functional features of the replisome are highly conserved from prokaryotes to eukaryotes. There is continuous DNA synthesis of the leading strand and discontinuous DNA synthesis of the lagging strand due to the antiparallel nature of DNA coupled with the fact that DNA polymerases progress only in the 5' to 3' direction. The lagging strand is synthesised in short separated segments called Okazaki fragments, which subsequently get ligated.

In budding yeast, the replicative helicase, Mcm2-7, and accessory factors, Cdc45 and GINS (Go, Ichi, Nii, and San; five, one, two, and three in Japanese), make up the CMG complex (Maiorano et al. 2006). Replication factor C (RFC), comprising of Rfc1-5, is the clamp-loading complex that loads proliferating cell nuclear antigen (PCNA) at ssDNA/dsDNA junctions (Majka and Burgers 2004). PCNA is a homotrimeric DNA clamp that acts as a processivity factor for DNA polymerases during replication. During replication, the replicative helicase, Mcm2-7, unwinds parental DNA to expose the two individual strands. Pol α -primase complex is a multi-protein complex composed of primase and polymerase activity. To initiate replication, the primase subunits of the Pol α -primase complex synthesises an oligomer of 7

to 12 ribonucleotides which then undergoes limited extension with deoxyribonucleotides by Pol α (Pellegrini 2012). DNA Polymerases δ and ϵ elongate the primers generated by Pol α in an accurate and processive manner (Kunkel 2004; Kunkel 2011). Pol δ is essential for synthesis of the lagging strand, whereas Pol ϵ is used to synthesise the leading strand (Pursell et al. 2007; Nick McElhinny et al. 2008; Kunkel 2011; Georgescu et al. 2014). On the lagging strand, the RNA primers are displaced by Pol δ and removed by flap endonucleases, FEN1 and Dna2 (Kang et al. 2000; Stewart et al. 2008; Stewart et al. 2009) (Figure 1.4).

1.2.2 Impediments to replication

Replication fork barriers (RFBs) on a DNA template can stall and sometimes collapse a replication fork. Replication fork stalling is defined as the stabilisation of an arrested replication fork by checkpoint proteins to retain the replisome components on the DNA while the RFB is being removed. The replication fork may resume progression after the RFB has been removed. On the other hand, replication fork collapse is defined as the dissociation of the replisome components from the DNA at an arrested fork. Replication fork resumption is impaired and replisome components have to be reloaded before replication can be restarted.

There are a variety of RFBs that can impede replication fork progression (Rothstein et al. 2000). These RFBs can be either accidental or programmed.

Accidental barriers exist in the form of lesions, within the DNA template, secondary structures in the DNA and protein-DNA barriers

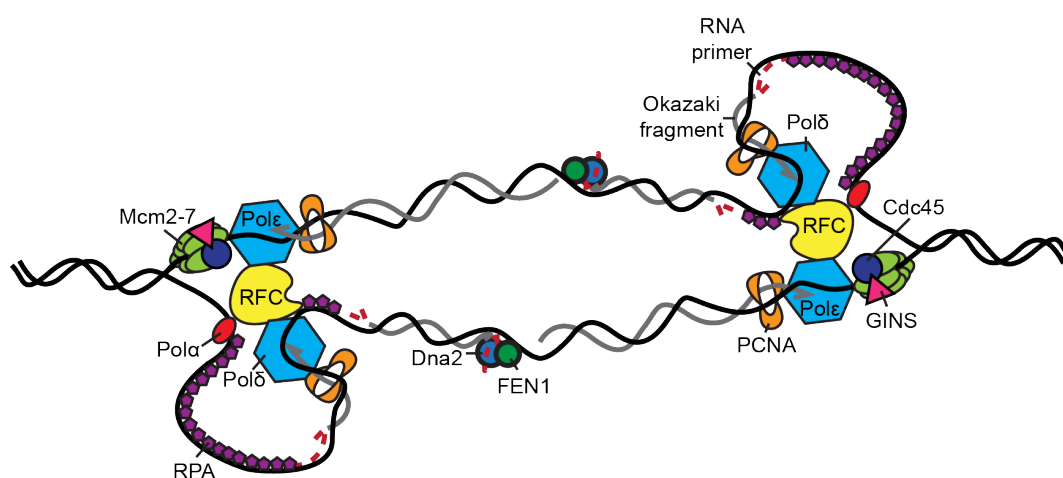


Figure 1.4 DNA replication

DNA replication is catalysed in a highly ordered manner by a multisubunit enzyme complex known as the replisome. The GINS complex maintains interactions within the replisome, which contains the Cdc45 and Mcm2-7 replicative helicase, the sliding clamp PCNA, and DNA polymerases Pol α , Pol δ and Pol ϵ . Pol α is the lagging strand primase, Pol δ is the lagging strand polymerase, while Pol ϵ is the leading strand polymerase. FEN1 and Dna2 are flap endonucleases that remove RNA primers displaced by Pol δ . Adapted from (McGlynn and Lloyd 2002b).

(Mirkin and Mirkin 2007). There are a number of different genotoxic agents that induce fork stalling DNA lesions. Methyl methanesulfonate (MMS) exposure results in the formation of 3-methyladenine (N3-MeA) adducts that block replication forks (Wyatt and Pittman 2006). Ultraviolet light (UV) induces cyclobutane pyrimidine dimers (CPDs) that can stall the replisome. Ionizing radiation (IR) causes strand breaks. Camptothecin (CPT) poisons topoisomerase I (Top1) and inhibits the re-ligation step such that the Top1-DNA covalent intermediate becomes longer lived at the 3' end of the nick (Hsiang et al. 1985). Nicks on the leading strand template can lead to the formation of a one-ended DSB as the leading strand polymerase runs off the template (Kuzminov 1999; Michel et al. 2001; Branzei and Foiani 2005) (see Figure 1.8). Contrarily, nicks on the lagging strand template can lead to the formation of a two-ended DSB since the synthesis of the lagging strand is discontinuous (Caldecott 2008). Hydroxyurea (HU) can temporarily pause replication forks by depleting the cellular deoxynucleotide triphosphate (dNTP) pool. Cisplatin induces ICLs, which prevent strand separation and impede the progression of the replicative helicase. Additionally, accidental barriers exist in the form of DNA secondary structures such as triplet repeats, G-quadruplex DNA and palindromic sequences that form hairpins. Some of these secondary structures interfere with strand separation, thus impeding replication (Samadashwily et al. 1997; Smith 2008; Mirkin 2013). Further, accidental barriers exist in the form of protein-DNA barriers such as transcription factors and RNA polymerases that are bound to the DNA template (Rothstein et al. 2000), for example replication fork stalling often occurs at highly transcribed

transfer RNA (tRNA) genes (Deshpande and Newlon 1996; Admire et al. 2006).

Remarkably, cells have evolved programmed protein-DNA barriers to synchronise the passage of replication forks across specific genomic loci to regulate important cellular functions. These programmed barriers are polar and block forks in only one direction. For example, the Tus-*ter* barrier in *E. coli* limits the encounter of two replication forks to a defined region of the chromosome to prevent the possibility of rolling circle replication and to aid the resolution of chromosome dimers (Khatri et al. 1989; Hill and Marians 1990; Hiasa and Marians 1994; Neylon et al. 2005; Bastia et al. 2008). *S. cerevisiae* ribosomal DNA (rDNA) regions contain the Fob1 protein-DNA barrier to prevent replication in the direction opposite to transcription, thus avoiding head-on collisions between replication and transcription machinery (Brewer et al. 1992; Kobayashi et al. 1992). Also, the replication termination sequence 1 (*RTS1*) in *S. pombe* enables mating-type switching (Dalgaard and Klar 2001; Vengrova et al. 2002; Egel 2004). A detailed description of mating-type switching and the *RTS1* RFB will be given in the next section.

1.2.2.1 Mating-type switching and the *RTS1* replication fork barrier

S. pombe exist as plus (P) or minus (M) mating-types. Mating-type switching relies on a genomic imprinting event that chemically modifies one strand of the DNA at the recipient *mat1* mating-type locus on chromosome II (Dalgaard and Klar 1999). Formation of the imprint depends on DNA replication occurring from the telomere to the centromere (Figure 1.5). The *mat1* cassette is transcriptionally active while the *mat2P* and *mat3M* cassettes are

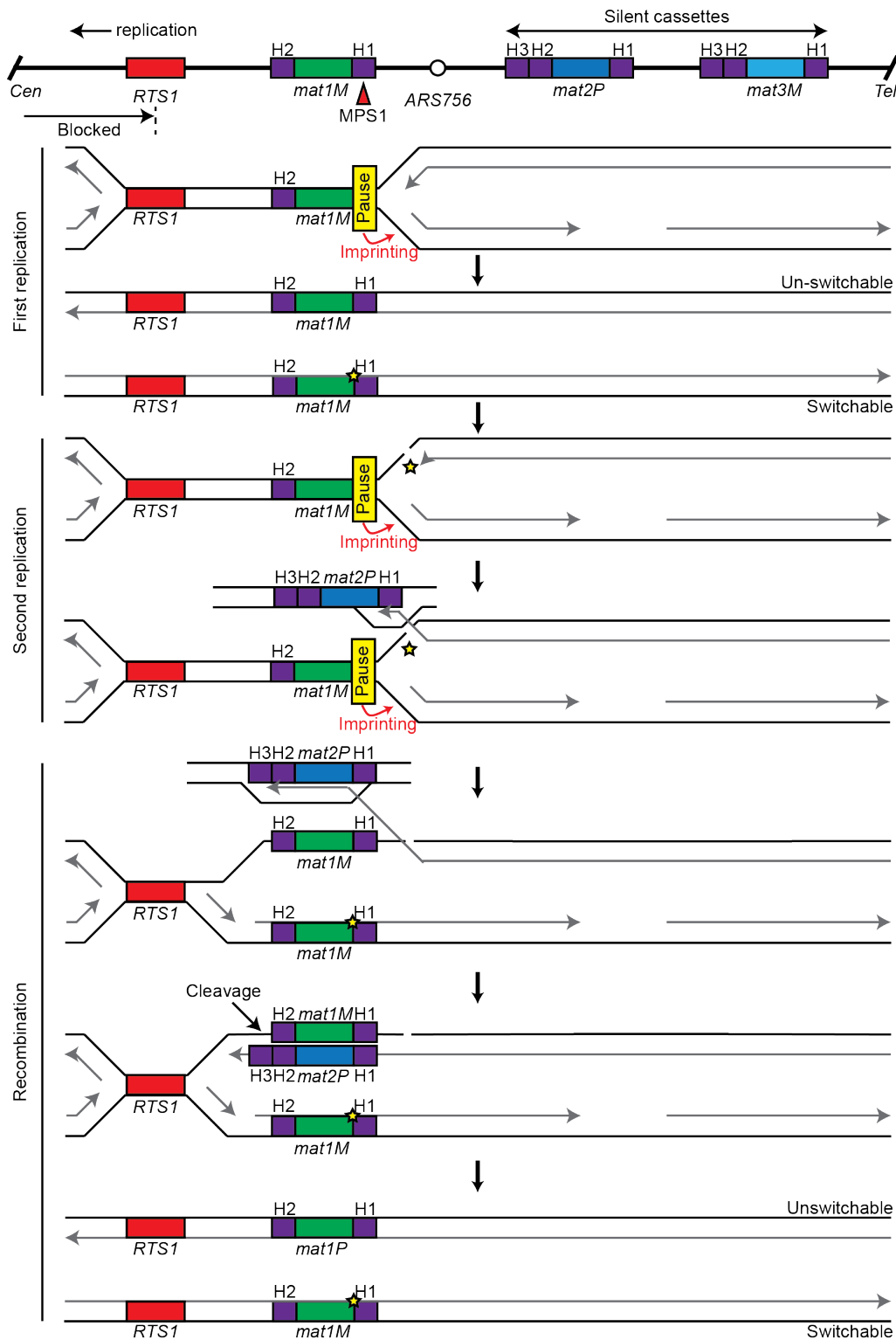


Figure 1.5 Mating-type switching in *S. pombe*

Cartoon depicting the mating-type locus on chromosome II. During mating-type switching, expressed mating-type at *mat1* can be replaced through gene conversion with *mat2P* or *mat3M*. Effective switching allows one of the two daughter cells formed in every generation to have a switched mating-type. Refer to text for more details. Adapted from (Lambert et al. 2007).

transcriptionally silent. H1 and H2 are homologous regions present on each cassette while H3 is specific to *mat2P* and *mat3M*. During the first replication, the fork traveling from the centromere to the telomere is blocked at *RTS1*. The fork traveling from the telomere to the centromere pauses at the mating-type pause site (MPS1) and an imprint (depicted by a yellow star in Figure 1.5) is formed by the incorporation of two ribonucleotides into the DNA during lagging strand synthesis. This imprint remains present in the DNA through to the next cell cycle. Thus, one daughter inherits a switchable chromatid while the other inherits an un-switchable chromatid (Figure 1.5). In the second round of replication, when the leading strand replication complex encounters the imprint, it leads to a DSB in the daughter cell that inherits the imprint. The 3' end of the DSB can invade the H1 box of one of the two transcriptionally silent donor loci, *mat2P* or *mat3M* (Figure 1.5). Gene conversion occurs, replacing the DNA at the *mat1* locus with an opposite mating-type cassette. Following that, the template of the leading strand is cleaved and re-synthesised using the newly synthesised leading strand as a template. Therefore, one daughter inherits a switched but un-switchable chromatid whereas the other daughter inherits a switchable chromatid (Arcangioli 1998; Dalgaard and Klar 1999; Arcangioli and de Lahondes 2000). Donor choice is governed by two portable recombination enhancers adjacent to *mat2P* and *mat3M* capable of promoting use the use of *mat2P* or *mat3M* even when they are transposed to an ectopic location (Jakociunas et al. 2013).

RTS1 is a polar protein-DNA barrier that ensures that *mat1* is replicated in the right orientation for imprinting, so that the ensuing mating-type switching can occur (Vengrova et al. 2002). *RTS1* contains two cis-acting sequences

(Dalgaard and Klar 2001). The first sequence, region B, is a ~450 bp sequence consisting of four repeated ~55 bp motifs bound by trans-acting Replication termination factor 1 (Rtf1). The second sequence, region A, is a ~60 bp purine-rich element located next to the first sequence and is bound by the trans-acting factor Rtf2 (Codlin and Dalgaard 2003). The two cis-acting sequences cooperate for a 4-fold enhanced barrier activity (Codlin and Dalgaard 2003; Eydmann et al. 2008). Rtf1 has two chimeric myb/SANT domains. One interacts with the *RTS1* repeated motifs and the enhancer region, while the other binds DNA weakly (Eydmann et al. 2008). Additionally, Rtf1 is a paralogue of Reb1, which is needed for Pol I transcription termination and rDNA replication barrier function (Packham et al. 1996; Rodriguez-Sanchez et al. 2011). Rtf2 stabilises the stalled fork at *RTS1* and prevents replication fork collapse, to enable DNA replication to be completed by a converging replication fork (Inagawa et al. 2009). Besides being replication progression complex components that travel with the replication fork, Swi1 and Swi3 form a complex that is needed for *RTS1* barrier activity, and also contributes to global fork protection (Dalgaard and Klar 2000; Noguchi et al. 2004) (Figure 1.6).

1.2.3 Overcoming replication fork barriers

Lesions, secondary structures and proteins bound to the DNA template all have the ability to stall a replication fork either by the inhibition of its replicative helicase or DNA polymerases. Accessory replicative helicases exist in all organisms to facilitate DNA replication across barriers. They also enable stalled replication forks to overcome DNA barriers before replication

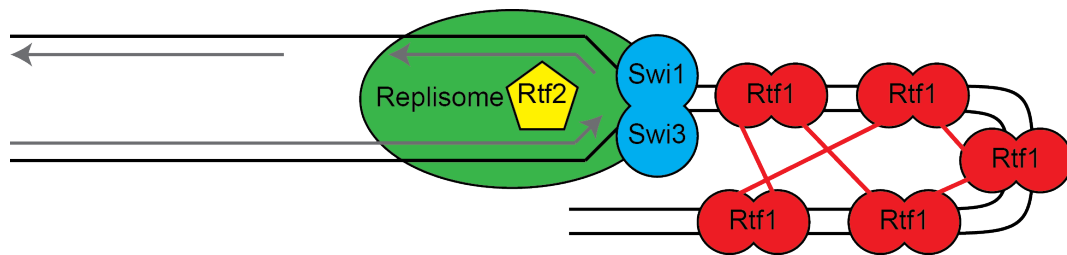


Figure 1.6 *RTS1* replication fork barrier

Cartoon depicting the termination of replication forks at the *RTS1* barrier, which is also dependent on the four trans-acting factors: Rtf1, Rtf2, Swi1 and Swi3.

forks collapse, thus allowing the resumption of replication and avoiding the dangers associated with fork restart and recombination (Keil and McWilliams 1993; Ivessa et al. 2003; Schmidt and Kolodner 2004; Torres et al. 2004; Guy et al. 2009; Gupta et al. 2013).

The Pif1 helicase family of 5' to 3' directed DNA helicases belongs to the super family IB helicases and is found in almost all eukaryotes from yeasts to humans, including some diverse bacteria. *S. cerevisiae* has two Pif1 family helicases, Pif1 and Rrm3, while *S. pombe*, like humans, contains only one Pif1 family helicase, Pfh1. It is thus possible that the role of Pfh1 in *S. pombe* has more similarities to the human Pif1 homologue than the *S. cerevisiae* Pif1 family helicases (Bochman et al. 2010). These ATP-dependent helicases have the ability to unwind duplex DNA and disrupt stable non-histone protein-DNA complexes (Lahaye et al. 1991; Lahaye et al. 1993; Zhou et al. 2000; Bochman et al. 2011). They have roles in replicating hard-to-replicate regions like mitochondrial, telomeric, centromeric, ribosomal and G-quadruplex DNA. Besides, they are important for Okazaki fragment maturation (Bochman et al. 2010; Chung 2014).

In *S. cerevisiae*, the Rrm3 helicase is required for normal rates of fork progression and the prevention of fork stalling (Ivessa et al. 2000; Ivessa et al. 2002; Ivessa et al. 2003; Azvolinsky et al. 2009). Rrm3 interacts with Pol2, the catalytic subunit of Pol ϵ , and the sliding clamp PCNA, suggesting that Rrm3 is a component of the replisome (Azvolinsky et al. 2006). It has also been shown by yeast two hybrid experiments that Rrm3 interacts with the origin recognition complex (ORC), demonstrating that Rrm3 is loaded very early on

in S phase (Azvolinsky et al. 2006; Matsuda et al. 2007). Cells deficient for Rrm3 exhibit replication defects, a hyper-recombination phenotype (Keil and McWilliams 1993) and require various fork processing pathways for survival (Schmidt and Kolodner 2004; Torres et al. 2004). It has also been reported that in *S. cerevisiae*, the Srs2 helicase is required for hairpin unwinding and fork progression (Anand et al. 2012). In *S. pombe*, Pfh1 performs a similar role to its homologue Rrm3, facilitating DNA replication through hard-to-replicate regions and suppressing genome instability (Sabouri et al. 2012; Steinacher et al. 2012).

1.2.4 Roles that homologous recombination play in supporting replication

Besides repairing damaged DNA, HR also plays important roles in facilitating the timely and efficient replication of DNA. These roles are discussed below.

1.2.4.1 Fork protection

During replication, recombination machinery is required to escort replication forks to maintain robust DNA replication. When replication is perturbed, HR is also required to either stabilise the stalled fork for replication resumption, or to maintain the stalled fork in a structure to allow its merging with a converging fork.

In *Xenopus*, it has been shown via electron microscopy that in the absence of RAD51, ssDNA gaps accumulate at moving replication forks. These ssDNA gaps result from the uncoupling between leading and lagging strand synthesis. In the absence of any induced DNA damage, when RAD51 is

absent, half of all replication forks contain at least 200 nucleotides of ssDNA at the moving fork (Hashimoto et al. 2010). This provides evidence that recombination machinery escorts fork progression for efficient DNA replication.

In mammalian cells, BRCA2 and the ubiquitylated FA protein FANCD2, protect stalled forks by stabilising the newly formed RAD51 filaments (Schlacher et al. 2011; Schlacher et al. 2012). In the absence of RAD51 or BRCA2, MRE11 extensively resects nascent DNA strands at interrupted forks. This suggests that recombination proteins have a specific role in protecting the integrity of newly synthesised DNA at stalled replication forks. It has also been shown via 2D gel electrophoresis that in the rDNA region of mouse embryonic fibroblasts, replication forks treated with HU became unstable leading to fork breakage in the absence of BRCA2 (Lomonosov et al. 2003).

In *S. cerevisiae*, replication termination zones tend to overlap with known RFBs, suggesting that fork stalling sites are regions where fork termination is likely to occur (Fachinetti et al. 2010). Also, it has been shown in *S. pombe* that when recombination is impaired, fork termination at a collapsed fork leads to cell death, demonstrating that the merging of an opposing replication fork cannot sufficiently rescue a collapsed fork (Lambert et al. 2005; Lambert et al. 2010). It is possible that during fork termination, recombination proteins are required to stabilise a stalled fork, to hold it in a suitable topology for incoming forks to merge. It is thus also likely that fork termination sites can become hot spots of recombination when termination is interrupted (Steinacher et al. 2012).

1.2.4.2 Replication fork restart

In the event that a replication fork collapses or breaks, recombination can reinstate the replisome to restart replication at a non-replication origin site.

When a replication fork collapses but does not break, HR proteins can promote the regression of the stalled fork, also known as fork reversal, into a chicken foot structure. A chicken foot is a four-way DNA junction that resembles a HJ where the two nascent strands are unwound from their templates and annealed to each other, whilst the parental DNA strands re-anneal (Higgins et al. 1976; Postow et al. 2001; Sogo et al. 2002). This provides a platform for HR proteins to load on to perform strand invasion whereupon replication proteins can reload to restart replication (Liu et al. 1999) (Figure 1.7A). These regressed fork structures are more readily detected in checkpoint deficient *cds1Δ* fission yeast or *rad53Δ* budding yeast cells than in wild-type cells and have thus been deemed pathological (Lopes et al. 2001; Hu et al. 2012). However, there has been direct observation of stalled fork restart via fork regression in the T4 replication system by Manosas and colleagues, corroborating the idea that fork regression is used as a fork restart strategy (Manosas et al. 2012). Besides, in yeast, *Xenopus laevis* egg extracts and mammalian cells, poly (ADP-ribose) polymerase activity promotes fork reversal following exposure to low doses of CPT, providing evidence that fork reversal can be detected under non-pathological conditions (Ray Chaudhuri et al. 2012).

Fork regression and branch migration to form a HJ, as well as the resetting of the regressed fork can be catalysed by DNA helicases and translocases. In

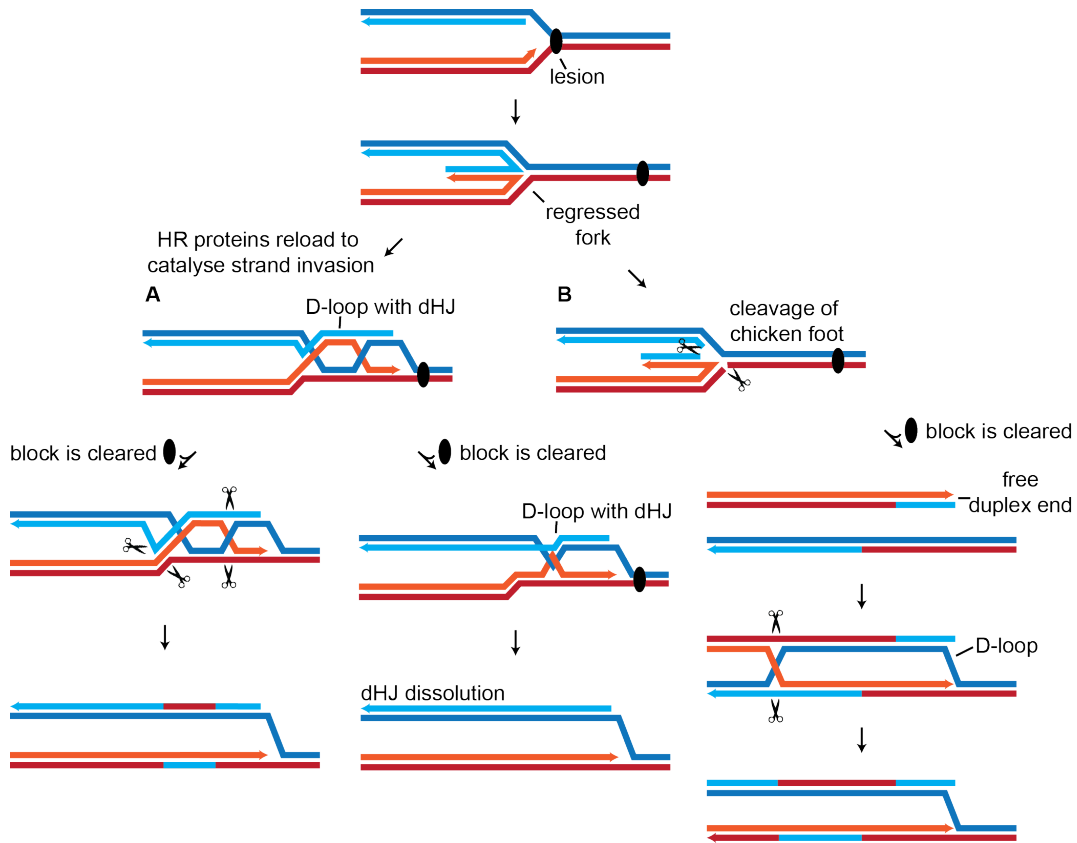


Figure 1.7 Replication fork restart

Fork regression at a fork stalled at a lesion (black oval) might allow its restart in two ways. (A) First, the dsDNA spooled out from the chicken foot may recombine with the homologous sequences in the reannealed parental strands to form a D-loop with a dHJ. Replication machinery can then reload on the forked DNA structure to restart replication. After the block is cleared, the dHJ can be resolved by endonucleolytic cleavage or dHJ dissolution. (B) Second, the chicken foot structure can be cleaved. Cleavage of the chicken foot structure may facilitate unmasking and removal of the block. The free duplex end can recombine with the homologous sequence on the other branch of the cleaved chicken foot structure to form a D-loop. Endonucleolytic cleavage of the HJ formed at the D-loop and reloading of the replication machinery at the fork would reconstitute an active replisome.

eukaryotes, it has been shown *in vitro* that fork regression and branch migration can be driven by DNA helicases such as Fml1, Mph1 and FANCM (Gari et al. 2008b; Sun et al. 2008; Atkinson and McGlynn 2009; Whitby 2010; Zheng et al. 2011). In conjunction with Rad54, Rad51 can also regress replication forks (Bugreev et al. 2011). Although Rqh1 and BLM have also been shown to promote regression of a replication fork *in vitro* (Ralf et al. 2006), it is believed that they primarily reset chicken foot structures to limit recombination caused by cleavage of the chicken foot structure (Doe et al. 2000; Karow et al. 2000). Additionally, WRN can protect replication forks from Mus81-dependent fork breakage, possibly by catalysing fork resetting (Ammazzalorso et al. 2010; Machwe et al. 2011; Murfuni et al. 2013). On the contrary, it is thought that reversed forks are structures that protect stalled forks from cleavage by Mus81 (Neelsen and Lopes 2015). The repair of stalled or regressed replication forks by cleavage runs the risk of having inappropriate recombination between the broken end of the fork and a homologous sequence somewhere else in the genome (Figure 1.7B). The likelihood of that happening is high given the large proportion of repetitive sequences in eukaryotic genomes. It is hence unsurprising that nucleases like Mus81 which have the ability to cleave stalled or regressed replication forks are strictly regulated (Froget et al. 2008).

As mentioned above, DSBs can arise through replication fork run-offs at ssDNA nicks in the template DNA. This results in a broken replication fork with one sister chromatid physically removed from the fork structure. DNA damage like ICLs also arrest forks and are cleaved by endonucleases, leading to a broken fork (Moriel-Carretero and Aguilera 2010; Long et al.

2011). In a similar way to BIR, HR is able to rebuild a replisome at broken forks to allow the resumption of DNA synthesis, a process termed recombination-dependent replication (RDR) (Figure 1.8). In yeast, this process requires HR proteins like Rad51, Rad52, Rad55 and Rad57 (Hashimoto et al. 2010). Components of the replisome like the leading and lagging strand apparatus, DNA polymerases α , δ and ϵ , and the CMG replicative helicase are also required for this process (Lydeard et al. 2010). Proteins associated with the pre-replication complex assembly at replication origins are however not required for BIR (Lydeard et al. 2010). It has been shown in *S. cerevisiae* that BIR strand exchange can occur in a Rad52 dependent manner, independent of Rad51 (Bosco and Haber 1998; Davis and Symington 2004). Unlike replication forks assembled from replication origins, BIR replication forks require Pol32, a subunit of Pol δ (Gerik et al. 1998; Lydeard et al. 2010), and its long-range synthesis is dependent on Pif1 (Wilson et al. 2013). Additionally, it seems that DNA synthesis via BIR is more error-prone and less processive compared to conventional DNA replication (Deem et al. 2011; Anand et al. 2014; Sakofsky et al. 2014). It is likely that BIR evolved to be less processive so that repair is predisposed to engaging the second end of a DSB, to prevent the replication of an entire chromosome arm which could lead to LOH (Smith et al. 2007). Interestingly, it is unclear how closely BIR studies model broken forks *in vivo*. BIR studies measure strand invasion of a broken DNA fragment into an intact duplex DNA molecule, whereas replication fork breakage *in vivo* most likely gives rise to a broken end and a gapped sister chromatid.

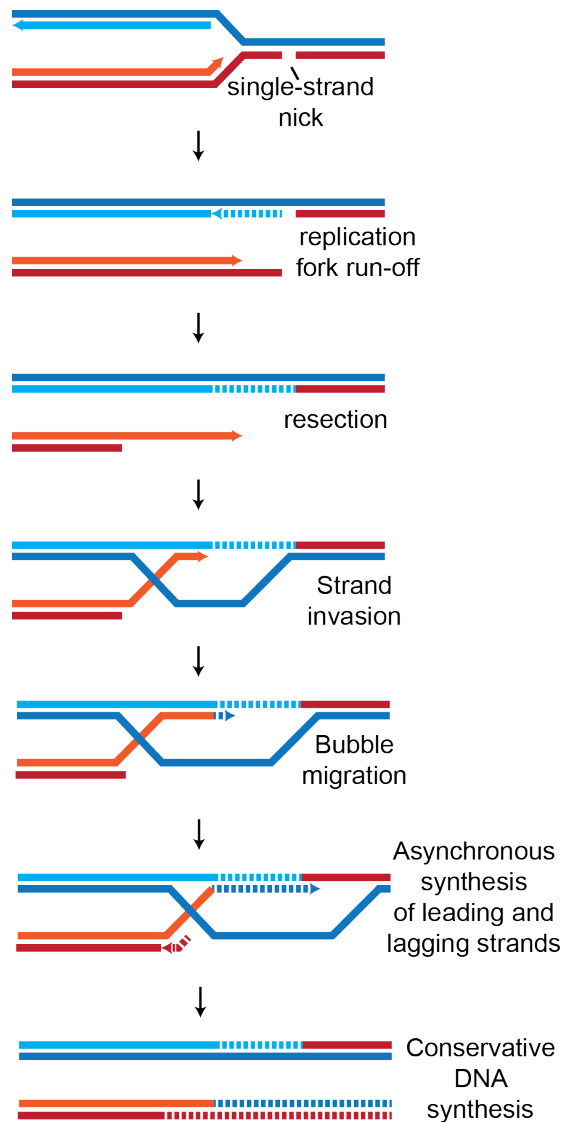


Figure 1.8 Recombination-dependent replication

A nick in the leading strand template can lead to replication fork run-off, which results in a broken replication fork. Following the breakage of the replication fork, the one-sided DSB end is resected. HR proteins load onto the 3' end of the ssDNA and invades a homologous DNA sequence. Replisome components reload at the D-loop to restore the replication fork. This leads to a migrating bubble that persists for potentially hundreds of kilobases down the length of the chromosome leading to conservative DNA synthesis

1.2.4.3 Template switch mediated lesion bypass

One way a replication fork could respond to a lesion on the leading strand template is via template switching, followed by the subsequent re-priming of DNA synthesis downstream of the lesion. This process involves the transient uncoupling of leading and lagging strand polymerases so that the lagging strand polymerase can proceed beyond the position in which the leading strand polymerase is blocked (Higuchi et al. 2003). This way, the extended lagging strand can then serve as a template for the prematurely terminated leading strand via a fork regression process. The lesion on the leading strand template can therefore be bypassed by employing the nascent lagging strand as a template. Theoretically, no mutations are introduced during template switching as the lagging strand contains the same information as the “original” template strand of the leading strand. The chicken foot structure can then be resolved either by the re-setting of the regressed fork or by HR in which the 3' end of the newly synthesised leading strand invades the parental template at a point downstream of the lesion (McGlynn and Lloyd 2002a) (Figure 1.9).

The second way a lesion can be bypassed via template switching occurs without fork regression. This involves the strand invasion of ssDNA gaps, instead of DNA ends, to initiate template switching. In this process, parental strands re-anneal, exposing the non-damaged newly synthesized chromatid, which acts as a template to the impeded nascent strand. Non-canonical dHJs, which form at a late stage of template switching before being processed to hemicatenanes, have been visualised in budding yeast during gap filling by template switching (Giannattasio et al. 2014).

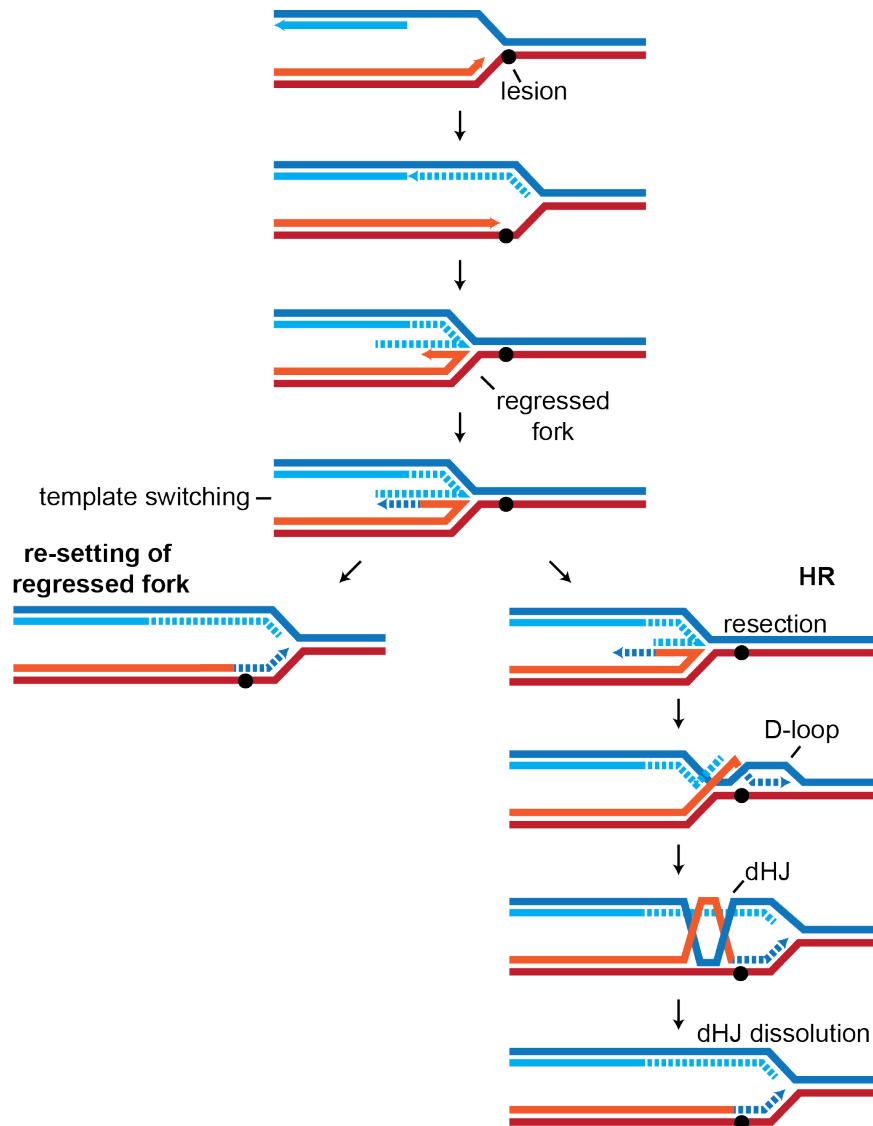


Figure 1.9 Template switch mediated lesion bypass

A replication fork is arrested due to a lesion on the leading strand template. Leading and lagging strand synthesis uncouples. The replication fork could regress forming a HJ. Template switching occurs where the nascent lagging strand serves as a template for the leading strand. Chicken foot structure is resolved either by re-setting of the regressed fork or via HR in which the 3' end of the newly synthesised leading strand invades the parental template at a point downstream of the lesion.

1.2.4.4 Post-replicative repair

In *E. coli*, yeast and mammalian cells, ssDNA gaps can form when a stalled polymerase skips a block to continue replication (Rupp and Howard-Flanders 1968; Lehmann 1972; Meneghini 1976; Heller and Marians 2006a; Heller and Marians 2006b; Elvers et al. 2011). This is only relevant to barriers that affect one template strand and not barriers that block both strands of the DNA template. When either the leading or the lagging polymerase is impeded, the unimpeded polymerase can skip the barrier on its template strand and reinitiate synthesis downstream of the barrier. When the lesion is on the lagging strand template, the lesion would only block the extension of one Okazaki fragment, to which a replisome can respond by the simple re-priming of synthesis downstream of the lesion (Langston and O'Donnell 2006). This leaves a ssDNA gap behind the replication fork which can be filled via post-replicative translesion synthesis (TLS) (involving the use of specialised translesion polymerases that can facilitate the insertion of bases opposite damaged nucleotides) or HR (Lopes et al. 2006). In post-replicative HR, the gap is filled by a template switch mechanism using the replicated sister chromatid as the template. This process involves the invasion of the sister chromatid by the 3' end of the gapped strand to form a D-loop (Figure 1.10). The 3' end is extended by the DNA replicative polymerase δ and its accessory subunit Pol32 to fill the gap. The D-loop can then be displaced, resolved, or dissolved by the concerted action of Sgs1 and Top3 (Mankouri et al. 2011) (Figure 1.10). It is however still unclear whether such gap repair is closely

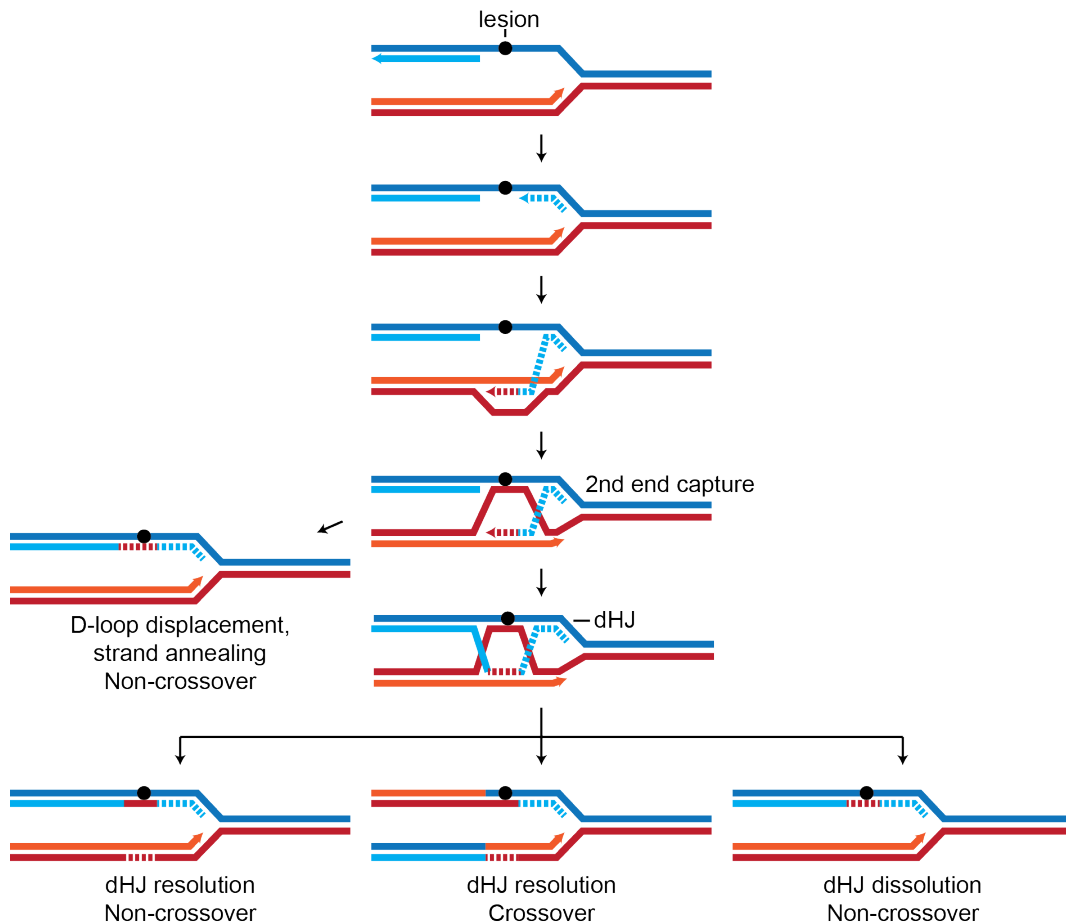


Figure 1.10 Post-replicative repair

Lesions that are skipped during DNA replication, particularly in the lagging strand, can lead to ssDNA gaps in the chromosome. These gaps can be filled in through a post-replicative repair process that is dependent on recombination factors. The 3' end of the gapped strand can invade the nascent leading strand, which is used as a template for filling in the gap. The recombination intermediate is then resolved in a variety of ways resulting in different outcomes.

coupled to DNA replication, or occurs some time after the passage of the replication fork.

Various data indicate that post-replication gap repair is a prominent mechanism for dealing with template blocking lesions. For example, DNA fibre analysis in MMS treated budding yeast has shown that ssDNA gaps accumulate when Rad51 has been disrupted (Alabert et al. 2009), and similarly electron microscopic analysis of damaged replication forks in budding yeast and *Xenopus* have shown that ssDNA gaps accumulate at and behind the replication fork on both the leading and lagging strand, especially when post-replicative repair pathways have been ablated (Byun et al. 2005; Lopes et al. 2006). Additional evidence of post-replicative HR includes the detection of joint-molecules between sister-chromatids by 2D gel electrophoresis in S-phase cells upon MMS treatment (Liberi et al. 2005) and following UV-C irradiation, which in the latter case have been shown to depend on RPA, Rad52, Rad55, Rad57, Exo1 and Pol δ but not Pol ϵ (Costes and Lambert 2012; Giannattasio et al. 2014).

1.2.5 Replication fork termination

As alluded to earlier (see section 1.2.1), DNA replication initiates bi-directionally from origins that fire during S phase. The completion of replication involves the fusion of these replication forks with forks approaching from adjacent origins in a process known as replication termination or replication fork merging. In eukaryotes, fork merging seems to happen stochastically within 4 kb zones (Zhu et al. 1992). There is a strong association between replication termination sites and RFB sites (Greenfeder

and Newlon 1992b; Fachinetti et al. 2010). For example, the *RTS1* barrier at the fission yeast mating-type locus and the Fob1 barrier at the budding yeast rDNA locus (Brewer and Fangman 1988; Dalgaard and Klar 2001). Additionally, accidental RFBs that exist in replication slow zones, centromeres, tRNA genes, and sites where there is frequent collision of transcription and replication machinery, induce fork stalling and thus fork termination although to a lesser extent than programmed RFBs (Greenfeder and Newlon 1992a; Deshpande and Newlon 1996; Cha and Kleckner 2002; Lemoine et al. 2005; Admire et al. 2006; Azvolinsky et al. 2006). These programmed and accidental RFBs at termination sites can trigger HR that can lead to genomic rearrangements and chromosome breakage (Ahn et al. 2005; Lambert et al. 2005; Kobayashi 2006; Lambert et al. 2010). As such, a group of specialised helicases, the Pif1 family DNA helicases have evolved a sweepase function to dismantle protein-DNA barriers ahead of replication forks (Ivessa et al. 2002; Ivessa et al. 2003). Further, cells deploy topoisomerases to resolve torsional stress in the DNA that builds up as forks terminate (Wang 2002).

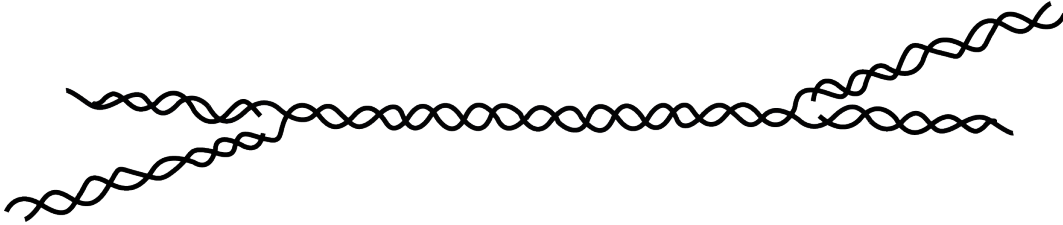
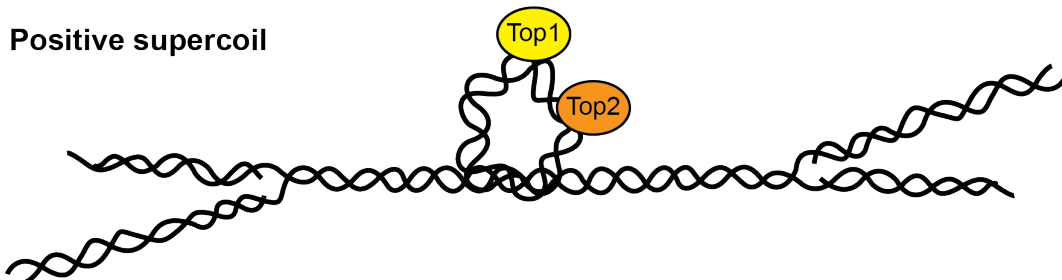
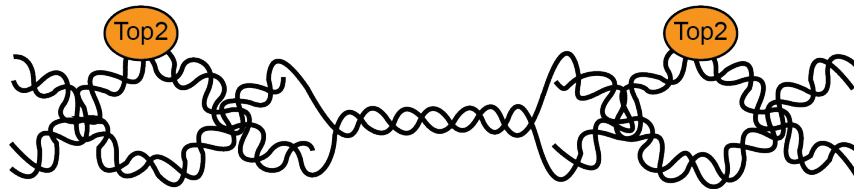
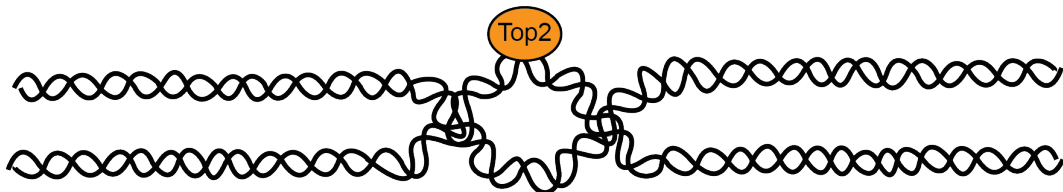
1.2.5.1 Pif1 helicases

As mentioned in section 1.2.3, budding yeast has two Pif1 helicase family proteins, Rrm3 and Pif1. Although Rrm3 and Pif1 affect replication across many of the same substrates their effects on these substrates are dissimilar and occasionally opposing. Pif1 facilitates fork stalling at RFBs whereas Rrm3 facilitates replication past RFBs (Ivessa et al. 2000). Rrm3 is a part of the replisome and promotes fork progression through non-histone protein-DNA

complexes (Ivessa et al. 2002; Ivessa et al. 2003). In budding yeast, Rrm3 facilitates fork progression across termination regions, and X-shaped molecules (detected by 2D gel electrophoresis) accumulate at termination sites when Rrm3 is absent (Fachinetti et al. 2010). Similarly in fission yeast, X-shaped molecules accumulate at termination sites in the absence of Pfh1 and these have been shown to be converging replication forks rather than recombination intermediates (Steinacher et al. 2012). Such data have lead to the proposal that Pfh1/Rrm3 are needed to ensure the successful completion of DNA replication, especially when other proteins impede the path of the replisome.

1.2.5.2 Topoisomerases

DNA develops positive supercoils that get converted into precatenanes as forks merge (Fields-Berry and DePamphilis 1989). *In vitro* and *in vivo* experiments have demonstrated the involvement of both type IB (Top1) and type II (Top2) topoisomerases in replication fork merging (DiNardo et al. 1984; Wang 2002; Baxter and Diffley 2008; Cuvier et al. 2008). In *S. cerevisiae*, together with Rrm3, Top1 and Top2 travel with the replication fork to relieve torsional stress generated (Azvolinsky et al. 2006; Bermejo et al. 2007). DNA generates positive supercoiling ahead of the fork which is removed by Top1 (mainly) and Top2 (Wang 2002), while precatenanes formed behind the fork are disentangled by Top2 (Lucas et al. 2001; Wang 2002). As forks converge and fuse, catenated sister chromatids are resolved by Top2 (Fachinetti et al. 2010) (Figure 1.11). Although type IA (Top3) topoisomerase has been implicated in the resolution of sister chromatid junctions at termination sites, it

Converging replication forks**Positive supercoil****Precatenane****Catenated sister chromatids****Figure 1.11 Topological transitions during replication fork merging**

As replication forks converge, positive supercoiling is generated ahead of the forks which is mainly removed by Top1 (mainly) and Top2. Precatenanes formed behind the fork are disentangled by Top2. As forks converge, catenated sister chromatids are resolved by Top2.

has been shown that Top3 is not required for fork fusion or resolution of torsional strains (Fachinetti et al. 2010). When fork fusion occurs, the leading and lagging strand polymerases from the opposing forks meet, physically occupying the remaining unreplicated DNA. It has been proposed that polar RFBs guarantee that at least one of the two forks resumes replication with asymmetrical leading and lagging strands to ensure that the two pairs of nascent DNA strands do not fuse concurrently (Fachinetti et al. 2010). This is supported by the observation that at termination sites, fork stalling is asymmetric (Gruber et al. 2000). Similar to the assembly of proteins at replication origins, the disassembly of proteins during fork termination is controlled in a universal complex mechanism in eukaryotic cells. During fork termination, ubiquitylation of the Mcm7 subunit of the CMG helicase causes Cdc48 segregase to associate with CMG to disassemble the helicase (Maric et al. 2014; Moreno et al. 2014). In budding yeast, the ubiquitylation of Mcm7 depends on the F-box protein Dia2 (Maric et al. 2014).

1.2.5.3 Consequences of failing to complete replication

At common fragile sites where the distance between origins is greater than in the rest of the genome, in the event that one or both forks fail, there is a lesser tendency for late firing of origins or cryptic origin firing to complete replication in a timely manner. Additionally, RFBs at termination regions can prevent stretches of DNA from being replicated. In the event that stretches of unreplicated DNA persist into mitosis or termination is disrupted, it will lead to the formation of ultrafine anaphase bridges in mammalian cells and ultimately

to DNA breakage (Chan et al. 2007; Chan et al. 2009; Jacome and Fernandez-Capetillo 2011).

The final stages of replication fork termination, requires the timely and orderly orchestration of various catalytic and enzymatic activities. These include the fusion of asymmetrical leading and lagging strands, the disassembly of replisomes (Maric et al. 2014; Moreno et al. 2014) and the decatenation of sister chromatids (DiNardo et al. 1984). Given the complexity of this process, it is conceivable that the final stages of fork merging is prone to problems that could lead to the formation anaphase bridges, and potentially DNA breakage and recombination. Such problems have been observed under pathological conditions. For example, the impedance of DNA replication with LacI bound to a *lacO* array to form a persistent non-programmed bi-directional protein-DNA barrier in fission yeast and mammalian cells (Jacome and Fernandez-Capetillo 2011; Sofueva et al. 2011), and the disruption of fork termination when the replication helicase Pfh1 in fission yeast is absent (Steinacher et al. 2012).

Many aspects of fork termination remain unclear. It is unknown whether the normal process of fork merging is prone to problems or whether the coordination and execution of termination events are so robust that they rarely give rise to problems. This is something that I address later in Chapter 3 of this dissertation.

1.3 FANCM family of DNA helicases/translocases

The FANCM family of DNA helicases are DNA junction-specific helicases/translocases that play key roles in the processing of perturbed replication forks and recombination intermediates. They have been implicated in the recruitment of the FA core complex to DNA damage sites, the bypass of DNA lesions by template switching, and crossover avoidance during DSB repair by recombination (Whitby 2010). Additionally, they have been shown to catalyse replication fork reversal, D-loop dissociation, and DNA branch point migration *in vitro* (Whitby 2010).

1.3.1 The FANCM family members

The FANCM family of proteins are superfamily 2 (SF2) DEAH-box helicases with members in archaea and eukaryotes. Mph1 from *S. cerevisiae* was the first member of this family to be characterised (Scheller et al. 2000). The gene was named *mph1* (mutator phenotype), since cells deleted of *mph1* had an increased spontaneous mutation rate (Schurer et al. 2004; Banerjee et al. 2008). The second member to be identified was Hef (helicase-associated endonuclease for fork structured DNA) from *Pyrococcus furiosus* (Komori et al. 2002). Besides containing an N-terminal Mph1-like helicase domain (DEAH box helicase domain), Hef also has an ERCC4-like endonuclease domain and a tandem helix-hairpin-helix (HhH)₂ domain in its C-terminal region, which closely resembles that found in members of the XPF/MUS81 nuclease family (Komori et al. 2002; Nishino et al. 2005; Lestini et al. 2010). Interestingly, Hef is the only member of the FANCM family with a nuclease activity and has the ability to cleave an array of branched and nicked DNA

substrates, including replication fork-like structures. Hef's (HhH)₂ domain enables it to homo-dimerise and thereby bind to two duplex arms within a branched DNA substrate concurrently.

The human orthologue FANCM was isolated as the Fanconi anaemia A-associated polypeptide with a mass of 250 kDa (FAAP250) using an antibody to the FANCA protein (Meetei et al. 2005). FAAP250 was subsequently identified in a FA patient carrying bi-allelic mutations in this particular gene and thereafter the protein was renamed as FANCM (Meetei et al. 2005). FANCM was also identified in chicken DT40 cells as the Hef orthologue, which also functions as part of the FA core complex in chickens (Mosedale et al. 2005). Like Hef, FANCM contains a SF2 DEAH-box helicase domain, a ERCC4-like nuclease domain and a (HhH)₂ domain, however, the ERCC4-like nuclease domain in both human and chicken FANCM is non-functional (Meetei et al. 2005) (Figure 1.12).

1.3.2 The involvement of FANCM in the Fanconi Anaemia repair pathway

FA is a rare X-linked or autosomal recessive disease characterised by chromosomal fragility, heightened cancer predisposition, congenital abnormalities and bone marrow failure (D'Andrea 2003; D'Andrea and Grompe 2003; Kennedy and D'Andrea 2005; Mace et al. 2005; Niedernhofer et al. 2005; Gurtan and D'Andrea 2006; Mirchandani and D'Andrea 2006). A hallmark of cells from FA patients is hypersensitivity to ICL agents like cisplatin, mitomycin C (MMC) or diepoxybutane (DEB) (Sasaki 1975; German et al. 1987; Auerbach 1988). The primary role of the FA pathway is in

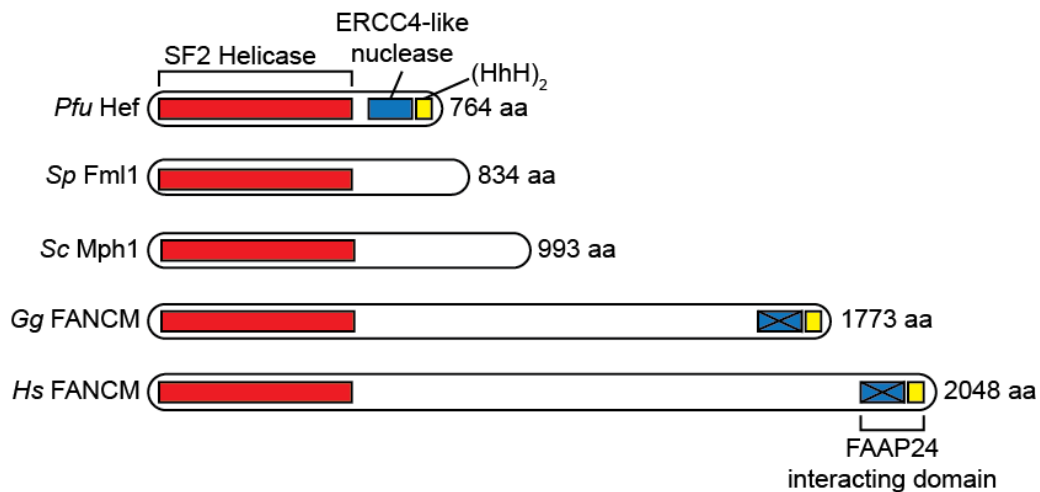


Figure 1.12 FANCM family of DNA helicases/translocases

Schematic showing conservation of protein domain organisation in different FANCM family members. The SF2 helicase, ERCC4-like nuclease and Helix hairpin Helix (HhH)₂ domains are indicated. Abbreviations: *Pfu*, *Pyrococcus furiosus*; *Sp* *Schizosaccharomyces pombe*; *Gg* *Gallus gallus*; *Hs*, *Homo sapiens*; *Sc*, *Saccharomyces cerevisiae*. The ERCC4-like nuclease domains in *Hs* FANCM and *Gg* FANCM are catalytically inactive and this has been indicated by a cross in the schematic. *Hs* FANCM interacts with FAAP24 via its ERCC4-like nuclease and (HhH)₂ domains.

coordinating various repair activities that involve the three classic repair pathways, NER, HR and TLS, in the removal of ICLs (Niedzwiedz et al. 2004).

The FA network comprises at least 16 genes (*FANCA*, *FANCB*, *FANCC*, *FANCD1*, *FANCD2*, *FANCE*, *FANCF*, *FANCG*, *FANCI*, *FANCJ*, *FANCL*, *FANCM*, *FANCN*, *FANCO*, *FANCP* and *FANCQ*) and all FA patients that have been tested contain a mutation in one or more of these genes (Wang 2007; Moldovan and D'Andrea 2009; Soulier 2011; Osorio et al. 2013). In addition, five FA associated proteins (FAAP100, FAAP24, Mhf1, Mhf2 and FAN1), which have not been linked to mutations in FA patients have been identified (Ciccio et al. 2007; Ling et al. 2007; Kee and D'Andrea 2010; MacKay et al. 2010; Singh et al. 2010; Smogorzewska et al. 2010; Yan et al. 2010). The FANC network of 16 genes and 5 FA associated genes can be divided into three groups. The first group constitutes the FA core complex, comprising *FANCA*, *FANCB*, *FANCC*, *FANCE*, *FANCF*, *FANCG*, *FANCL*, *FANCM*, FAAP100, FAAP24, Mhf1 and Mhf2. FANCM recruits the FA core complex to stalled replication forks, to facilitate the monoubiquitination of a second group of proteins, FANCD2 and FANCI by the E3 ubiquitin ligase, FANCL (Kennedy and D'Andrea 2005; Dorsman et al. 2007; Sims et al. 2007; Smogorzewska et al. 2007; Wang 2007; Moldovan and D'Andrea 2009). Together, the FANCI-FANCD2 heterodimer is known as the ID complex. Monoubiquitination of the ID complex results in its recruitment to sites of DNA damage to form DNA repair foci (Smogorzewska et al. 2007; Alpi et al. 2008). In these damage foci, the ID complex interacts with a third group of proteins FANCD1 (BRCA2), FANCJ (BRIP1), FANCN (PALB2) and FANCO (RAD51C) (Hussain et al. 2004; Wang et al. 2004; Xia et al. 2006; Zhang et al. 2009). DNA repair

proteins like RAD51 and PCNA have been detected at the damage foci (Geng et al. 2010; Long et al. 2011). Besides, it has been shown that monoubiquitinated FANCD2 physically interacts with BRCA2, BRCA1 and RAD51C at DNA repair sites (Taniguchi et al. 2002; Hussain et al. 2004; Wang et al. 2004). It has been reported that when monoubiquitination of the ID complex is disrupted, DNA crosslink repair and HR are compromised (Nakanishi et al. 2005; Smogorzewska et al. 2007).

FAAP24 is a 24 kDa, 253 amino acid protein that shares 20 % homology with the C-terminal ERCC4-like and (HhH)₂ domains of FANCM. Similar to FANCM, the nuclease domain of FAAP24 is not functional. Heterodimerisation of FANCM and FAAP24 is mediated by the C-terminal region of FANCM. *In vitro*, the C-terminal fragment of FANCM (FANCM¹⁷²⁷⁻²⁰⁴⁸) in complex with FAAP24 binds ssDNA, dsDNA, fork-shaped DNA and has a preference for splayed arm DNA. This ability to bind intermediates of DNA replication and repair suggests that a complex of FAAP24 and FANCM may serve as a sensor of DNA damage to recruit the FA core complex to stalled replication forks. In HeLa cells, FAAP24 knockdown leads to sensitivity to ICL agents and disrupts FANCD2 monoubiquitination (Ciccia et al. 2007). Furthermore, the FANCM-FAAP24 complex is required for UV and CPT resistance, and for crossover recombination suppression (Rosado et al. 2009; Singh et al. 2009).

It appears that the FA pathway evolved at a late stage in evolution since most of its protein components (exceptions being FANCM, FANCI, MHR23 and FANCD1) are present only in vertebrates. The conservation of FANCM

homologues from archaea to humans, suggests that the FANCM protein has functions in addition to the FA pathway. Indeed, work from our laboratory has confirmed a role for the *S. pombe* Fml1 protein in HR in overcoming ICLs, limiting crossing over during mitotic and meiotic DSB repair, fork reversal and D-loop disruption (Sun et al. 2008; Lorenz et al. 2012).

1.3.3 Overcoming crosslinks

ICLs are the most deleterious of DNA lesions as they consist of covalent linkages between two DNA template strands, preventing DNA unwinding and posing formidable blocks to DNA replication and transcription (Moldovan and D'Andrea 2009). If not repaired or bypassed appropriately, they have the potential to cause cell death (Akkari et al. 2000; Scharer 2005). Surprisingly, DNA combing has shown that replisomes can travel across ICLs with the aid of FANCM's translocase activity and its Mhf1 and Mhf2 partner proteins, leaving the ICL behind for post-replicative repair (Huang et al. 2013). Also, it has been demonstrated in *Xenopus* egg extracts that the repair of an ICL can occur when two replication forks converge on it (Raschle et al. 2008). Because ICLs affect both strands of the DNA, they cannot be simply repaired through excision but instead require the coordination and concerted action of several repair pathways including NER, TLS and HR (Grossmann et al. 2001; McHugh and Sarkar 2006; Mirchandani and D'Andrea 2006; Sarkar et al. 2006; Lehoczky et al. 2007).

During ICL repair, it is conceivable that FANCM-FAAP24 and the ID complex are involved in recognition of the stalled fork since they bind to branched substrates *in vitro*. This is followed by the activation of the ATR-CHK1

kinases, which phosphorylate the FA core and ID complex in turn to activate them. As mentioned above, the activated FA core complex then monoubiquitinates the ID complex to initiate repair of the ICL (Figure 1.13A). It remains unclear how ICLs are repaired however, it is plausible that FANCM reverses the stalled fork for cleavage of the leading strand template via MUS81-EME1 and XPF-ERCC1 to unhook the ICL lesion (Yuan et al. 2010; Zhang and Walter 2014) (Figure 1.13B). BRCA1-FANCD1 (BRIP1) and other helicases may facilitate DNA unwinding around the ICL for XPF-ERCC1 cleavage (Patel and Joenje 2007; Thompson and Hinz 2009). The unhooked ICL is then bypassed by TLS while the FA complex holds the unhooked DNA strand together (Figure 1.13C). RAD51 recombinases are recruited to the 3' ssDNA overhang by FANCD1 (BRCA2) and FANCD2 (PALB2) to generate the nucleoprotein filament for strand invasion and re-establishment of the replication fork. BRCA1-FANCD1 encourages strand invasion by unwinding dsDNA (Figure 1.13D). Finally the HJ is resolved and DNA replication restarts (Figure 1.13H).

On the other hand, ICL incision can also occur on the lagging strand as seen in *Xenopus* (Raschle et al. 2008; Knipscheer et al. 2009). The first incision of the lagging strand is likely to be carried out by SLX1-SLX4, which has a preference for 5' flaps (Zhang and Walter 2014) (Figure 1.13E). The nascent leading strand is then extended via TLS to bypass the ICL (Raschle et al. 2008). XPF-ERCC1 could make the second incision to unhook the ICL (Zhang and Walter 2014) (Figure 1.13F). Finally the replication fork can be re-established by strand invasion of the 3' overhang of the broken arm (Figure 1.13G). It is likely that NER is subsequently used to remove the unhooked ICL

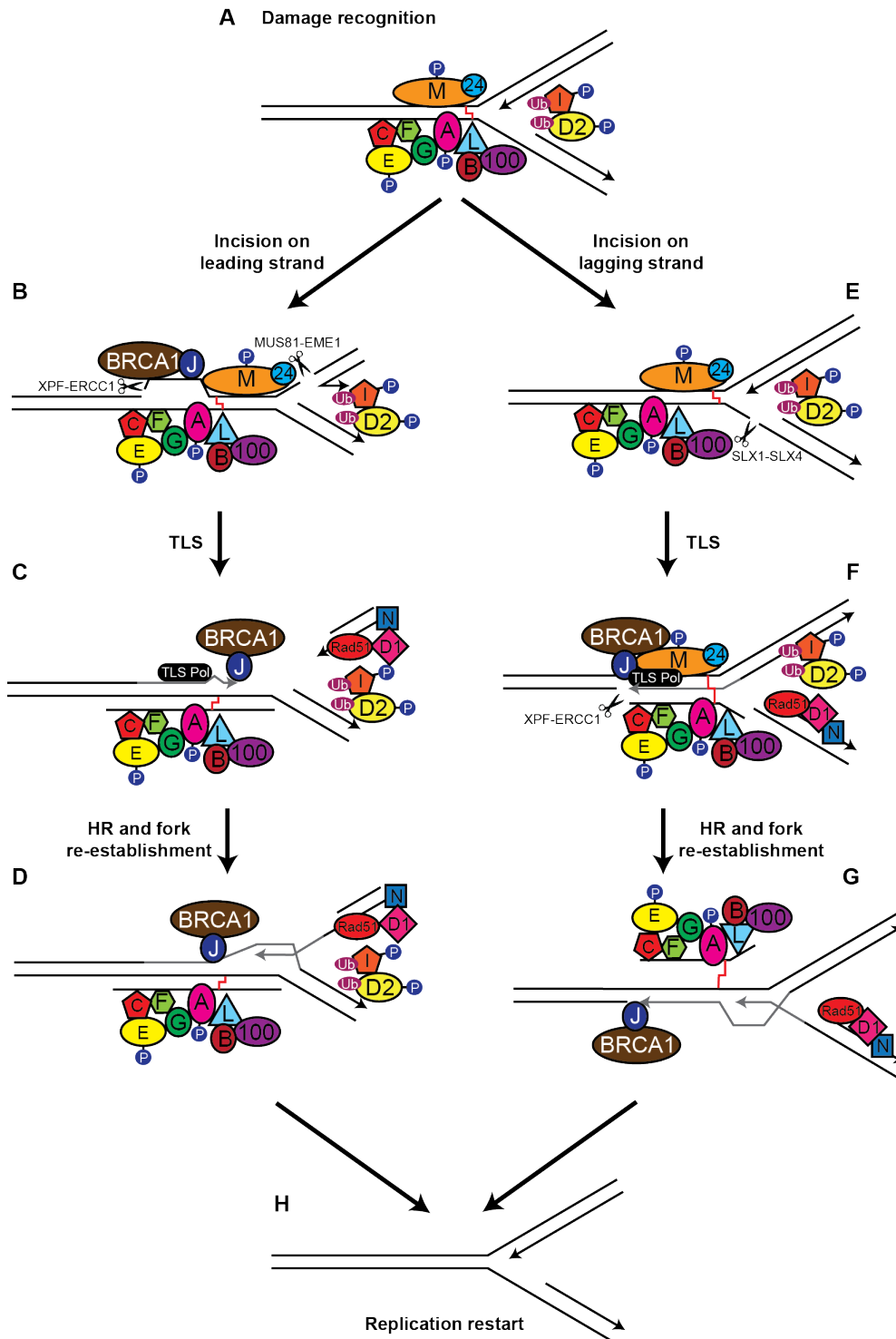


Figure 1.13 The Fanconi anaemia pathway in ICL repair

Cartoon depicting ICL repair involving the FA pathway, NER, TLS and HR. Letter 'P' in dark blue circles: Phosphorylation; Letters 'Ub' in dark red ovals: monoubiquitination; red zigzag line: ICL; scissors: endonucleases; all other letters in various shapes: Different FA complementation groups; newly synthesised DNA strands are indicated in grey. Refer to text for more details. Adapted from (Yuan et al. 2010).

after DNA replication. The HJ is resolved and DNA replication restarts (Figure 1.13H).

1.3.4 Replication fork reversal

As mentioned above (see section 1.2.4.2), Fml1, Mph1 and FANCM can regress and branch migrate replication forks in an ATPase-dependent manner *in vitro* (Gari et al. 2008b; Sun et al. 2008; Whitby 2010; Zheng et al. 2011; Nandi and Whitby 2012). As alluded to earlier, regressed fork structures provide a platform for HR proteins to load on to initiate recombination dependent replication restart (McGlynn and Lloyd 2002b). It has been shown in fission yeast that when *fml1* is deleted or has its ATPase domain mutated, HR is reduced during fork blockage at an *RTS1* barrier (Sun et al. 2008; Nandi and Whitby 2012). Furthermore, it has been reported that in mammalian cells, FANCM is required to stabilise and stimulate restart of stalled forks (Moldovan and D'Andrea 2009).

1.3.5 D-loop dissociation and crossover avoidance

As alluded to earlier (see section 1.1.2.3), the resolution of recombination intermediates can generate two types of recombinants, crossovers and non-crossovers (Paques and Haber 1999). Mitotic crossover can lead to LOH or gross chromosomal rearrangements, both of which are hallmarks of cancer. To prevent this, cells have evolved mechanisms to bias resolution of recombination intermediates towards non-crossovers (Lorenz and Whitby 2006). The FANCM family of proteins amongst other helicases have been

implicated in limiting crossovers by dissociating D-loops to promote the SDSA sub-pathway.

The first evidence for this was the observation that FANCM deficient murine and chicken DT40 cells displayed increased spontaneous sister chromatid exchange (Bakker et al. 2009; Rosado et al. 2009). This suppression of spontaneous sister chromatid exchange in chicken DT40 is dependent on FANCM's helicase activity (Rosado et al. 2009). Deletion of *fml1* in *S. pombe* results in a four-fold increase in crossover frequency during the repair of a plasmid gap (Sun et al. 2008). Also, in *S. cerevisiae*, the effect of *mph1* deletion on the frequency of crossing over during the repair of a HO endonuclease-induced site-specific DSB by recombination results in a two-fold increase in the frequency of crossovers during recombination between allelic sites and a three-fold increase in the frequency of crossovers when recombination is between ectopic sites (Prakash et al. 2009). Helicase dead *mph1* mutants are not able to suppress the elevated level of crossovers in a *mph1* deletion mutant, suggesting that the helicase activity is required in dissociating D-loops (Prakash et al. 2009).

Furthermore, *in vitro* Fml1, Mph1 and FANCM have been observed to dissociate an 80/90-mer oligonucleotide that has annealed to supercoiled circular DNA by RecA or Rad51 (Gari et al. 2008a; Sun et al. 2008; Prakash et al. 2009). *In vitro* Fml1 and Mph1 have been observed to unwind the strand that has invaded a synthetic D-loop assembled from complementary oligonucleotides (Prakash et al. 2009). Specifically, Mph1 has been shown to dissociate D-loops in the presence of other recombination proteins like Rad51

(Prakash et al. 2009). As alluded to earlier, the ability of the FANCM family members to dissociate D-loops is dependent on their ability to hydrolyse ATP. Neither the helicase dead Mph1^{D209N}, nor FANCM^{K117R} is able to catalyse this reaction (Gari et al. 2008a; Prakash et al. 2009). Interestingly, the ATPase dead *fml1*^{K99R} and *fml1*^{D196N} mutants exhibit higher levels of crossovers during DSB repair than the *fml1*Δ null mutant, indicating that in the absence of Fml1's motor activity, its binding to the DNA can hinder alternative crossover avoidance mechanisms (Nandi and Whitby 2012). Besides mitosis, it has been established that in plants and fission yeast that FANCM and Fml1 also drive the non-crossover pathway in meiosis (Crismani et al. 2012; Lorenz et al. 2012).

1.4 FANCM-associated histone-fold proteins 1 and 2

Weidong Wang and his colleagues identified two novel histone-fold proteins, Mhf1 (FANCM-associated Histone-Fold Proteins 1) and Mhf2 that co-purify with FANCM (Yan et al. 2010). Because Mhf1 and Mhf2 were independently identified as centromeric proteins, they were also given the names CENP-S (centromere-associated protein S) and CENP-X respectively (Foltz et al. 2006; Amano et al. 2009). However, for simplicity I will refer to them as Mhf1 and Mhf2, or collectively as MHF.

1.4.1 Mhf1 and Mhf2 act cooperatively with FANCM in the Fanconi Anaemia DNA repair pathway

Unlike many FA pathway proteins, MHF is conserved from yeast to human (Singh et al. 2010; Yan et al. 2010; Nishino et al. 2012; Tao et al. 2012; Yang

et al. 2012; Wang et al. 2013; Sugahara et al. 2014). Both MHF and FANCM are constitutively located almost exclusively in chromatin, both in the absence and presence of DNA damage (Singh et al. 2010). Upon the induction of ICLs in HeLa cells, MHF is rapidly recruited to damage sites in S phase cells, and the recruitment of MHF is required for FANCM recruitment (Yan et al. 2010). MHF and FANCM act in the same pathway for the promotion of FANCD2 monoubiquitination and for the suppression of spontaneous sister chromatid exchange (Singh et al. 2010; Yan et al. 2010).

The MHF heterodimer binds dsDNA and contributes to FANCM's chromatin targeting. It has been shown that the MHF complex stimulates FANCM's replication fork reversal and HJ branch migration activities *in vitro* (Singh et al. 2010; Yan et al. 2010). It has also been shown that in fission yeast, the MHF complex works together with Fml1 to stimulate HR at blocked replication forks and promote crossover avoidance during mitotic and meiotic repair of DSBs (Yan et al. 2010; Lorenz et al. 2012; Bhattacharjee et al. 2013).

In MHF knockdown HeLa cells, besides exhibiting hypersensitivity to ICLs, cells are also hypersensitive to MMS and CPT (Singh et al. 2010; Yan et al. 2010), phenotypes unrelated to the depletion of other FA core components. Additionally, although integral to the FA pathway, 75 % of MHF heterodimers exist in association with FANCM, independent of the FA core complex (Yan et al. 2010). These observations, together with the fact that orthologues of FANCM and MHF are present in yeast (unlike the other FA core components), indicate roles independent of the FA pathway, which are likely to be conserved in all eukaryotes.

1.4.2 The different complexes that Mhf1 and Mhf2 form at the centromere

Structural studies have revealed that the MHF complex exists and functions as a heterotetramer made up of two Mhf1-Mhf2 heterodimers (Nishino et al. 2012; Tao et al. 2012; Yang et al. 2012; Fox et al. 2014; Zhao et al. 2014) (Figure 1.14A). It has a similar architecture to the heterotetramers of histones H3 (or its variants) and H4. Additionally, the secondary structure orientations and overall structure of Mhf1 and Mhf2 themselves resemble canonical histones H3 and H4, respectively (Yang et al. 2012). The Mhf1-Mhf2 heterodimer and heterotetramer are held together by conserved residues, which form salt bridges, hydrogen bonds, electrostatic interactions and numerous hydrophobic contacts (Yang et al. 2012). In *S. cerevisiae*, a series of MHF mutants with an altered oligomeric state were hypersensitive to MMS, demonstrating that the integrity of the MHF heterotetrameric architecture is required for its biological function in DNA repair (Yang et al. 2012). In human, it was also demonstrated that the MHF heterotetrameric state was essential for the proper targeting and/or stable localization of the MHF tetramer at the centromere (Tao et al. 2012). Moreover, when Mhf1 was absent in chicken, or Mhf1's DNA binding ability was disrupted, there was reduced localisation of the outer kinetochore protein complex Ndc80, implying that the DNA binding activity of Mhf1 is critical for the assembly of outer kinetochore proteins (Nishino et al. 2012).

The positively charged patches on the convex side of MHF are important for DNA binding. DNA is condensed by its binding to both sides of MHF

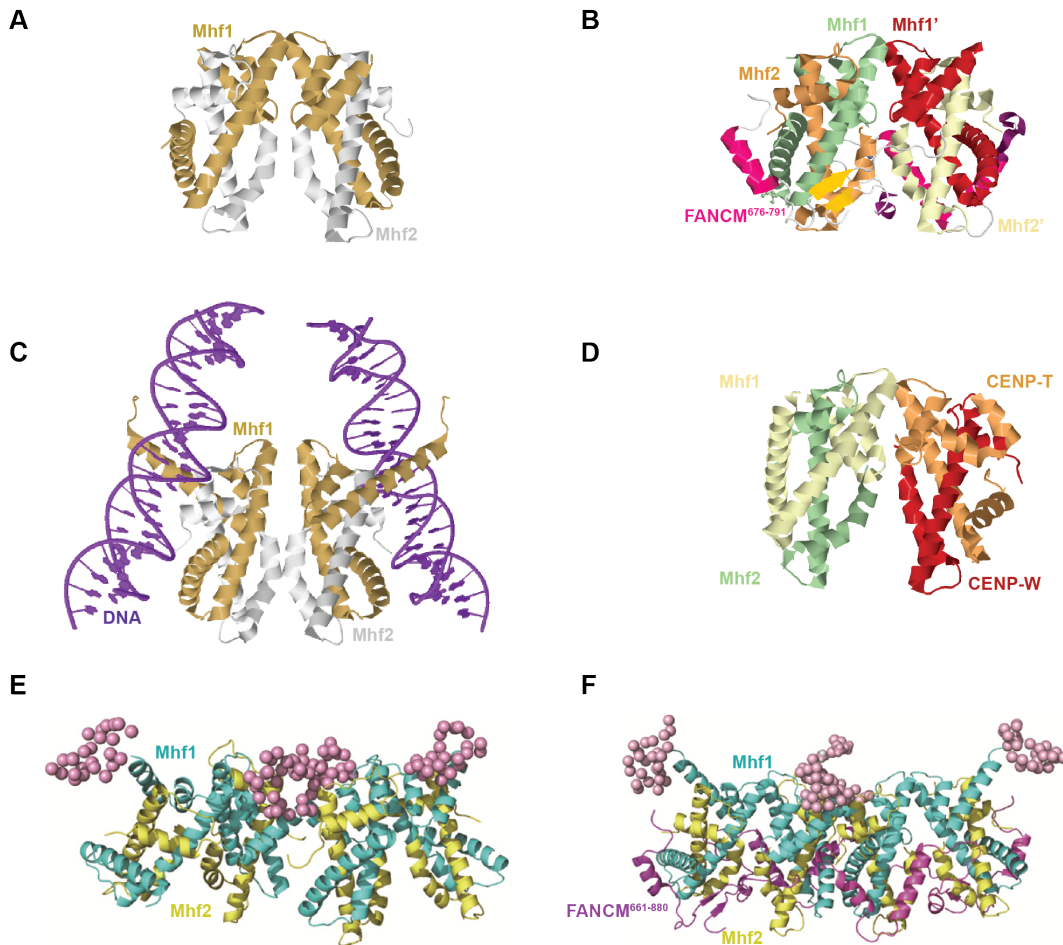


Figure 1.14 The different complexes that Mhf1 and Mhf2 form at the centromere

(A) Ribbon diagram representation of the human Mhf1-Mhf2 tetramer. Mhf1 is coloured in brown; and Mhf2 is coloured in grey (PDB ID: 4DRA) (Tao et al. 2012). (B) Ribbon diagram representation of the overall structure of the human MHF-FANCM676–791 complex. Mhf1 is coloured in green and red; Mhf2 is coloured in orange and cream; and FANCM676–791 is coloured in pink and yellow (PDB ID: 4E45) (Fox 3rd et al. 2014). (C) Ribbon diagram representation of the overall structure of human MHF-DNA complex. Mhf1 is coloured in brown; and Mhf2 is coloured in grey (PDB ID: 4NE1) (Zhao et al. 2014). (D) Ribbon diagram representation of the chicken Mhf1-Mhf2-CENP-T-CENP-W complex. Mhf1 is coloured in cream; Mhf2 is coloured in green; CENP-T is coloured in orange; and CENP-W is coloured in red (PDB ID: 3VH5) (Nishino et al. 2012). (E) Ribbon diagram representation of the overall structure of human Mhf1-Mhf2 octamer. Mhf1 is coloured in blue; Mhf2 is coloured in yellow; and missing loops are represented as dummy residues coloured in pink (Wang et al. 2013). (F) Ribbon diagram representation of the overall structure of the human Mhf1-Mhf2 octamer in complex with FANCM661–880. Mhf1 is coloured in blue; Mhf2 is coloured in yellow; FANCM661–880 is coloured in magenta and missing loops are represented as dummy residues coloured in pink (Wang et al. 2013).

(Zhao et al. 2014). Fox and colleagues reported that on its own, MHF binds preferentially to linear dsDNA, whereas when MHF is bound to the C-terminal of FANCM, it binds preferentially to branched DNA (replication fork or HJ) (Fox et al. 2014). In contradiction to this, Zhao and colleagues reported that regardless of the presence of FANCM, human MHF complexes prefer branched DNA to linear dsDNA because it engages the two adjacent arms of a DNA junction (Zhao et al. 2014) (Figure 1.14C). This ability of MHF to bind DNA is critical for enhancing FANCM's DNA branch migration activity, FANCD2 monoubiquitination in the FA pathway and resistance to DNA damaging agents, CPT and MMC in HeLa cells (Zhao et al. 2014).

MHF interacts with a region on the C-terminal side of FANCM's helicase domain (Yan et al. 2010; Tao et al. 2012; Fox et al. 2014). Crystal structures of the MHF heterotetramer with the truncated FANCM^{676–791} fragment (containing residues with the MHF interacting domain) reveal a pentameric complex consisting of one MHF heterotetramer and one truncated FANCM^{676–791} polypeptide stabilised by zinc atom-ligand binding (Tao et al. 2012; Fox et al. 2014) (Figure 1.14B). Contrary to Fox and colleagues' report that upon FANCM binding, there is asymmetric remodelling of the MHF DNA-binding surface and increase in positive charges to favour a switch in the DNA-binding preference from linear dsDNA to branched DNA (Fox et al. 2014), Tao and colleagues reported that there is no allosteric change in MHF upon FANCM binding (Tao et al. 2012). Instead, Tao and colleagues saw that FANCM binding changes its electrostatic surface potential and creates a new DNA-binding site on the MHF-FANCM complex (Tao et al. 2012). Both hydrophobic interactions in the MHF-FANCM interface (which are important for stabilising

the complex) and DNA-binding surfaces of the MHF-FANCM complex are important for normal activation of the FA network and suppression of SCEs. In particular, the MHF residues involved in DNA-binding have a bigger contribution than the FANCM DNA-binding residues (Fox et al. 2014). Furthermore, in Hela cells, the centromeric localisation of FANCM is greatly reduced when Mhf1 is suppressed (Tao et al. 2012).

As alluded to above, apart from being DNA repair proteins in conjunction with FANCM, Mhf1 and Mhf2 are centromeric proteins and they promote the assembly of the kinetochore. In this respect, a MHF heterodimer associates with another heterodimer of histone-fold proteins CENP-T and CENP-W, to form the Mhf1-Mhf2-CENP-T-CENP-W heterotetramer (Figure 1.14D). *In vitro*, the Mhf1-Mhf2-CENP-T-CENP-W heterotetramer binds and supercoils DNA. In chicken DT40 cells, mutants disrupted for heteromerization or DNA binding displayed compromised kinetochore assembly (Nishino et al. 2012).

More recently, using small angle X-ray scattering (SAXS), it has been reported that MHF can also exist as an octamer of two MHF tetramers positioned side by side (Wang et al. 2013) (Figure 1.14E and Figure 1.14F). They reveal that the higher oligomeric state generates large positively charged patches undetected by crystal structures. These patches stabilise its binding to dsDNA and it was postulated that the MHF octamer provides a 'landing pad' for the whole FA core complex to assemble on the DNA (Wang et al. 2013).

1.4.3 Centromere, kinetochore, spindle pole body

In all eukaryotes, faithful duplication of the entire genome followed by high fidelity chromosome segregation of the duplicated genome is essential for the precise transfer of genetic information from one generation to the next. A large multi-protein complex, known as the kinetochore, at the centromere, facilitates and regulates the timing and accuracy of chromosome segregation. The fundamental role of the kinetochore is to link the chromosomes to the dynamic microtubules (composed of tubulin dimers), thereby forming the mitotic spindle. In eukaryotes, the mitotic spindle emanates from the spindle pole body or centrosome and attaches to the centromeres of chromosomes via the kinetochore. The kinetochore is made up of the inner and the outer kinetochore. The inner kinetochore attaches to the centromeric DNA while the outer kinetochore attaches to the microtubules. Besides acting as a point of attachment for the mitotic spindle, the kinetochore also plays an important role in sensing errors in chromosome attachment to the spindle. The spindle assembly checkpoint proteins sense attachment and force to ensure that all chromosomes have a stable bipolar attachment before authorising the transition from metaphase to anaphase (Bouck et al. 2008; Cheeseman and Desai 2008; Welburn and Cheeseman 2008; Santaguida and Musacchio 2009).

Budding yeasts have (116 to 120 bp long) point centromeres, defined by three conserved sequences: centromere DNA element I (CDEI), CDEII and CDEIII (Clarke and Carbon 1980; Fitzgerald-Hayes et al. 1982). On the other hand, fission yeast and higher eukaryotes have longer regional centromeres with

multiple microtubule attachment sites per centromere and no sequence conservation (Chikashige et al. 1989; Baum et al. 1994; Pidoux and Allshire 2004). Fission yeast centromeres (40 to 100 kb long) consist of central core region 1 (cnt1), cnt2 and cnt3, bordered on either side by inverted repeat sequences L (imrL) and imrR, which are flanked by outer repetitive sequences, outer repeat L (otrL) and otrR (Allshire 1995; Pidoux and Allshire 2004). Human centromeres are much longer (0.5 to 5.0 mega bases (Mb)) and contain 1 to 4 Mb of 171 bp α -satellite repeats (Choo 2001).

Although there is no conservation of centromeric DNA sequence between species, other than in budding yeasts, much of the centromeric components required to assemble a functional kinetochore are conserved. At present, 16 proteins have been identified as centromere-associated network (CCAN) components. These clusters of proteins have been divided into several subgroups based on biochemical and genetic analyses. They include the CENP-C group; the CENP-T, CENP-W group; the CENP-H, CENP-I, CENP-K (CENP-L, CENP-M, CENP-N) group; the CENP-O, CENP-P, CENP-Q, CENP-R, CENP-U group; and the Mhf1, Mhf2 group (Perpelescu and Fukagawa 2011) (Figure 1.15).

Upon entry into mitosis, chromosomes condense and the constriction forms at the centromere. This region of a chromosome is defined by the incorporation of a histone H3 variant CENP-A (Henikoff and Dalal 2005; Black and Bassett 2008) (Figure 1.16). CENP-A nucleosomes are the hallmarks that define centromeres epigenetically and they act as a scaffold onto which other kinetochore proteins assemble. CENP-A is conserved from yeast to

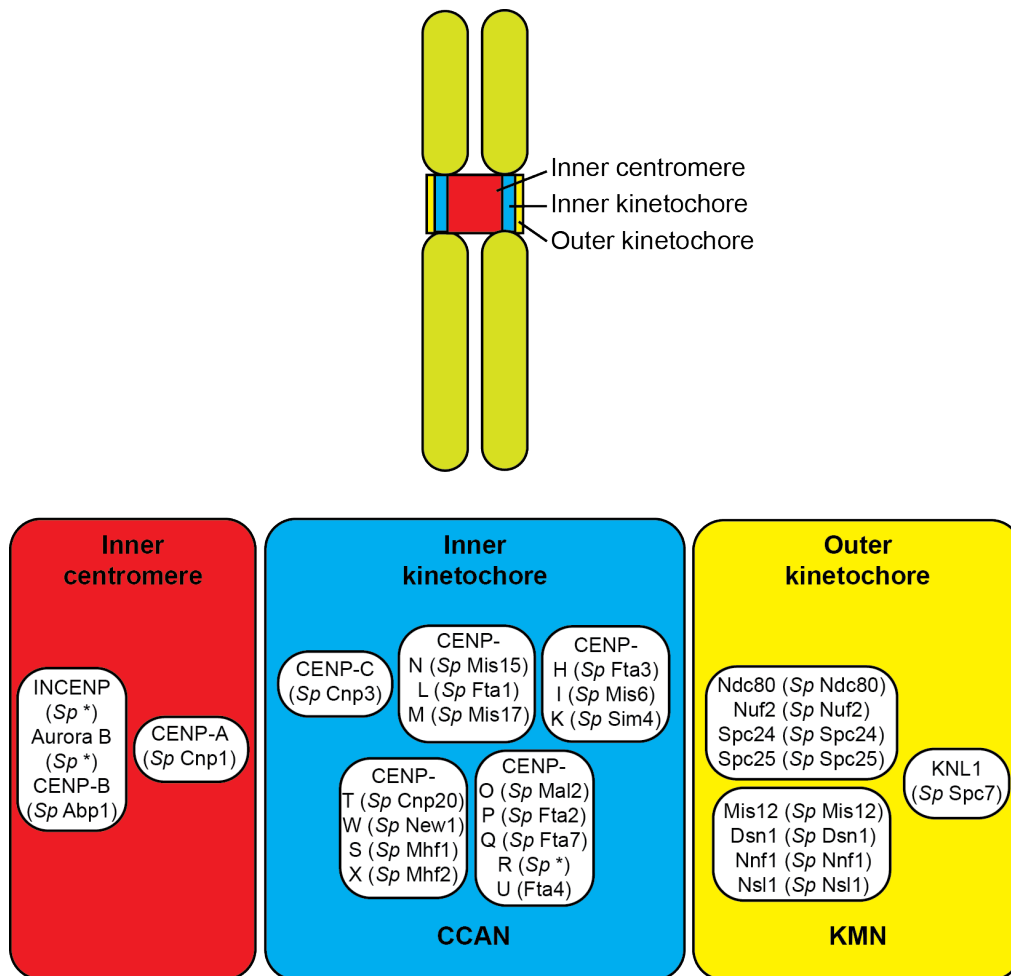


Figure 1.15 Schematic model of trilaminar structure of the kinetochore

During late G2 or early prophase, kinetochores conglomerate onto newly replicated sister chromatids. These structures disassemble after the completion of mitosis or meiosis. The inner centromere (red) is the area spanning the sister-chromatids, which is defined by the chromosome passenger protein complex (CPC) such as INCENP, Aurora B, CENP-B, borealin, survivin and condensin. Under an electron microscope, kinetochores appear as a trilaminated structure consisting of a middle electron-light layer flanked by two dense layers. The inner kinetochore (blue), also known as the CCAN, consists of five groups of CENPs: CENP-C, CENP-T-W-S-X, CENP-N-L-M, CENP-H-I-K and CENP-O-P-Q-R-U. The CCAN proteins assemble directly onto centromeric chromatin following CENP-A nucleosome deposition. The Ndc80 complex (Ndc80, Nuf2, Spc24 and Spc25), Mis12 complex (Mis12, Dsn1, Nnf1 and Nsl1) and KNL1, form the outer kinetochore (yellow). A fibrous corona comprising CENP-E and CENP-F lies beyond the outer kinetochore. *S. pombe* homologues are in parentheses. Asterisks indicate that homologues in *S. pombe* have not been identified/do not exist.

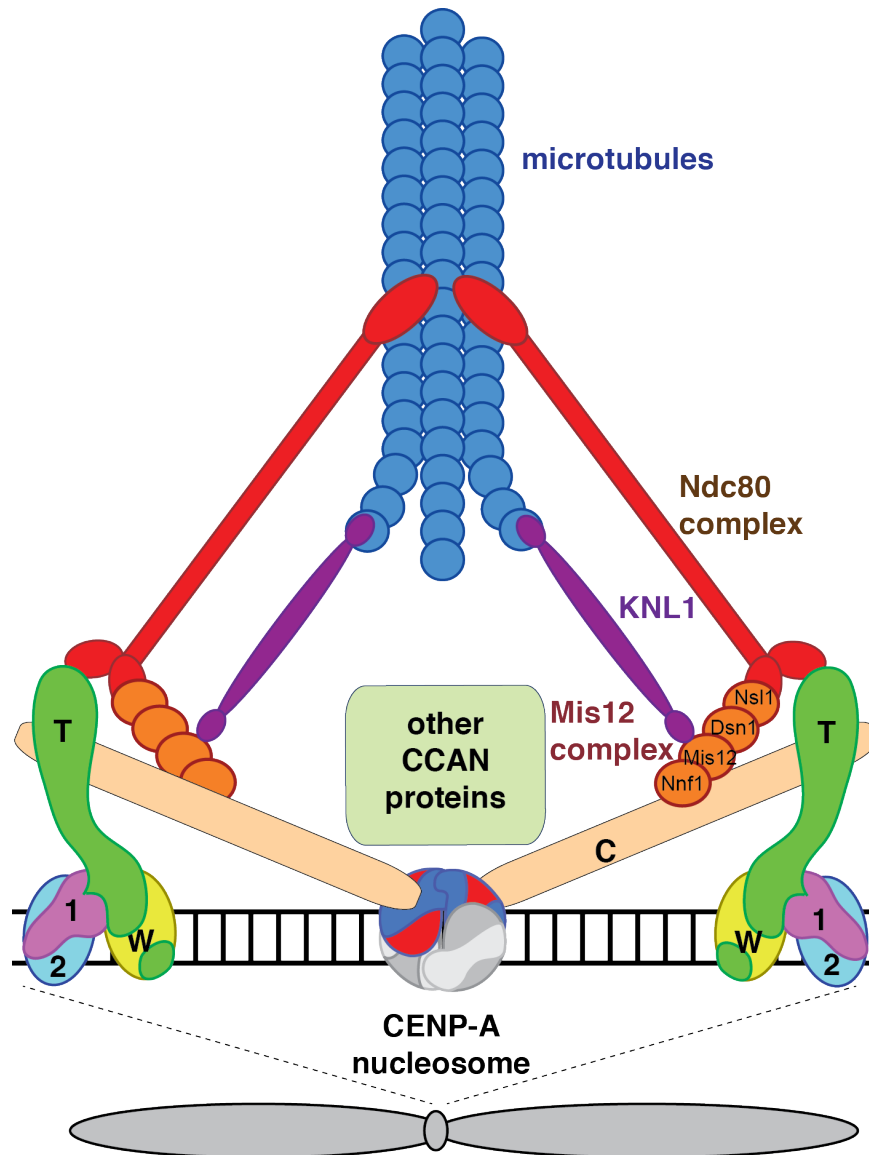


Figure 1.16 Cartoon depicting the kinetochore structure

Centromeric chromatin is linked to the microtubules in two ways. Firstly, CENP-C directly interacts with CENP-A nucleosome and binds to the Nnf1 subunit of the Mis12 complex. The Mis12 complex in turn interacts with the Ndc80 complex, which binds to the microtubules forming the first connection from the centromeric chromatin to the microtubules. The Mis12 subunit of the Mis12 complex also binds to the microtubules through KNL1, providing another interface for kinetochore-microtubule interaction. Secondly, the N-terminal of CENP-T in the Mhf1-Mhf2-CENP-T-CENP-W complex, which wraps centromeric DNA, binds to the Ndc80 complex, which in turn binds to the microtubules. The green box depicts the remaining CCAN proteins, which have no structural data. Adapted from (Osman and Whitby 2013).

mammals, and its absence is lethal, although a 90 % depletion in mammalian cells can be tolerated. CENP-A is essential for the localisation of almost all components of the kinetochore. It has been reported that when CENP-A or the CENP-A chaperon HJURP (Holliday junction recognition protein) is artificially tethered to non-centromeric DNA through the *lacO*-*LacI* system in mammalian and *Drosophila* cells, kinetochores are able to assemble on the non-centromeric DNA (Barnhart et al. 2011; Mendiburo et al. 2011). Furthermore, in *Xenopus* egg extracts kinetochores assemble on CENP-A polynucleosomes, but not on H3 polynucleosomes (Guse et al. 2011). CENP-C is an inner kinetochore DNA-binding protein essential for kinetochore assembly (Gascoigne et al. 2011; Przewloka et al. 2011; Screpanti et al. 2011). It acts as one of the two separate mutually exclusive connections between the centromeric chromatin and the outer kinetochore (Gascoigne et al. 2011; Przewloka et al. 2011; Screpanti et al. 2011) (Figure 1.16). It anchors the centromeric chromatin to the outer kinetochore by connecting to CENP-A embedded within the centromeric nucleosomes on one end, and to the Nnf1 subunit of the Mis12 complex, through the Mis12 complex to the Spc24-Spc25 subunits of the Ndc80 complex on the other end. The Ndc80 complex is an outer kinetochore component that binds directly to the microtubules (Malvezzi et al. 2013) (Figure 1.16). Loss of CENP-C in human cells leads to small or absent kinetochores (Tomkiel et al. 1994). CENP-C localisation is dependent on CENP-A in budding and fission yeast and it interacts with CENP-A in budding yeast (Howman et al. 2000; Van Hooser et al. 2001). Structural studies have shown that CENP-C contains a central motif, a CENP-C motif, and a C-terminal dimerization motif. In budding and

fission yeast, CENP-C directly recruits CENP-L to the kinetochore and that CENP-L overexpression in *cenp-c* deletion cells suppresses its chromosome segregation defects, hinting that the main function of CENP-C might be for CENP-L recruitment (Tanaka et al. 2009).

Besides CENP-C, CENP-T is the other component that acts as a mediator between centromeric chromatin and the outer kinetochore (Figure 1.16). CENP-T is a histone-fold protein and forms a complex with Mhf1, Mhf2 and CENP-W in which DNA wraps around to form a nucleosome-like structure (see section 1.4.2) (Nishino et al. 2012). CENP-T and CENP-W has been reported to be crucial for cell viability, whereas Mhf1 and Mhf2 are less important for survival in DT40 cells (Hori et al. 2008; Amano et al. 2009). Mammalian and budding yeast CENP-T have been reported to directly interact with the Ndc80 complex in a phosphorylation and cell cycle dependent manner, providing the connection between the chromatin and the microtubules (Bock et al. 2012; Schleiffer et al. 2012; Nishino et al. 2013). Artificial tethering of CENP-T to the Ndc80 complex in budding yeast enables the segregation of mini-chromosomes without centromeres, demonstrating that primary function of CENP-T is in recruiting the Ndc80 complex (Schleiffer et al. 2012). Furthermore, ectopically localising the N-terminal of CENP-T and CENP-C to chromatin using the *lacO*-*LacI* system in human cells is adequate for recruiting centromeric proteins to form a pseudokinetochore to enable chromosome segregation (Gascoigne et al. 2011). During anaphase, CENP-T competitively binds the Ndc80 complex in budding yeast, inhibiting the binding of the Ndc80 complex to the other KMN components (Bock et al. 2012; Schleiffer et al. 2012). Indeed, CENP-T has been reported to stretch

physically under tension from the kinetochore, potentially explaining how forces generated at the microtubules get converted into motion at the sister chromatids (Suzuki et al. 2011).

KMN is a key outer kinetochore component comprising KNL1 (Desai et al. 2003; Kiyomitsu et al. 2007; Cheeseman et al. 2008); the Mis12 complex with four subunits Mis12, Dsn1, Nnf1 and Nsl1 (Kline et al. 2006); and the Ndc80 complex with four other subunits Ndc80, Nuf2, Spc24 and Spc25 (Janke et al. 2001; Wigge and Kilmartin 2001; McClelland et al. 2003; Bharadwaj et al. 2004). Recent reports suggest a role for KNL1 in surveying the state of kinetochore-microtubule interactions (Espeut et al. 2012). It has a conserved microtubule-binding domain at its N-terminal and this binding is regulated by its phosphorylation by Aurora B (Cheeseman et al. 2006; Welburn et al. 2010). It binds to the Mis12 subunit of the Mis12 complex on the other end, providing another interface for kinetochore-microtubule interaction. The Mis12 complex acts as a platform for the formation of the KMN complex and connects the outer kinetochore to the inner kinetochore through direct binding with CENP-C and the Ndc80 complex (Screpanti et al. 2011). The Ndc80 complex assists the load-bearing microtubule attachment (Powers et al. 2009). Ndc80-Nuf2 and Spc24-Spc25 have a globular head and a long coiled-coil domain, forming a dumb-bell-shaped tetramer via their coiled-coil domains (McClelland et al. 2004; Wei et al. 2005). The globular head domain of the Spc24-Spc25 dimer connects the Ndc80 complex to the inner kinetochore via direct contact with the Mis12 complex and CENP-T (Petrovic et al. 2010; Malvezzi et al. 2013; Nishino et al. 2013) (Figure 1.16).

Whilst it is clear that Mhf1 and Mhf2 work in conjunction with FANCM to promote DNA repair and recombination, as well as at the kinetochore for proper chromosome segregation, there has been speculation on whether FANCM supports centromeric function or whether the other CCAN proteins support DNA repair and recombination (Osman and Whitby 2013). It has been postulated that the high level of inter-repeat sequence identity within the centromere is maintained by recombination (possibly requiring FANCM) between inter-repeats to form covalently closed DNA loops which help direct the assembly of CENP-A nucleosomes at the centromeres. Additionally, it has been shown that FANCM is recruited to centromeres in an MHF-dependent manner in HeLa cells, supporting the idea that FANCM has a role at the centromere (McFarlane and Humphrey 2010; Tao et al. 2012). Despite that, the deletion of MHF in fission yeast leads to poor cell viability caused by a high rate of chromosome mis-segregation, whereas the deletion of Fml1 appears to have little effect on centromere function (Bhattacharjee et al. 2013; Osman and Whitby 2013). On the other hand, Zeitlin and colleagues have reported the recruitment of CENP-A, CENP-T, CENP-U and CENP-N to non-centromeric DNA DSBs induced by I-SceI endonuclease cleavage alongside recombination proteins like RAD51 in human and mouse cells (Zeitlin et al. 2009). The precise role of FANCM in recombination at the centromere and the role of these CCAN proteins at DSBs remain unsolved, however, these models and ideas potentiate new avenues for investigation to provide greater insight into genome stability.

1.5 Aim of study

At the outset the two main aims of my DPhil work were to: 1) investigate whether replication fork termination is frequently associated with recombination; and 2) determine whether a putative octameric form of MHF was biologically important.

To begin, I sought to position a *RTS1* direct repeat recombination reporter (Ahn et al. 2005) at a site with a cluster of replication origins, resulting in shorter distances and therefore times between replication fork initiation and termination (Chapter 3). Through this, I aimed to answer some of the fundamental questions of whether termination is often associated with recombination.

In the next part of my dissertation, I aimed to determine whether the octameric form of MHF was biologically important by mutating three Mhf2 amino acids in fission yeast that disrupt the octamer configuration when mutated *in vitro* (Chapter 4). As will become apparent, during the course of my investigation, I noticed that one of the “octamerisation” mutants also exhibits hypersensitivity to both MMS and cisplatin, suggesting a defect in promoting Fml1 function. This led my investigation of MHF to evolve to reassess the importance of the physical interaction of the “octamerisation” mutant with Fml1 (Chapter 5). Furthermore, epistasis analysis between the “octamerisation” mutants and mutants defective in centromere function revealed a synergistic interaction between Dcr1 and MHF, initiating my investigation of whether Dcr1 influences recombination at stalled replication forks in fission yeast (Chapter 7).

Lastly, since MHF has a centromeric role in chicken and human cells (Amano et al. 2009; Schleiffer et al. 2012), I also examined the *mhf* Δ mutants for phenotypes consistent with a defect in the kinetochore. Further investigation led me to uncover a novel function for it in controlling the initiation of septation in fission yeast (Chapter 6).

Chapter 2: Materials and Methods

2.1 Media

2.1.1 *E. coli* media

All *E. coli* strains were grown in Luria-Bertani (LB) (10 g Tryptone, 5 g yeast extract (YE), 5 g NaCl) broth or on LB-agar (15 g/l agar) at 37 °C, unless otherwise stated. Media were supplemented with antibiotics where appropriate, namely ampicillin (final concentration 50 µg/ml), carbenicillin (final concentration 50 µg/ml) and chloramphenicol (final concentration 20 µg/ml) (Sambrook and Russell 2001). Antibiotics were filtered (0.2 µm Milipore syringe filters) and stored as 1000 X stocks at -20 °C (Sambrook and Russell 2001).

2.1.2 *S. pombe* media

General molecular genetic techniques for *S. pombe* are based on established protocols (Sabatinos and Forsburg 2010).

YE (30 g D-Glucose and 5 g Yeast Extract in 1 L Milli-Q water) and YE containing all supplements (YES) (YE supplemented with 225 mg/l uracil, leucine, lysine, histidine and adenine each) were used as complete medium. Edinburgh Minimal Media with 3.7 mg/ml L-glutamic acid monosodium salt (EMMG) (3 g potassium hydrogen phthalate, 2.2 g anhydrous Na₂HPO₄, 3.7 g sodium glutamate, 30 g D-glucose, 20 ml 50 X salts, 1 ml 1000 X vitamins, 0.1 ml 10000 X minerals made up to 1 L with sterile Milli-Q water) was used as minimal media and supplemented with amino acids as appropriate. When necessary, 18 g/l Difco™ agar was added to the media.

Strains that are auxotrophic for one or more of the following: uracil, leucine, lysine, histidine, arginine, and/or adenine, require the respective supplements at 225 mg/l for growth on minimal media. For selection of strains carrying antibiotic resistance, media was supplemented with geneticin (G418, final concentration 100 mg/l), nourseothricin (clonNat, final concentration 100 mg/l) or hygromycin B (final concentration 200 mg/l).

To differentiate between Ade⁺ and Ade⁻ colonies, cells were plated onto YE with low adenine (YELA), where concentration of adenine is 10 mg/l, which allows Ade⁺ colonies to appear white and Ade⁻ colonies red.

Ade⁺ recombinants were selected on YE supplemented with 225 mg/l uracil, 225 mg/l leucine, 225 mg/l lysine, 225 mg/l histidine and 0.2 mg/ml guanine (YE-ade+guanine). Guanine prevents uptake of residual adenine (Osman and Whitby 2009).

Recipes for medium used in this study are provided in Table 2.1.

2.2 Plasmid construction

All restriction enzymes used in this study were supplied by New England Biolabs (Ipswich, MA). Oligonucleotides for polymerase chain reaction (PCR) amplification were custom-made and supplied by Sigma-Genosys (Table 2.2) (Haverhill, Suffolk). PCR reactions were performed using either a Hybaid (OmniGene) (Woburn, MA) or Biometra thermocycler (Göttingen, Germany). The plasmids used in this study are listed in Table 2.3, and the construction of plasmids is presented in Table 2.4.

2.2.1 Site-directed mutagenesis

Site-directed mutagenesis was performed using the QuikChange® Site-Directed Mutagenesis Kit (Stratagene, La Jolla, CA) following the manufacturer's manual. PCR reactions were performed using a Biometra thermocycler (Göttingen, Germany).

2.2.2 Random mutagenic PCR

Random mutagenic PCR was performed using the GeneMorph® II Random Mutagenesis Kit (Agilent Technologies) following the manufacturer's manual. PCR reactions were performed using a Biometra thermocycler (Göttingen, Germany).

To generate a conditional *mhf1/2* mutant (described in section 6.2.2), I generated a library of *mhf1* and *mhf2* random mutations using random mutagenic PCR amplification, with primers oMW1427 and oMW1443 on plasmid pMW910 for *mhf1*; and primers oMW1433 and oMW1444 on plasmid pMW912 for *mhf2* (Table 2.2 and Table 2.3). pMW910 and 912 are derivatives of pFA6-kan^R containing *mhf1/mhf2* and *mhf1/mhf2* 3' flanking genomic DNA cloned on the other side of the kan^R cassette (Table 2.3). These were constructed for targeted gene replacement at the *mhf1* and *mhf2* loci, respectively. The mutagenized PCR product was digested with Sall-HF and Ascl, and ligated back into pMW910 and pMW912 backbones (replacing the wild-type copy of the gene and a 3HA tag) for *mhf1* and *mhf2* respectively (Table 2.3). The resultant plasmids were next transformed into *E. coli* to create mutant libraries each consisting of ~5000 transformants. The plasmid

mutant libraries were isolated from the *E. coli* transformants following amplification and subject to restriction digest (with XmnI and EcoRV-HF for *mhf1*; and NaeI and EcoRV-HF for *mhf2*) to produce fission yeast targeting constructs.

2.2.3 *E. coli* transformation

50 to 100 ng plasmid DNA was transformed into 100 to 150 μ l chemically competent *E. coli* DH5 α cells using the standard 42 °C heat-shock treatment for bacterial transformation (Sambrook and Russell 2001). Cells were plated on LB medium containing appropriate selective antibiotic(s) and grown at 37 °C overnight.

2.3 *S. pombe* strain construction

2.3.1 *S. pombe* transformation

S. pombe was transformed using a standard lithium acetate transformation protocol (Bahler et al. 1998). Cells were washed thoroughly with sterile Milli-Q water and resuspended in 0.1 mM lithium acetate (pH 7.5) with TE (10 mM Tris-HCl [pH 7.5], 1 mM EDTA (ethylenediaminetetraacetic acid)), in a 1:1 ratio. 1 to 10 μ g of gel purified linearized DNA and 20 μ g of carrier DNA (sonicated salmon sperm DNA) were mixed with 100 μ l of cells and incubated at room temperature for 10 minutes. 260 μ l of 0.1 mM lithium acetate, TE and 40 % PEG were then added to the mixture at 30 °C for 2 hours. 43 μ l of dimethylsulfoxide (DMSO) was added to the mixture and the cells were heat-shocked at 42 °C for 5 minutes. The transformed cells were plated in 1:1 and 1:10 dilutions, either directly onto selective media with antibiotics (for

nourseothricin selection) or, plated onto selective media without antibiotics and incubated at 30 °C for 48 hours before replica plating the transformants onto complete media with antibiotics (for geneticin or hygromycin B selection). Transformants were screened for correct integration of the DNA construct by colony PCR (described in section 2.3.2).

2.3.2 *S. pombe* colony PCR

Where possible, PCR primers were designed such that one primer anneals to the parental chromosomal sequence flanking the target site while the second primer anneals within the inserted DNA, on both the 5' and 3' ends of the insert. Where the product was less than ~2.5 kb, PCR primers which anneal to the parental DNA 5' and 3' of the insert, were also used to confirm an insertion or deletion.

Colony PCRs were performed by boiling a yeast colony, at 100 °C for 2 minutes, to release the chromosomal DNA. This was followed by amplification of specific DNA fragments, with Taq polymerase (New England Biolabs). All colony PCRs were carried out alongside a wild-type control. Where appropriate, PCR products were sequenced to confirm the presence or absence of point mutations. At least 6 independent transformants were subsequently tested for growth and genotoxin sensitivity to determine the reproducibility of the phenotype.

2.3.3 *S. pombe* crosses

Cells with opposite mating types were mixed and grown on malt extract agar (MEA) at 25 °C for 2 to 3 days. Spores were released by overnight digestion

with glucylase at 30 °C. Released spores were then washed with sterile Milli-Q water, and plated either directly onto selective media (for nourseothricin, uracil, leucine, histidine, and/or arginine selection), or onto complete media (for geneticin or hygromycin B selection) and incubated at 30 °C for 5 days before replica plating the transformants onto appropriate selective media.

2.4 DNA analysis

2.4.1 *S. pombe* genomic DNA extraction

Small-scale preparations for PCR or southern blot analyses were made from 10 ml overnight cultures of *S. pombe*. Harvested cells were spheroplasted by incubating in 1 ml of Citrate Phosphate buffer (40 mM EDTA, 50 mM Na₂HPO₄, 55 mM citric acid and 1.2 M sorbitol) containing 30 units of Yeast Lytic Enzyme (MP Biomedical) for 1 hour at 37 °C. The efficiency of spheroplasting was assessed by microscopic examination of cells mounted in 2 % sodium dodecyl sulphate (SDS). Spheroplasts were resuspended in 0.55 ml TE (10 mM Tris-HCl [pH 8.0], 1 mM EDTA) with 1 % SDS and incubated at 65 °C for 1 hour. 175 µl of 5 M potassium acetate was then added and the sample was centrifuged at 4 °C for 15 minutes at 13000 rpm. The supernatant was retained and an equal volume of isopropanol was added to precipitate the DNA. After pelleting the DNA by centrifugation, the DNA was washed in 70 % ice-cold ethanol, resuspended in 50 µl of TE containing 20 µg/ml RNaseA and incubated at 65 °C for 10 minutes. Proteins were removed by two rounds of phenol/chloroform/isoamyl alcohol (25:24:1) extraction, and the DNA was stored at -20 °C.

To isolate genomic DNA for analysis of replication intermediates on two-dimensional (2D) gels, 500 ml *S. pombe* cultures were grown overnight at 30 °C to a concentration of 1×10^7 cells/ml. Cells were then killed by addition of 0.1 % sodium azide (Sigma-Aldrich) and washed in water before being stored as pellets at -80 °C. Subsequently, cells were spheroplasted in an equal volume of CEPES buffer (400 mM EDTA, 120 mM Na₂HPO₄, 40 mM citric acid, 1.2 M sorbitol) containing 10 mg/ml *Trichoderma harzianum* lysing enzymes (Sigma-Aldrich), 0.625 mg/ml *Arthrobacter luteus* lyticase (Sigma-Aldrich) for 1 hour at 37 °C and 10 mM dithiothreitol (DTT). Spheroplasted cells were embedded in 2 % Bio-Rad Low Melt Agarose (Bio-Rad). To degrade proteins, plugs were incubated two times overnight in 2 ml NDS-PK buffer (495 mM EDTA, 10 mM Tris-HCl [pH 7.5], 1 % N-lauroylsarcosine), containing 1 mg/ml Proteinase K at 50 °C. To inactivate residual Proteinase K, plugs were incubated in 10 ml TE with 0.5 mM phenylmethylsulphonylfluoride (PMSF) on a rocking platform for 1 hour at room temperature, and subsequently washed three times in 20 ml TE for 1 hour each, on a rocking platform. Plugs were then washed another three times in 20 ml 1 X CutSmart buffer (New England Biolabs), melted at 65 °C for 15 to 20 minutes, and then incubated with 1 % β-agarase (New England Biolabs) for 2 hours at 42 °C. Extracted DNA was digested with 300 units of EcoNI, with 100 µg RNaseA overnight at 37 °C. Another 75 units of EcoNI was added and kept incubating the next day. DNA was then enriched for replication intermediates on benzoylated naphtholyated DEAE (diethylaminoethanol) (BND)-cellulose (Sigma-Aldrich). 0.666 g per sample of BND cellulose was washed 3 X with Milli-Q water and 3 X with BND buffer 1

(0.3 M NaCl, 10 mM Tris-HCl [pH 7.5], 1 mM EDTA). BND cellulose was then left to resuspend in BND buffer 1 for 4 hours at room temperature. A mixture of the BND cellulose and BND buffer 1 was then added to a BND cellulose column 1 ml at a time until each tube has 5 ml of the mixture. DNA samples were equilibrated to a final concentration of 0.3 M NaCl and loaded onto BND cellulose columns. dsDNA was eluted by the addition of 3 ml of BND buffer 2 (0.8 M NaCl, Tris-HCl [pH 7.5], 1 mM EDTA) and allowed to drip through. ssDNA was eluted by the addition of 3 ml of pre-warmed BND buffer 3 (1 M NaCl, 10 mM Tris-HCl [pH 7.5], 1 mM EDTA, 1.8 % (w/v) caffeine and allowed to drip through. After which, 3 ml of isopropanol and 10 µg tRNA was added to the DNA, mixed gently and incubated overnight at -20 °C. After pelleting the DNA by centrifugation, the DNA was washed in 70 % ice-cold ethanol 2 X and left to dry. DNA was redissolved in 40 µl of BND buffer 4 (10 mM NaCl, 10 mM Tris-HCl [pH 7.5], 0.1 mM EDTA).

2.4.2 Two-dimensional gel electrophoresis

2D gel analysis was performed based on the previously described method of (Brewer and Fangman 1987). In the first dimension, replication intermediates were separated on the basis of the fragments' sizes by running DNA samples on a 0.4 % agarose gel in 1 X TBE without ethidium bromide to ensure uninterrupted migration. The first dimension was run at 40 V for 24 hours. The second dimension was run in a 1.2 % agarose gel in 1 X TBE with 0.3 µg/ml ethidium bromide under continuous buffer (1 X TBE with 0.3 µg/ml ethidium bromide) recirculation at 4 °C to separate intermediates on the basis of their

shape. The second dimension was run at 120 V for 18 hours, until the front dye has migrated 8 cm.

2.4.3 Southern blotting

Before blotting, gels were gently shaken at room temperature in 0.25 M HCl for 20 minutes to depurinate the DNA and then gently shaken in transfer solution (0.5 M NaOH, 1.5 M NaCl) for 10 minutes. DNA was transferred from gels onto positively-charged nylon membranes (Hybond-XL, GE Healthcare, Little Chalfont, UK) by capillary transfer (Sambrook and Russell 2001). After dismantling the blot, membranes were washed with 2 X saline sodium citrate (SSC) (0.3 M sodium citrate, 0.03 M NaCl buffer) for 10 minutes and stored at -20 °C. Pre-hybridisation was done at 65 °C for 30 to 60 minutes in modified Church and Gilbert Buffer (0.5 M Phosphate buffer [pH 7.2], 7 % (w/v) SDS, 10 mM EDTA). Hybridisation was performed at 65 °C overnight as described (Sambrook and Russell 2001) in a Grant Boekel hybridization oven (HIR10M). Bands of interest were detected by radiolabeling the probe with [α^{32} -P]dCTP (Perkin Elmer) using an Amersham Rediprime II Random Prime Labelling System (GE Healthcare) kit following the manufacturer's instructions. The probe used was an EcoNI-XbaI restriction fragment of the *his3* gene from pFOX2. Following two washes with 2 X SSC, 1 % SDS and another wash with 0.1 X SSC, 1 % SDS, blots were exposed to phosphoscreens and visualised using a Fuji FLA3000 phosphoimager (FujiFilm, Tokyo, Japan). Blots were then analysed using the Image Gauge software (FujiFilm).

2.5 Biochemistry techniques

A list of buffers and solutions for biochemistry techniques is presented in Table 2.5.

2.5.1 Expression and Purification of Mhf1 and His-Mhf2^{D87A}

A one litre culture of *E. coli* BL21-RIL cells (Novagen) transformed with pMW891 (D87A) was grown with aeration at 25 °C (New Brunswick Scientific shaking incubator at 250 rpm) in LB broth containing 50 µg/ml ampicillin and 20 µg/ml chloramphenicol to an A₆₀₀ of 0.5. Mhf1 and His-Mhf2^{D87A} was induced by adding isopropyl-1-thio-D-galactopyranoside (IPTG) to a final concentration of 0.5 mM, following which the cells were grown with aeration at 25 °C for a further 5 hours. The cells were then harvested by centrifugation, resuspended in 20 ml Buffer H (50 mM potassium phosphate, pH 8.0, 0.3 M NaCl, 10 % glycerol) and frozen at -80 °C. Protein expression was analysed by SDS-PAGE (SDS-polyacrylamide gel electrophoresis). To purify the complex, the frozen cells were defrosted and mixed with 1 % Triton X-100, 14 mM β-mercaptoethanol and protease inhibitors (EDTA-free protease inhibitor cocktail tablets (Roche)) before putting them through a French pressure cell at 19000 p.s.i. The lysates were cleared by centrifugation at 19000 g for 40 minutes at 4 °C, and the supernatant was loaded directly onto a 1 ml nickel-nitrilotriacetic acid (Ni-NTA) Superflow column (Qiagen) that had been pre-equilibrated with Buffer H. The column was then washed with 60 ml of Buffer H plus 20 mM imidazole before eluting bound Mhf1 and His-Mhf2^{D87A} with Buffer H plus 200 mM imidazole into three 1 ml fractions. The second and third fractions were pooled and loaded directly onto a HiLoad 16/60

Superdex 200 gel filtration column (Amersham Biosciences), which was then developed with 120 ml of Buffer A (50 mM Tris-HCl, pH 8.0, 1 mM EDTA, 1 mM DTT, 10 % glycerol) plus 300 mM NaCl. 60 x 2 ml fractions were collected and the peak of Mhf1 and His-Mhf2^{D87A} eluted in fractions 35 to 43 as determined by SDS-PAGE with staining by Coomassie blue. Fractions 38 and 39 were pooled and stored as aliquots at -80 °C.

2.5.2 Fml1⁶⁰⁴⁻⁸³⁴ + Mhf1 and His-Mhf2^{D87A} interaction assay

Fragments of Fml1, consisting of amino acids 604 to 834 fused to maltose binding protein (MBP), were immobilized on 10 µl amylose beads by incubating them together for 2 hours at 4 °C on a rotating platform. The beads were subsequently washed with Buffer I [0.05 M NaCl] before incubating them with the Mhf1 and His-Mhf2^{D87A} protein complex for 3 hours at 4 °C on a rotating platform. The beads were then extensively washed in Buffer I with various NaCl concentrations [0.05 M, 0.2 M, 0.3 M and 0.5 M] (3 x 20 minutes) before eluting the bound proteins by boiling the beads in 28 µl of 1 % SDS.

2.5.3 Electrophoretic mobility shift assay

X-12 substrates (described in (Whitby and Lloyd 1998)) for the MHF-DNA binding reactions were 5'-end-labelled with γ -³²P using T4 polynucleotide kinase and gel purified as described before (Wong, 2013). Reactions (20 µl) contained 0.1 nM of radiolabelled DNA in 25 mM Tris-Cl pH 8.0, 1 mM DTT, 0.1 mg/ml bovine serum albumin (BSA), 120 mM NaCl and 6 % glycerol. MHF was added as indicated and the mixture was incubated on ice for 15 min and

separated on 4 % (29:1) polyacrylamide gels in a 1 X low ionic strength buffer (6.7 mM Tris-Cl 8.0, 3.3 mM sodium acetate, 2 mM EDTA). Both buffer and gel were pre-cooled at 4 °C, for an hour, and electrophoresis was carried out at room temperature. Gels were dried on 3MM Whatman paper (Sigma-aldrich), exposed to phosphoscreens, which were subsequently scanned using a Fuji FLA3000 phosphoimager (FujiFilm, Tokyo, Japan) and analysed using the Image Gauge software (FujiFilm).

2.5.4 SDS-polyacrylamide gel electrophoresis

SDS-PAGE was performed using resolving gels containing 12 % (29:1) acrylamide (375 mM Tris-Cl 8.8, 0.1 % w/v SDS) and stacking gels containing 5 % (29:1) acrylamide (125 mM Tris-Cl 6.8, 0.1 % w/v SDS). Protein samples were mixed with 4 X Laemmli sample buffer (250 mM Tris-HCl [pH 6.8], 10 % v/v β-mercaptoethanol, 8 % SDS, 0.1 % Bromophenol Blue, and 40 % v/v glycerol) and boiled at 95 °C for 5 minutes prior to loading alongside a protein marker (either Prestained protein Marker, Broad Range, New England Biolabs; or Fermentas PageRuler™ Unstained protein ladder, Fermentas). Gels were run at 40 mA for 1 hour 40 minutes and subsequently stained with Coomassie blue. Stained gels were imaged using a Gel DocXR+Imaging system (Bio-Rad).

2.6 Genetic assays

2.6.1 Genotoxin sensitivity spot assays

Logarithmically growing cells from liquid cultures of *S. pombe* were harvested, washed and resuspended in sterile Milli-Q water at a density of

2×10^7 cells/ml. Subsequent ten-fold dilutions from 2×10^6 cells/ml to 2×10^4 cells/ml were made. Aliquots (10 μ l) of the cell suspensions were spotted onto YES plates containing various concentrations of chemical genotoxins (MMS, HU, CPT, TBZ (thiabendazole), Cisplatin) or irradiated with various doses of UV light using a Stratalinker (Stratagene) as specified. In each spot assay, the designated 1 (undiluted) spot represents 2×10^7 cells, whereas 0.1, 0.01, and 0.001 spots represent 2×10^6 , 2×10^5 , 2×10^4 cells respectively. Plates were typically incubated at 30 °C for 4 days unless otherwise stated. All spot assays were done on the same plate. Where indicated, the spot assays were done on the same plate but were cropped and placed next to each other for ease of presentation.

Crude spot assays were done by inoculating colonies of the same size, from different strains in 1 ml Milli-Q water. Subsequent ten-fold dilutions from 10^{-1} , to 10^{-3} were made. Aliquots (10 μ l) of the cell suspensions were spotted onto YES plates containing various concentrations of chemical genotoxins (MMS, HU, CPT, TBZ, Cisplatin) or irradiated with various doses of UV light using a Stratalinker (Stratagene) as specified. Plates were typically incubated at 30 °C for 4 days unless otherwise stated.

2.6.2 Direct repeat recombination assay

Direct repeat recombination was assayed by measuring the frequency of Ade⁺ recombinants as described (Osman and Whitby 2009).

Strains were initially plated onto minimal media lacking histidine and containing only low levels of adenine (EMMG-his/LA). These were grown at

30 °C for about 6 days. One colony from each strain was then resuspended in 1 ml sterile Milli-Q water and serially diluted in 10-fold steps. Appropriate dilutions of cells were plated onto YES+5S plates and incubated at 30 °C for 5 to 6 days. Single colonies from each strain to be assayed were then resuspended in sterile Milli-Q water. Approximately 10^2 cells were plated onto YELA plates to assess cell viability. Approximately 10^4 to 10^5 cells were plated onto YE-ade+guanine plates, to score for Ade⁺ recombinants. The frequency of recombinants was calculated by counting the colonies on the plates typically after 5 to 6 days of incubation at 30 °C unless otherwise stated. Plates with Ade⁺ recombinants were then replica plated onto minimal media lacking adenine and histidine (EMMG-ade-his) to estimate the proportion of conversion-type recombinants (Ade⁺His⁺). After 2 days of incubation at 30 °C, the frequency of conversion-type recombinants was calculated by counting colonies.

In the case of the Chromosome I telomere strains, where expression of adenine in the deletion-type recombinants (Ade⁺His⁻) was poor, colonies on the YE-ade+guanine plates were counted after 11 to 12 days of incubation at 30 °C.

2.6.3 Mitotic DSB repair assay

The mitotic DSB repair assay was performed as previously described (Sun et al. 2008).

pAN1 (plasmid used as substrate for gap repair was digested with NcoI to linearize it and then resolved on a 0.8 % agarose gel and isolated using a

QIAquick Gel Extraction Kit (Qiagen). Logarithmically growing strains were transformed by lithium acetate transformation (described in section 2.3.1) with either 5 µg of linearised or uncut pAN1, omitting carrier DNA. Cells were plated onto minimal media lacking uracil (EMMG-ura) and incubated at 30 °C for 6 days. The transformants were then counted to determine the relative transformation efficiency of the cut versus the uncut plasmid, which would give an indication of how well the cut plasmid was repaired.

300 transformants derived from transformation with the cut plasmid were subsequently patched onto EMMG-ura plates and incubated for 2 days at 30 °C before replica plating onto minimal media lacking both adenine and uracil (EMMG-ura-ade) to score the number of Ade⁺ recombinants amongst the Ura⁺ transformants. The patched transformants on EMMG-ura were also replica plated onto non-selective YELA plates, which after 2 days at 30 °C were re-replica plated onto YELA. Following the two rounds of growth on non-selective media, the YELA patches were replica plated onto plates containing 5' fluorotic acid (FOA), which counter-selects Ura⁺ cells. Growth on FOA indicated that the Ura⁺ containing linear fragment did not get integrated into the chromosome (i.e. it was a non-crossover) since it was readily lost without selection. Poor growth on FOA indicated that the Ura⁺ containing linear fragment was not easily lost due to its integration into the chromosome via crossover recombination. The percentage of Ura⁺ transformants that were *ade6*⁺ as well as the percentage of Ura⁺Ade⁺ transformants that were crossovers was calculated. 3 assays were performed for each strain to derive mean values.

2.6.4 Meiotic crossover assay

Strains of opposite mating types were freshly grown on minimal media with low adenine (EMMG+LA), washed once with 1 ml of 0.145 M NaCl, resuspended in 0.5 ml 0.145 M NaCl, spotted (50 µl) onto MEA plates, and incubated at 25 °C for 3 days. A quarter of the cell material from each spot was washed once with 1 ml Milli-Q water, resuspended in 0.5 ml Milli-Q with 15 µl gluculase, and incubated at 30 °C overnight. Released spores were then washed 3 X with sterile Milli-Q water and counted. 200, 250 and 300 spores from each cross were plated onto three YELA plates each. 20000, 25000 and 30000 spores were also plated onto three YE-ade+guanine plates each. Plates were incubated at 30 °C for 5 days. All the colonies on these plates were counted. The percentage of spore viability was calculated by:

$$\frac{(\text{Total no. colonies that grow on all three YELA plates})}{(\text{Total no. spores plated onto all three YELA plates})}$$

The percentage of gene conversion was calculated by:

$$\frac{(\text{Total no. colonies that grow on all three YE-ade+guanine plates})}{[(\text{Total no. colonies that grow on all three YELA plates}) * 100]}$$

The YELA plates with closest to 200 colonies were replica plated onto EMMG-ura, EMMG-his, and EMMG-ura-his plates. The YE-ade+guanine plates with closest to 200 colonies were replica plated onto EMMG-ura-ade, EMMG-ade-his, and EMMG-ade-ura-his plates. Plates were incubated at 30 °C for 2 days.

All colonies were counted. The percentage of crossovers associated with gene conversion at *ade6-3083* was then calculated by:

$$\frac{(\text{Total no. colonies that are Ura}^{-}\text{His}^{-} \text{ and Ura}^{+}\text{His}^{+})}{(\text{Total no. of colonies that grow on YE-ade+guanine plate with closest to 200 colonies})}$$

6 assays were performed for each strain, from which mean values were calculated.

2.7 Cell biology techniques

2.7.1 FACS analysis

Cells were fixed with pre-chilled (at -20 °C) 70 % ethanol, pelleted and then washed with 1 ml sterile Milli-Q water. They were then washed with 1 ml 50 mM Na citrate buffer (filter sterilised), resuspended in 0.5 ml of 50 mM Na citrate containing 0.1 mg/ml RNaseA, and incubated at 37 °C for 2 hours. 0.5 ml 50 mM Na citrate containing 4 µg/ml propidium iodide (Sigma P4107) was then added to the samples. Samples were sonicated for 10 minutes using a Decon FS300b sonicator. Samples were then analysed using a FACS Calibur and Cell Quest Pro software (BD Bioscience).

2.7.2 Microscopy

Cells from an exponentially growing culture in YES were harvested and fixed with 70 % ethanol for subsequent microscopy.

The fixed cells (from sections 5.2.5, 6.2.1, 6.2.4, 6.2.5, 6.2.6 and 6.2.7) were stained with DAPI (4',6-diamidino-2-phenylindole), or SYBR® green and Calcofluor white, and analysed using an Eclipse TE2000-U microscope (Nikon), equipped with a X100/1.4 oil PlanApo objective and appropriate filter sets to detect blue, green and red fluorescence. Black and white images were acquired with a Cool-Snap HQ² Turbo1394 camera (Photometrics) controlled by MetaMorph software v. 7.7.3.0 (Molecular Devices, CA). Images were pseudo-coloured and overlaid using Adobe Photoshop CS5 v12 (Adobe Systems, CA).

The fixed cells (from sections 6.2.6 and 6.2.7) were stained with DAPI and analysed using an inverted Olympus IX71 microscope controlled by a DV Elite Core using DeltaVision softWoRx 5.5.0 software using DAPI and GFP (green fluorescent protein) filters (Applied Precision Inc., Issaquah, WA). Images were taken with an EvolveTM 512 EMCCD camera (Photometrics, Tucson, AZ) using an oil-immersed Olympus 100 X UPlanSApo objective with a NA of 1.40. A stack of 16 focal planes at a step-size of 0.3 µm was taken for each image.

2.8 Data analysis

2.8.1 Image processing and analysis

Raw images from the DV Elite Core were subsequently denoised with Priism with the dimensionality set to “3D:xyz” for three iterations (A. Buades 2005). Denoised images were then deconvolved with softWoRx 5.5.0 software with the additive method for 12 cycles (Applied Precision). Foci were scored

manually and separately for each fluorescent channel. Foci were distinguished as being a minimum of 2000 units brighter than background intensity levels with a minimum of a 2 x 2 pixel area.

2.8.2 Statistics

Microsoft Office 2011 Excel 14.0 was used to perform F-tests to establish whether variances between samples were equal or unequal. Two sample t-tests were then used to determine the statistical significance of differences between mean values. Matlab was used to perform two sample Kolmogorov-Smirnov tests to determine the statistical significance of differences between distributions. *P* values below 0.05 were considered significant (5 % significance interval), and *P* values below 0.01 were considered highly significant (1 % significance interval).

The Thompson tau technique was used to eliminate official outliers in the direct repeat recombination assay samples.

Table 2.1 Media for *S. pombe*

Complete media	
<u>YE (1 L)</u> 30 g D-Glucose 5 g Yeast extract (18 g Difco agar) Make up to 1 L with Milli-Q water. Autoclave.	<u>YELA</u> YE supplemented with: 225 mg/l uracil 225 mg/l leucine 225 mg/l lysine 225 mg/l histidine 10 mg/l adenine
<u>YES</u> YE supplemented with: 225 mg/l uracil 225 mg/l leucine 225 mg/l lysine 225 mg/l histidine 225 mg/l adenine	<u>YE-ade+guanine</u> YE supplemented with: 225 mg/l uracil 225 mg/l leucine 225 mg/l lysine 225 mg/l histidine 4 ml 50 mg/ml guanine solution Guanine solution: 50 mg/ml guanine in 1 M NaOH. Filter sterilize before use.
<u>YES+5S</u> YE supplemented with: 450 mg/l uracil 450 mg/l leucine 450 mg/l lysine 450 mg/l histidine 450 mg/l adenine	

Minimal media	
<u>EMMG (1 L)</u> 3 g Potassium hydrogen phthalate 2.2 g Anhydrous Na ₂ HPO ₄ 3.7 g Sodium glutamate 30 g D-glucose 20 ml 50 salts 1 ml 1000 X vitamins 0.1 ml 10000 X minerals (18 g Difco agar) Make up to 1 L with Milli-Q water. Autoclave.	<u>50 X salts</u> 52.5 g MgCl ₂ ·6H ₂ O 50 g KCl 2 g NaSO ₄ 0.735 g CaCl ₂ ·2H ₂ O <u>1000 X vitamins</u> 10 g nicotinic acid 10 g inositol 1 g panthotenic acid 10 mg biotin
<u>10000 X vitamins</u> 10 g citric acid 5 g boric acid 4 g MnSO ₄ 4 g ZnSO ₄ ·7H ₂ O 2 g FeCl ₂ ·6H ₂ O 1 g KI 0.4 g CuSO ₄ ·5H ₂ O	<u>EMMG-his</u> EMMG supplemented with: 225 mg/l uracil 225 mg/l leucine 225 mg/l lysine 225 mg/l arginine 225 mg/l adenine
<u>EMMG-his/LA</u> EMMG supplemented with: 225 mg/l uracil 225 mg/l leucine 225 mg/l lysine 225 mg/l arginine 10 mg/l adenine	<u>EMMG-ade-his</u> EMMG supplemented with: 225 mg/l uracil 225 mg/l leucine 225 mg/l lysine 225 mg/l arginine

<u>EMMG-ura</u> EMMG supplemented with: 225 mg/l leucine 225 mg/l lysine 225 mg/l histidine 225 mg/l arginine 225 mg/l adenine	<u>EMMG-ura-ade</u> EMMG supplemented with: 225 mg/l leucine 225 mg/l lysine 225 mg/l histidine 225 mg/l arginine
<u>EMMG-ura-his</u> EMMG supplemented with: 225 mg/l leucine 225 mg/l lysine 225 mg/l arginine 225 mg/l adenine	<u>EMMG-ade-ura-his</u> EMMG supplemented with: 225 mg/l leucine 225 mg/l lysine 225 mg/l arginine
<u>EMMG+LA</u> EMMG supplemented with: 225 mg/l uracil 225 mg/l leucine 225 mg/l lysine 225 mg/l histidine 225 mg/l arginine 10 mg/l adenine	

FOA (1 L)

6.7 g yeast nitrogen base

30 g glucose

750 µl 10 M NaOH

3.7 g Sodium glutamate

Make up to 500 ml with Milli-Q water. Autoclave.

225 mg/l uracil

225 mg/l leucine

225 mg/l lysine

225 mg/l histidine

225 mg/l arginine

225 mg/l adenine

18 g Difco agar

Make up to 500 ml with Milli-Q water. Autoclave.

After autoclaving both, 1 g of FOA is dissolved in the yeast nitrogen base/glucose solution, and afterwards mixed with the agar.

MEA (1 L)

30 g malt extract

20 g agar

pH > 5.5

Dissolve ingredients in Milli-Q water. Autoclave.

Table 2.2 List of oligonucleotides used in this study

Name	Oligonucleotide Sequence (5'→3')
oMW285	AATGTACGGGCGACAGTC
oMW717	AGAAGAAAGCGATCGATGAG
oMW848	GGACGAGGCAAGCTAAAC
oMW1004	AGAAAGGCTGCTGGAATGTC
oMW1248	TATTACATATGGAGCAGGAAAGCGAAC
oMW1249	TTGGATCCTAACTAAAGTCAAGGGCTAG
oMW1348	ATTTAGTCGACAGTACAAAAGGATAACC
oMW1427	ATAGTCGACGAACGCTGATCCTGGATTTC
oMW1431	ATAGTCGACTCGTTCAATGCTGCCGGCTG
oMW1433	TAGAGCTCGCTGATACTAAATGGAGACG
oMW1443	GGCGCGCCTTATGTTGTACTGTTTTCTTTC
oMW1444	ATGGCGCGCCCTAACTAAAGTCAAGGGCTAG
oMW1507	TATGAAGAGAAAAAGAACGCAATCATGTCATCTTCTGAA
oMW1508	TTCAGAAGATGACATGATTGCGTTCTTTTTCTCTTCATA
oMW1509	GAAAACGGTTCAACCACAGGTTGTGCTTTTATGTTGTCGA A
oMW1510	TTCGACAACATAAAAGCACAACTGTGGTTGAACCGTTTT C
oMW1517	GAAAATGGCATCGCAGCTGCACTAGCCCTTGACTTTAGT
oMW1518	ACTAAAGTCAAGGGCTAGTGCAGCTGCGATGCCATTTTC
oMW1519	GCAGCTCAACTAGCCCTTGCATTTAGTTAGGGCGCGCCA
oMW1520	TGGCGCGCCCTAACTAAATGCAAGGGCTAGTTGAGCTGC
oMW1591	GGCATCGCAGCTGCACTAGCCCTTGCATTTAGTTAGGGC GCG
oMW1592	CGCGCCCTAACTAAATGCAAGGGCTAGTGCAGCTGCGAT GCC

oMW1636	TTACAAGCAAGTCGTACAGTC
oMW1637	TTAGTCAACAACTGCTCGCTC
oMW1638	AGAGGATAAGTCTAACTTAC
oMW1639	TGAAACCAACATCTATGAACG

Table 2.3 List of plasmids used in this study

Name	Vector	<i>E. coli</i> Marker	<i>S. pombe</i> Marker	Description	Purpose
pJN1	pFA6	Ampicillin	<i>kanMX6</i>	pFA6- <i>mhf1</i> ^{5'UTR-<i>mhf1</i>^{D75V}-<i>kan</i>^R-<i>mhf1</i> 3'UTR}	Targeting construct for generating point mutation in <i>S. pombe</i> .
pJN2	pFA6	Ampicillin	<i>kanMX6</i>	pFA6- <i>mhf2</i> ^{5'UTR-<i>mhf2</i>^{S59A}-<i>kan</i>^R-<i>mhf2</i> 3'UTR}	Targeting construct for generating point mutation in <i>S. pombe</i> .
pJN3	pFA6	Ampicillin	<i>kanMX6</i>	pFA6- <i>mhf2</i> ^{5'UTR-<i>mhf2</i>^{Q83A}-<i>kan</i>^R-<i>mhf2</i> 3'UTR}	Targeting construct for generating point mutation in <i>S. pombe</i> .
pJN4	pFA6	Ampicillin	<i>kanMX6</i>	pFA6- <i>mhf2</i> ^{5'UTR-<i>mhf2</i>^{D87A}-<i>kan</i>^R-<i>mhf2</i> 3'UTR}	Targeting construct for generating point mutation in <i>S. pombe</i> .
pJN5	pFA6	Ampicillin	<i>kanMX6</i>	pFA6- <i>mhf2</i> ^{5'UTR-<i>mhf2</i>^{S59A, Q83A, D87A}-<i>kan</i>^R-<i>mhf2</i> 3'UTR}	Targeting construct for generating point mutation in <i>S. pombe</i> .
pFOX2	pUC8	Ampicillin	<i>his3+</i>	pUC8- <i>his3</i> ⁺ - <i>ade6</i> -L469	For generating <i>ade6</i> direct repeat strains.
pAN1	pREP42	Ampicillin	<i>ura4+</i>	Contains a copy of <i>ade6</i> (with a interstitial deletion of a 153 bp <i>NcoI</i> fragment) cloned between the <i>PstI</i> and <i>SacI</i> sites of pREP42 to replace <i>nmt</i> promoters.	Plasmid gap repair assay.
pMW700	pUC8	Ampicillin	<i>his3+</i>	pUC8- <i>his3</i> ⁺ - <i>RTS1</i> - <i>IO-ade6</i> -L469	For generating <i>ade6</i> direct repeat strains.
pMW701	pUC8	Ampicillin	<i>his3+</i>	pUC8- <i>his3</i> ⁺ - <i>RTS1</i> - <i>AO-ade6</i> -L469	For generating <i>ade6</i> direct repeat strains.

pMW884	pET14b	Ampicillin	NIL	<i>mhf2</i> cDNA amplified from a <i>S. pombe</i> cDNA library using primers oMW1248 and oMW1249 and cloned as NdeI-BamHI fragment into pET14b	For generating pMW891 (D87A)
pMW889	pT7-7	Ampicillin	NIL	Predicted <i>mhf1</i> cDNA artificially synthesised (DNA 2.0) and subcloned as a NdeI-BamHI fragment into pT7-7	For generating pMW891 (D87A)
pMW891 (D87A)	pET14b	Ampicillin	NIL	BglII-Sall T7 promoter- <i>mhf1</i> cDNA fragment from pMW889 sub-cloned into BglII-Sall sites in pMW884 to make a co-expression plasmid for Mhf1 and His-Mhf2 in which both genes are expressed from separate T7 promoters. Site directed mutagenesis was subsequently used to generate the <i>mhf2</i> ^{D87A} point mutation.	Transformation of BL21RIL for Mhf1-HisMhf2 ^{D87A} purification
pMW910	pFA6	Ampicillin	<i>kanMX6</i>	pFA6- <i>mhf1</i> 5'UTR- <i>mhf1</i> -3HA- <i>kan^R</i> - <i>mhf1</i> 3'UTR	Used as a template for random mutagenesis of <i>mhf1</i> with oMW1427 and oMW1443
pMW912	pFA6	Ampicillin	<i>kanMX6</i>	pFA6- <i>mhf2</i> 5'UTR- <i>mhf2</i> -3HA- <i>kan^R</i> - <i>mhf2</i> 3'UTR	Used as a template for random mutagenesis of <i>mhf2</i> with oMW1433 and oMW1444
pMW914	pFA6	Ampicillin	<i>kanMX6</i>	pFA6- <i>mhf1</i> 5'UTR- <i>mhf1</i> - <i>kan^R</i> - <i>mhf1</i> 3'UTR	Site-directed mutagenesis of <i>mhf1</i> with oMW1509 and oMW1510 to generate pJN1.
pMW915	pFA6	Ampicillin	<i>kanMX6</i>	pFA6- <i>mhf2</i> 5'UTR- <i>mhf2</i> - <i>kan^R</i> - <i>mhf2</i> 3'UTR	Site-directed mutagenesis of <i>mhf2</i> to generate pJN2, pJN3 and pJN4 (refer to Table 2.4 for details).

Table 2.4 Plasmid construction

Name	Construction	Source
pJN1	Site directed mutagenesis of <i>mhf1</i> on pMW914 to <i>mhf1</i> ^{D75V} , using oMW1509 and oMW1510	This study
pJN2	Site directed mutagenesis of <i>mhf2</i> on pMW915 to <i>mhf2</i> ^{S59A} , using oMW1507 and oMW1508.	This study
pJN3	Site directed mutagenesis of <i>mhf2</i> on pMW915 to <i>mhf2</i> ^{Q83A} , using oMW1517 and oMW1518.	This study
pJN4	Site directed mutagenesis of <i>mhf2</i> on pMW915 to <i>mhf2</i> ^{D87A} , using oMW1519 and oMW1520.	This study
pJN5	Site directed mutagenesis of <i>mhf2</i> ^{Q83A} on pJN3 to <i>mhf2</i> ^{Q83A, D87A} , using oMW1591 and oMW1592. This is followed by a second site directed mutagenesis of the plasmid containing <i>mhf2</i> ^{Q83A, D87A} to <i>mhf2</i> ^{S59A, Q83A, D87A} , using oMW1507 and oMW1508.	This study

Table 2.5 Buffers and solutions for biochemistry techniques

Stacking buffer (pH 6.8) (per 200 ml)	12 g Tris 96 ml 1 M HCl 80 ml sterile Milli-Q water
Resolving buffer (pH 8.8) (per 100 ml)	36.3 g Tris Add sterile Milli-Q water up to 100 ml
15 % Resolving gel (for 6 gels)	15 ml Ultra pure protogel 3.75 ml Resolving buffer 0.3 ml 10 % SDS 225 µl 10 % AMPS 25 µl TEMED 9.45 ml sterile Milli-Q water
Stacking gel (for 6 gels)	1.25 ml Ultra pure protogel 2.5 ml Stacking buffer 0.1 ml 10 % SDS 75 µl 10 % AMPS 7.5 µl TEMED 5.65 ml sterile Milli-Q water
SDS-PAGE Loading dye (~8 ml)	1 ml Stacking buffer 0.8 ml 100 % Glycerol 1.6 ml 10 % SDS 4 ml 1 % Bromophenol Blue 0.5 ml 0.2 M DTT
10 X SDS-PAGE Running buffer (1 L)	30.3 g Tris-HCl 144 g Glycine 10 g SDS Add sterile Milli-Q water up to 1 L
10 X Phosphate Buffered Saline pH 7.4 (1 L)	80 g NaCl 2 g KCl 14.4 g Na ₂ HPO ₄ 2.4 g KH ₂ PO ₄ Add sterile Milli-Q water up to 1 L

10 X Native buffer (1 L)	30 g Tris-HCl 144 g Glycine Add sterile Milli-Q water up to 1 L
Blot buffer (1 L)	100 ml 10 X Native buffer 200 ml Methanol 2 ml 10 % SDS Add sterile Milli-Q water up to 1 L
Buffer A (1 L)	50 ml 1 M Tris-HCl pH 8 2 ml 0.5 M DTT 5 ml 0.2 M EDTA 100 ml Glycerol Add sterile Milli-Q water up to 1 L Filter sterilize and de-gas
Buffer H (100 ml)	4.7 ml 1 M K ₂ HPO ₄ 0.3 ml 1 M KH ₂ PO ₄ 10 ml Glycerol 100 µl 10 M β-mercaptoethanol Add sterile Milli-Q water up to 1 L
4 X Buffer I (50 ml)	5 ml 1 M Tris-HCl pH 7.5 40 µl 0.5 M EDTA 200 µl 10 % NP40 0.0616 g DTT 25 ml 80 % Glycerol Add sterile Milli-Q water up to 50 ml
50 X Low ionic strength buffer (1 L)	335 ml 1 M Tris-HCl pH 8.0 200 ml 0.5 M EDTA pH 8.0 13.5 g Sodium acetate

Chapter 3: Investigating whether fork termination is a recombinogenic process

3.1 Introduction

To investigate what triggers recombination at a site-specific RFB, I employed the well-characterised site-specific *RTS1* barrier (Dalgaard and Klar 2001). *RTS1* is a very efficient programmed polar RFB, which almost completely blocks replication forks when ectopically positioned on chromosome III (Chr III) (Ahn et al. 2005; Lorenz et al. 2009). Intra-chromosomal recombination between direct repeats is a well-established technique to quantify recombination events in replicating cells (Klein 1995). Our laboratory has previously shown that replication fork blockage at the *RTS1* RFB induces recombination between a direct repeat of *ade6⁻* heteroalleles (Figure 3.1A). The *RTS1*-based direct repeat recombination reporter is made up of two *ade6⁻* heteroalleles with an intervening *his3⁺* gene and *RTS1* sequence. An Ade⁺ cell can only arise if recombination between the two different loss-of-function *ade6⁻* alleles (*ade6-L469* and *ade6-M375*) restores the wild-type *ade6⁺* gene. Hence, the frequency at which an Ade⁺ cell is generated reflects the prevalence of recombination at this site. Ade⁺ recombinants can be divided into two classes, conversion-types (Ade⁺His⁺) and deletion-types (Ade⁺His⁻) (Figure 3.1A). Since only Ade⁺ recombinants are scored, this assay only measures ectopic recombination that restores a functional *ade6⁺* allele, and not other forms of recombination, which would be genetically silent. Nevertheless, it provides a readout of a subclass of recombination events occurring at the site and has been used by our laboratory to quantify levels of recombination (Ahn et al. 2005).

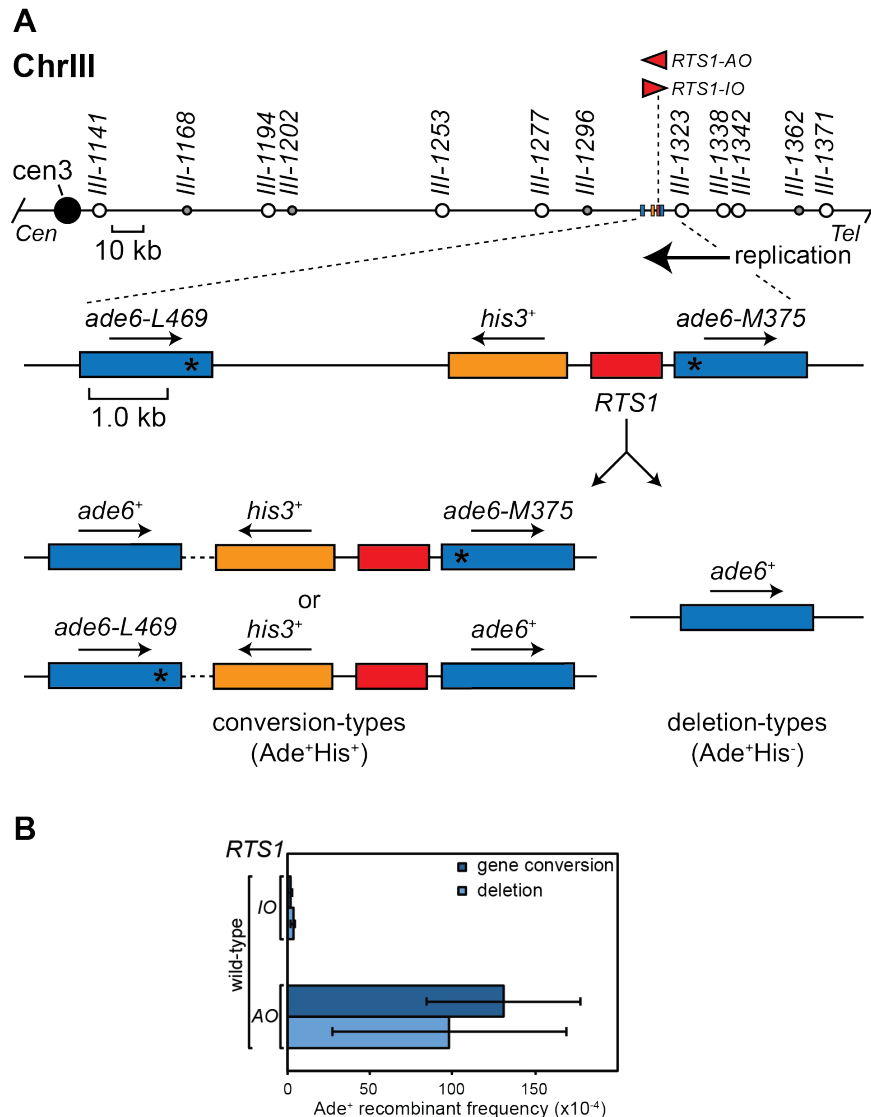


Figure 3.1 Direct repeat recombination at the endogenous *ade6* locus on chromosome III

(A) Schematic showing the location of the *ade6*⁻ intrachromosomal recombination reporter along with an intervening *his3*⁺ gene and *RTS1* polar RFB in relation to nearby replication origins at the endogenous *ade6* locus on chromosome III (ChrIII). Confirmed and likely origins are represented by white circles; dubious origins are represented by grey circles. Asterisks indicate the position of point mutations in *ade6-L469* and *ade6-M375*. Replication across the direct repeat recombination reporter occurs in the centromere-proximal direction. Thus, depending on the orientation of *RTS1*, it will either block (*RTS1-AO*) or not block (*RTS1-IO*) forks travelling in the centromere-proximal direction. Possible *ade6*⁺ recombinant products that may arise spontaneously or be induced by the perturbed replication fork at *RTS1-AO* include conversion- and deletion-types. (B) Bar chart showing the frequency of Ade⁺ recombinants in Chromosome III wild-type *RTS1-IO* and *RTS1-AO* strains after 5 to 6 days at 30 °C. Error bars represent $\pm$ one standard deviation. In order of presentation, the strains are MCW4712 and MCW4713.

Table 3.1 Direct repeat recombination frequencies at the endogenous *ade6* locus on chromosome III

Direct repeat recombination data presented in the same order as Figure 3.1B.

Genotype	<i>RTS1</i> orientation	Position of direct repeat	Number of colonies analysed	Total Ade ⁺ recombinant frequency ($\times 10^{-4}$) ^a	% Ade ⁺ His ⁺ recombinants ^a	Ade ⁺ His ⁺ recombinant frequency ($\times 10^{-4}$) ^a	Ade ⁺ His ⁻ recombinant frequency ($\times 10^{-4}$) ^a
wild-type	IO	Chr III	17	4.97 (± 2.03)	31.93 (± 12.84)	1.70 (± 1.46)	3.27 (± 1.28)
wild-type	AO	Chr III	24	229.01 (± 85.60)	59.82 (± 15.01)	130.91 (± 46.85)	98.10 (± 70.83)

^a The values in parentheses are the standard deviations about the mean.

At the endogenous *ade6* locus on chromosome III where the direct repeat recombination reporter is positioned, replication is essentially unidirectional in the telomere (*Tel*) to centromere (*Cen*) direction due to the relative positioning of replication origins flanking it (Ahn et al. 2005) (Figure 3.1A). Accordingly, one orientation of *RTS1*, known as the active orientation (*RTS1-AO*), will block most replication forks and induce high levels of recombination. On the other hand, the other orientation of *RTS1* will not block most replication forks and since the level of recombination it induces is as low as the spontaneous level of recombination, it is referred to as the inactive orientation (*RTS1-IO*).

At this locus, there is a ~80-fold stimulation of gene conversions and a ~30-fold stimulation of deletions in the *RTS1-AO* compared to the *RTS1-IO* (Ahn et al. 2005) (Figure 3.1B and Table 3.1). This induction of recombination between the direct repeats is dependent on Rad51 and Rad52 (Ahn et al. 2005). However, it is unclear whether this recombination occurs solely as a consequence of trying to restart the single blocked fork (Lambert et al. 2010), and/or as a consequence of replication fork merging at the stalled fork. Recent work from our laboratory showed that an impairment of fork termination at *RTS1*, by depletion of the Pif1 family DNA helicase Pfh1, strongly induces direct repeat recombination (Steinacher et al. 2012). Conceivably, even in the presence of Pfh1, fork termination is a process that is commonly assisted by HR proteins, causing fork termination sites to be recombination hotspots. If that were the case, replication fork stalling might give rise to potentially deleterious recombination by altering sites of fork termination. The aim of the work described in this study was to determine to

what extent replication fork termination as opposed to replication restart gives rise to recombination at the *RTS1* RFB.

3.2 Results

3.2.1 Setting up a system to study replication fork termination and replication fork restart

To establish a system in which the influence of replication fork merging at *RTS1* could be separated from that of replication restart, I decided to position the *RTS1* direct repeat recombination reporter at a site with a cluster of replication origins in the subtelomeric region on the left arm of chromosome I (Figure 3.2A and Table S3.1). In this region, origins *I-20* to *I-79* have a total origin efficiency percentage of 190.79 %, while origins *I-85* to *I-130* have a total origin efficiency percentage of 188.04 % (Figure 3.2A and Table S3.1). Since there is a high chance of forks firing on both sides of *RTS1*, I reasoned that at such a site, the high density of replication origins might result in shorter distances and therefore times between replication fork initiation and termination. Based on live cell imaging analysis of recombination protein recruitment at *RTS1*, we know that it takes approximately 10 minutes for Rad52 to load at the barrier following replication fork blockage (Nguyen et al. 2015). Therefore, if origins nearby *RTS1* fired from both sides, there would be insufficient time for replication fork restart to occur, and any induced recombination would therefore likely stem from replication fork termination.

Assuming similar origin firing times and a replication fork velocity of 3 kb/min, the time between replication fork blockage and convergence at *RTS1* at the

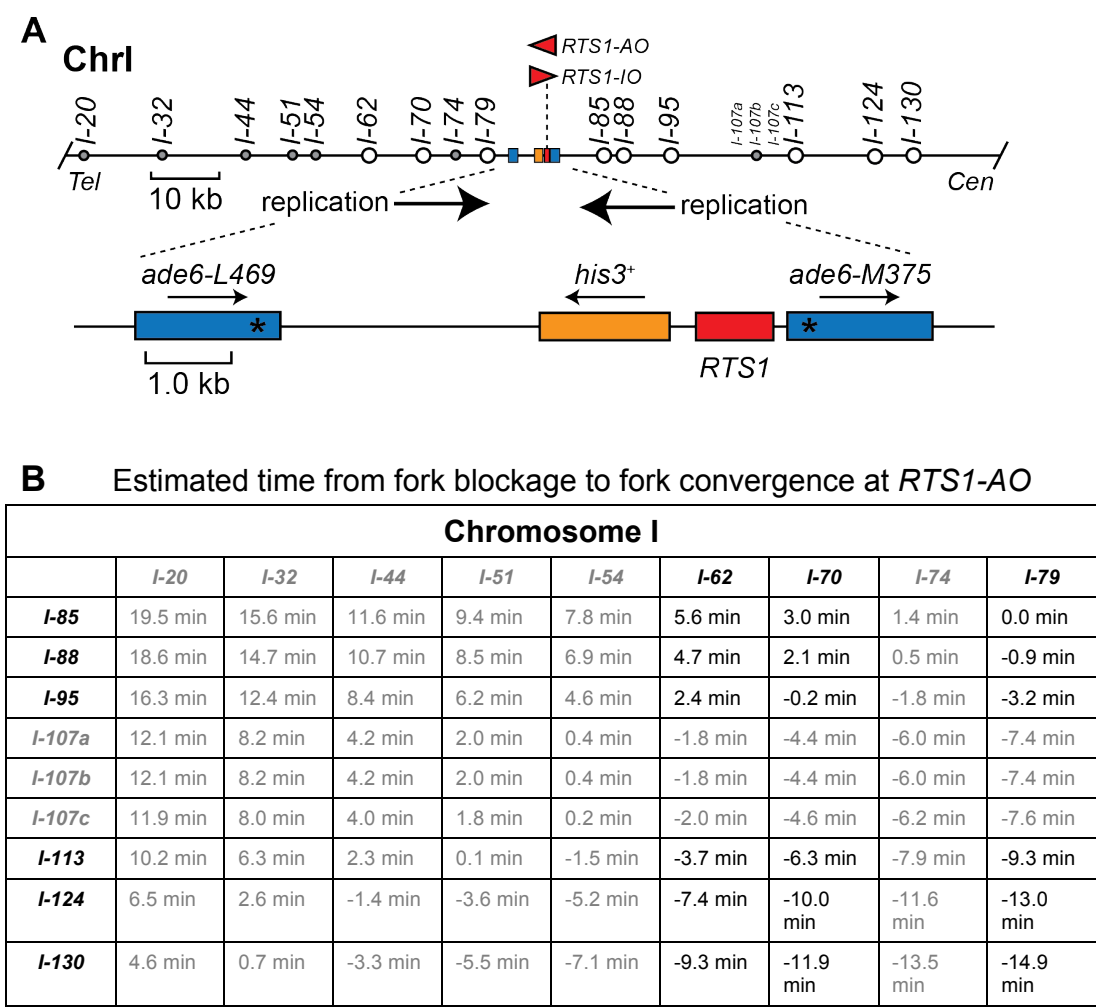


Figure 3.2 Experimental system for studying replication fork termination and replication fork restart at a site specific replication fork barrier positioned in the midst of a cluster of replication origins

(A) Schematic showing the location of the direct repeat recombination reporter in relation to nearby replication origins on chromosome I (ChrI). Confirmed and likely are represented by white circles; dubious origins are represented by grey circles. Origin details are described in Table S3.1. On chromosome I, replication across the direct repeat recombination reporter occurs from both directions. (B) Times are calculated from the distance between the midpoint of each origin (Siow et al. 2012) and a fork velocity of 3 kb/min, and assumes that each origin fires at the same time during S-phase. Timings involving only confirmed and/or likely origins are in black; timings involving dubious origins are in grey.

chromosome I site can be estimated (Figure 3.2B). As origin firing is stochastic, these times vary from cell-to-cell depending on which pair of origins fire. If any one of the origins between *I-51* to *I-79*, and any one of the origins between *I-85* to *I-113* fired at the same time, then in most cells, fork convergence will occur within 10 minutes of the first fork arriving at the barrier, which would be insufficient time for RDR to be initiated and give rise to an Ade⁺ recombinant. Origins between *I-51* to *I-79* have a very high total origin efficiency of 94.57 %, while origins between *I-85* to *I-113* also have a very high total origin efficiency of 132.27 %, indicating a very high probability of one of these origins on both sides of *RTS1* firing (Figure 3.2A and Table S3.1). On the other hand, in the rare case that origins from this region fire on only one side of *RTS1*, this could lead to a blocked fork with sufficient time for RDR to be initiated and give rise to an Ade⁺ recombinant.

As alluded to earlier, *RTS1* is a polar RFB and will block replication in an orientation-dependent manner. Although there is no predicted bias in replication proceeding across the recombination reporter on chromosome I, to distinguish the two orientations of *RTS1*, the orientation of *RTS1* that blocks forks moving towards the telomere will be referred to as the active orientation (*RTS1-AO*), whereas the orientation of *RTS1* that blocks forks moving towards the centromere will be referred to as the inactive orientation (*RTS1-IO*).

3.2.2 The direction of replication at the subtelomeric site on chromosome I is not strongly polarised within a population of cells

To determine the direction of replication across the direct repeat recombination reporter on chromosome I, I digested the genomic DNA from asynchronous cultures of the *RTS1-IO* and *RTS1-AO* strains with *Eco*NI, which cleaves the recombination reporter within the *his3⁺* and *ade6-M375* genes, resulting in a ~4.0 kb fragment (Figure 3.3A). Native two-dimensional (2D) gel electrophoresis analysis of replication intermediates in the *RTS1-IO* strain showed a Y-arc of replication intermediates and a pause (P) site (Figure 3.3B). The same pause site was detected in DNA from the *RTS1-AO* strain (Figure 3.3B). In addition to the Y-shaped replication intermediates, a spike of double Y-shaped molecules and a termination (T) signal were also detected on the 2D gels (Figure 3.3B). Molecules in this spike represent two opposing replication forks in the final stages of merging (Steinacher et al. 2012).

When the hybridisation signals of the pause sites were each quantified relative to that of the replicating DNA [Pause/ (Y-arc + Termination)], both *RTS1-IO* and *RTS1-AO* had similar intensities of 60.99 % and 68.30 % respectively (Figure 3.3C and Table 3.2). These data confirm that at this locus the direction of replication is not strongly polarised (i.e. replication can occur from either direction with a similar probability). Quantification of the proportion of forks that terminate relative to the amount of forks that pause [Termination/ (Termination + Pause)], shows a ~2-fold difference in *RTS1-IO* compared to *RTS1-AO* (Figure 3.3D and Table 3.2). This difference might be explained by

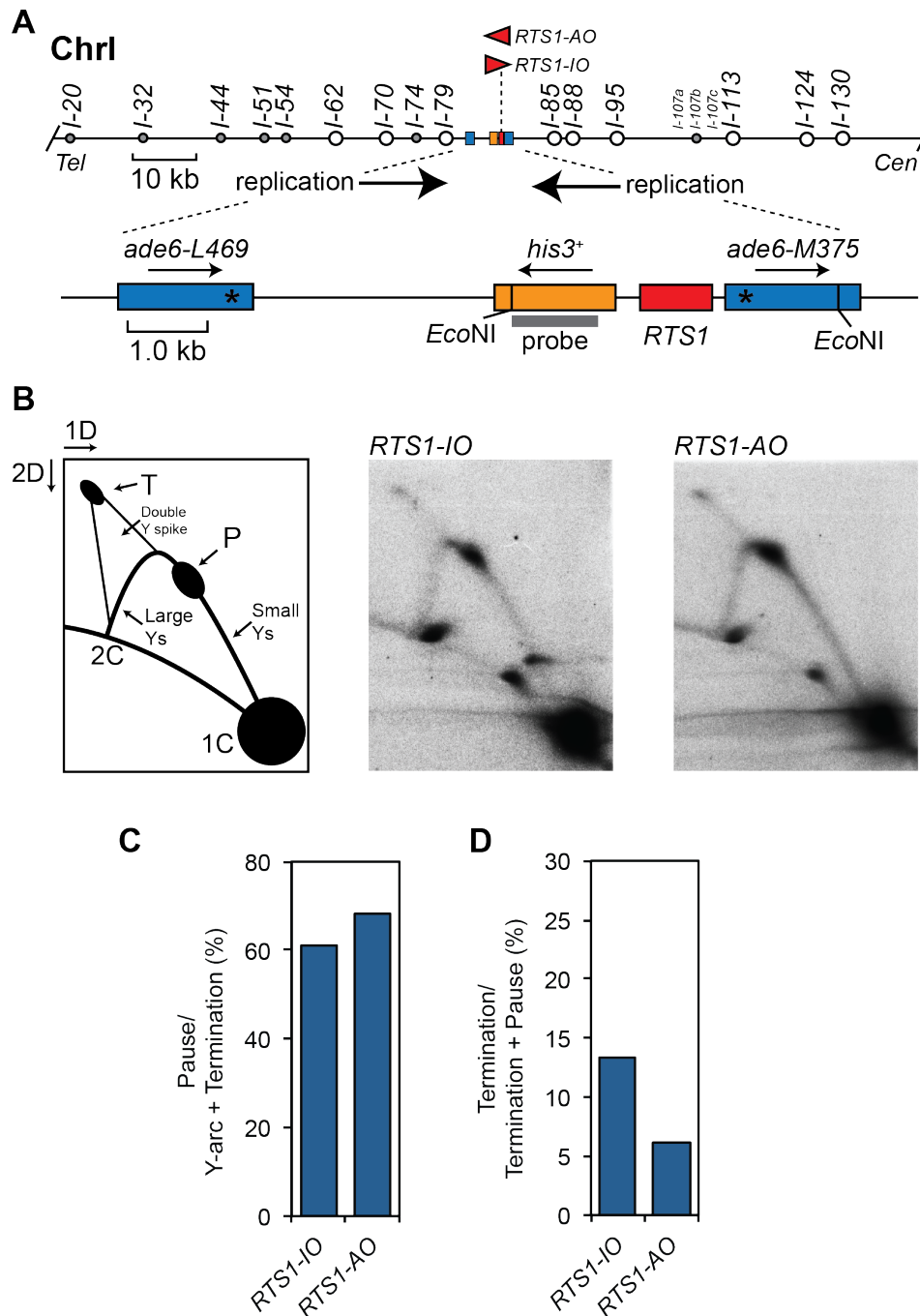


Figure 3.3 Replication across the direct repeat recombination reporter on Chromosome I occurs from both the centromere-proximal and the telomere-proximal directions

(A) Schematic showing the direct repeat recombination reporter on Chromosome I and the probe used for 2D gel analysis. (B) 2D gel analysis of replication intermediates in the *EcoNI* fragment shown in (A). The DNA is extracted from strains JN34 (*RTS1-IO*) and JN29 (*RTS1-AO*). (C) Amount of fork pausing at pause site (P) as a percentage of the total Y-arc and termination. (D) Amount of terminating replication forks (T) as a percentage of the total fork pausing and termination (P+T).

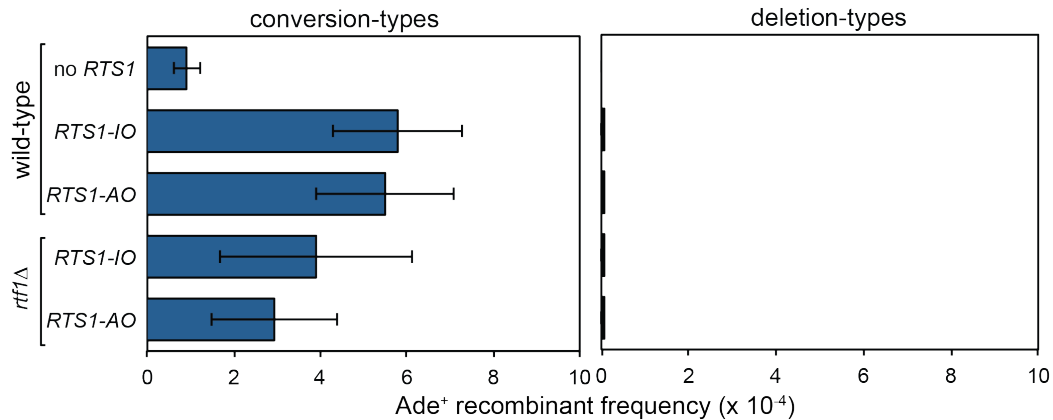
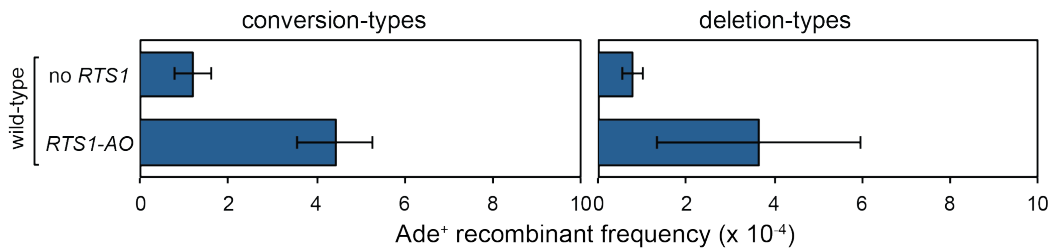
Table 3.2 2D gel analysis of replication intermediates in Chromosomes I and II

Genotype	<i>RTS1</i> orientation	Position of direct repeat	Pause/ Y-arc + Termination (%)	Termination/ Termination + Pause (%)
wild-type	<i>IO</i>	Chr I	60.99	13.40
wild-type	<i>AO</i>	Chr I	68.30	6.07
wild-type	<i>IO</i>	Chr II	53.17	7.59
wild-type	<i>AO</i>	Chr II	70.58	12.40
<i>pfh1-mt</i>	<i>AO</i>	Chr I	52.10	24.91

more of the blocked forks at *RTS1-AO* being able to restart and progress beyond the barrier. However, as my analysis is based on a single experiment it is equally possible that the difference is within the bounds of normal experimental variation. So, although I can conclude that replication termination does occur at both *RTS1-IO* and *RTS1-AO*, it remains uncertain whether restart is occurring.

3.2.3 *RTS1* at the subtelomeric site on chromosome I induces relatively little recombination

To determine whether replication fork blockage at *RTS1* positioned at the subtelomeric site on chromosome I induces recombination to the same extent as at the *ade6* locus on chromosome III, I assessed the frequency of direct repeat recombination at this site in the presence and absence of the RFB. In the absence of *RTS1*, the spontaneous frequency of Ade⁺ recombinants is approximately 0.92 in every 10000 viable cells, and surprisingly they are all conversion-types (Figure 3.4A and Table 3.3). As shown previously, replication fork blockage at *RTS1* at the endogenous *ade6* locus on chromosome III results in a ~80-fold stimulation of gene conversions and a ~30-fold stimulation of deletions (Figure 3.1B and Table 3.1). This sharp increase in recombination is however not seen at the subtelomeric chromosome I site. Instead, on chromosome I, the frequency of recombination increases only modestly (~6-fold) when *RTS1* is present. It is also approximately the same regardless of *RTS1* orientation, which is consistent with the observation that both orientations of the barrier cause similar amounts of replication fork blockage. Like in the absence of *RTS1*, virtually all

A**Chromosome I (Frequencies after 5 to 6 days at 30 °C)****B****Chromosome I (Frequencies after 11 to 12 days at 30 °C)****Figure 3.4 Direct repeat recombination on chromosome I**

(A) Bar chart showing the frequency of Ade⁺ recombinants in Chromosome I wild-type and *rtf1*Δ, no *RTS1*, *RTS1-IO* and *RTS1-AO* strains after 5 to 6 days at 30 °C. (B) Bar chart showing the frequency of Ade⁺ recombinants in Chromosome I wild-type no *RTS1* and *RTS1-AO* strains after 11 to 12 days at 30 °C. Error bars represent ± one standard deviation. In order of presentation, the strains are JN27, JN34, JN29, JN30, JN8, JN27 and JN29.

Table 3.3 Direct repeat recombination frequencies on chromosomes I and II

Direct repeat recombination data presented in the same order as Figure 3.4 and Figure 3.7.

Frequency of Ade ⁺ recombinants after 5 to 6 days at 30 °C											
Genotype	RTS1 orientation	Position of direct repeat	Number of colonies analysed	Total Ade ⁺ recombinant frequency (x10 ⁻⁴)		% Ade ⁺ His ⁺ recombinants		Ade ⁺ His ⁺ recombinant frequency (x10 ⁻⁴)		Ade ⁺ His ⁺ recombinant frequency (x10 ⁻⁴)	
				Mean ^a	P-value ^b	Mean ^a	P-value ^b	Mean ^a	P-value ^b	Mean ^a	P-value ^b
wild-type	no RTS1	Chr I	18	0.92 (±0.30)	-	100.00 (±0.00)	-	0.92 (±0.30)	-	0.0000 (±0.0000)	-
wild-type	IO	Chr I	17	5.80 (±1.48)	< 0.001	99.80 (±0.47)	0.790	5.79 (±1.49)	<0.001	0.0094 (±0.0204)	0.077
wild-type	AO	Chr I	16	5.49 (±1.59)	< 0.001	99.97 (±0.14)	0.296	5.49 (±1.60)	<0.001	0.0014 (±0.0054)	0.333
<i>rtf1Δ</i>	IO	Chr I	18	3.92 (±2.21)	< 0.001	99.73 (±0.80)	0.161	3.91 (±2.21)	<0.001	0.0088 (±0.0256)	0.161
<i>rtf1Δ</i>	AO	Chr I	12	2.96 (±1.45)	< 0.001	99.86 (±0.50)	0.227	2.96 (±1.45)	<0.001	0.0045 (±0.0156)	0.339
Frequency of Ade ⁺ recombinants after 11 to 12 days at 30 °C											
Genotype	RTS1 orientation	Position of direct repeat	Number of colonies analysed	Total Ade ⁺ recombinant frequency (x10 ⁻⁴)		% Ade ⁺ His ⁺ recombinants		Ade ⁺ His ⁺ recombinant frequency (x10 ⁻⁴)		Ade ⁺ His ⁺ recombinant frequency (x10 ⁻⁴)	
				Mean ^a	P-value ^a	Mean ^a	P-value ^b	Mean ^a	P-value ^b	Mean ^a	P-value ^b
wild-type	no RTS1	Chr I	9	1.98 (±0.28)	-	58.85 (±16.26)	-	1.19 (±0.42)	-	0.79 (±0.23)	-
wild-type	AO	Chr I	7	8.07 (±2.77)	0.001	58.25 (±14.59)	0.940	4.42 (±0.86)	<0.001	3.65 (±2.33)	0.017
Frequency of Ade ⁺ recombinants after 5 to 6 days at 30 °C											
Genotype	RTS1 orientation	Position of direct repeat	Number of colonies analysed	Total Ade ⁺ recombinant frequency (x10 ⁻⁴)		% Ade ⁺ His ⁺ recombinants		Ade ⁺ His ⁺ recombinant frequency (x10 ⁻⁴)		Ade ⁺ His ⁺ recombinant frequency (x10 ⁻⁴)	
				Mean ^a	P-value ^a	Mean ^a	P-value ^b	Mean ^a	P-value ^b	Mean ^a	P-value ^b
wild-type	no RTS1	Chr II	15	3.71 (±1.46)	-	36.76 (±12.79)	-	1.35 (±0.77)	-	2.36 (±1.31)	-
wild-type	IO	Chr II	15	5.50 (±0.91)	< 0.001	33.66 (±10.92)	0.724	1.85 (±0.64)	<0.001	3.65 (±0.91)	<0.001
wild-type	AO	Chr II	13	133.42 (±47.90)	< 0.001	24.60 (±8.90)	0.010	29.99 (±8.22)	<0.001	103.43 (±47.39)	<0.001
<i>rtf1Δ</i>	IO	Chr II	13	9.51 (±4.38)	< 0.001	56.28 (±20.18)	0.003	5.25 (±2.94)	<0.001	4.26 (±3.47)	0.042
<i>rtf1Δ</i>	AO	Chr II	16	6.09 (±2.67)	0.003	28.74 (±16.03)	0.204	1.79 (±1.76)	0.227	4.29 (±2.48)	0.003

^a The values in parentheses are the standard deviations about the mean.

^b P-values are derived from independent-sample t-tests comparing the mean values of the respective mutant to the corresponding mean value found in the control strain.

of the Ade⁺ recombinants are conversion-types. As an additional control I tested whether removal of the barrier protein Rtf1 reduced the level of recombinants back to spontaneous levels (Figure 3.4A and Table 3.3). Surprisingly the frequency of recombinants, although reduced slightly (between ~1.4 to 1.9-fold), remained significantly higher than in the strain with no *RTS1*. As *rtf1*Δ strains do not exhibit a general hyper-recombination phenotype (Figure 3.4A and Table 3.3), *RTS1* may retain some residual barrier activity in the absence of Rtf1, which could account for the increased frequency of recombination. However, this has not been observed at the *ade6* locus on chromosome III (unpublished data), and therefore it is conceivable that the presence of *RTS1* increases recombinant frequency simply by acting as a spacer that increases the probability of the two *ade6*⁺ repeats being juxtaposed in a way to favour recombination between them.

The absence of deletion-type recombinants was unexpected. One possible explanation for this was that the deletion-type recombinants (which would express *ade6*⁺ from the *ade6-L469* promoter) were not able to express *ade6*⁺ as well as the conversion-type recombinants (which would express *ade6*⁺ from the *ade6-M375* promoter). Conceivably, the deletion-type recombinants do not express *ade6*⁺ well because of an epigenetic effect where the surrounding DNA is non-permissive for the activity of the deletion-type *ade6*⁺ promoter. On the other hand, the conversion-type *ade6*⁺ promoter is well insulated from the epigenetic effect because it lies within a region of the recombination reporter. To investigate this, I extended incubation at 30 °C from 5 to 6 days, to 11 to 12 days so that any slower growing deletion-type Ade⁺ recombinants would have time to appear. Indeed, with an extended

period of growth, Ade⁺His⁻ recombinants appeared, which I confirmed as being genuine deletion-types by DNA sequencing (data not shown). With this revised protocol I was able to obtain an accurate value for the total Ade⁺ recombinant frequency (Figure 3.4B and Table 3.3). In the absence of *RTS1*, the frequency of conversion-type Ade⁺ recombinants is approximately 1.19 in every 10000 viable cells while that of deletion-types is approximately 0.79 in every 10000 viable cells. Replication fork blockage by *RTS1-AO* results in a modest increase in both conversion-type and deletion-type recombinant frequencies (~4-fold and ~5-fold respectively) (Figure 3.4B and Table 3.3). Unfortunately due to time constraints, I did not assay the *RTS1-IO* strain. However, since a similar amount of paused forks (Figure 3.3B, Figure 3.3C and Table 3.2) and Ade⁺ recombinant frequencies after 5 to 6 days at 30 °C (Figure 3.4A and Table 3.3) were observed in both *RTS1-IO* and *RTS1-AO* orientations, I suspect that the *RTS1-IO* recombinant frequency will be similar to that of the *RTS1-AO* strain after 11 to 12 days at 30 °C. Assuming that at least one of the origins between *I-51* to *I-79*, and one between *I-85* to *I-113*, fire at approximately the same time, then my data are consistent with the idea that replication fork blockage at *RTS1* primarily induces recombination if there is sufficient time for RDR to occur prior to fork convergence.

3.2.4 Can recombination be strongly triggered at subtelomeric sites?

As discussed above, the lack of induction of recombination at the subtelomeric site on chromosome I following replication fork blockage by *RTS1* can be explained by a lack of time for RDR to occur before fork convergence (Figure 3.1B, Figure 3.4, Table 3.1 and Table 3.3). However,

other possibilities are imaginable, for example RFB-induced recombination might be repressed at subtelomeric regions due to exclusion of recombinases from the site or some peculiar effect of the subtelomere (Bisht et al. 2008). To address this question, I inserted the *RTS1* direct repeat recombination reporter at another site with a cluster of replication origins in the subtelomeric region on chromosome II (Figure 3.5A and Table S3.1). In this region, origins *II-61* to *II-86b* have a total origin efficiency of 83.56 %, while for origins *II-90* to *II-111* it is 99.38 % (Figure 3.5A and Table S3.1). Like chromosome I, chromosome II has a high density of replication origins and if at least one of the indicated origins on each side of *RTS1* fire, fork convergence will occur within 10 minutes of the first fork arriving at the barrier (Figure 3.5B).

To determine the direction of replication across the direct repeat recombination reporter on Chromosome II, I analysed replication intermediates from asynchronous cultures of the *RTS1-IO* and *RTS1-AO* by 2D gel analysis as described in section 3.2.2 (Figure 3.6A). DNA from the *RTS1-IO* strain exhibited a Y-arc of replication intermediates and a modest pause site (P) (Figure 3.6B), whereas from the *RTS1-AO* strain a similar Y-arc is evident but with a much stronger pause site (Figure 3.6B). When the hybridisation signals of the pause sites were each quantified relative to that of the total Y-arc and termination signals, the *RTS1-IO* and *RTS1-AO* had intensities of 53.17 % and 70.58 % respectively (Figure 3.6C and Table 3.2). These data indicate that both orientations of *RTS1* lead to fork pausing and termination, and it is plausible that more forks are blocked in the *RTS1-AO* direction than in the *RTS1-IO* direction (Figures 3.6C and D, and Table 3.2).

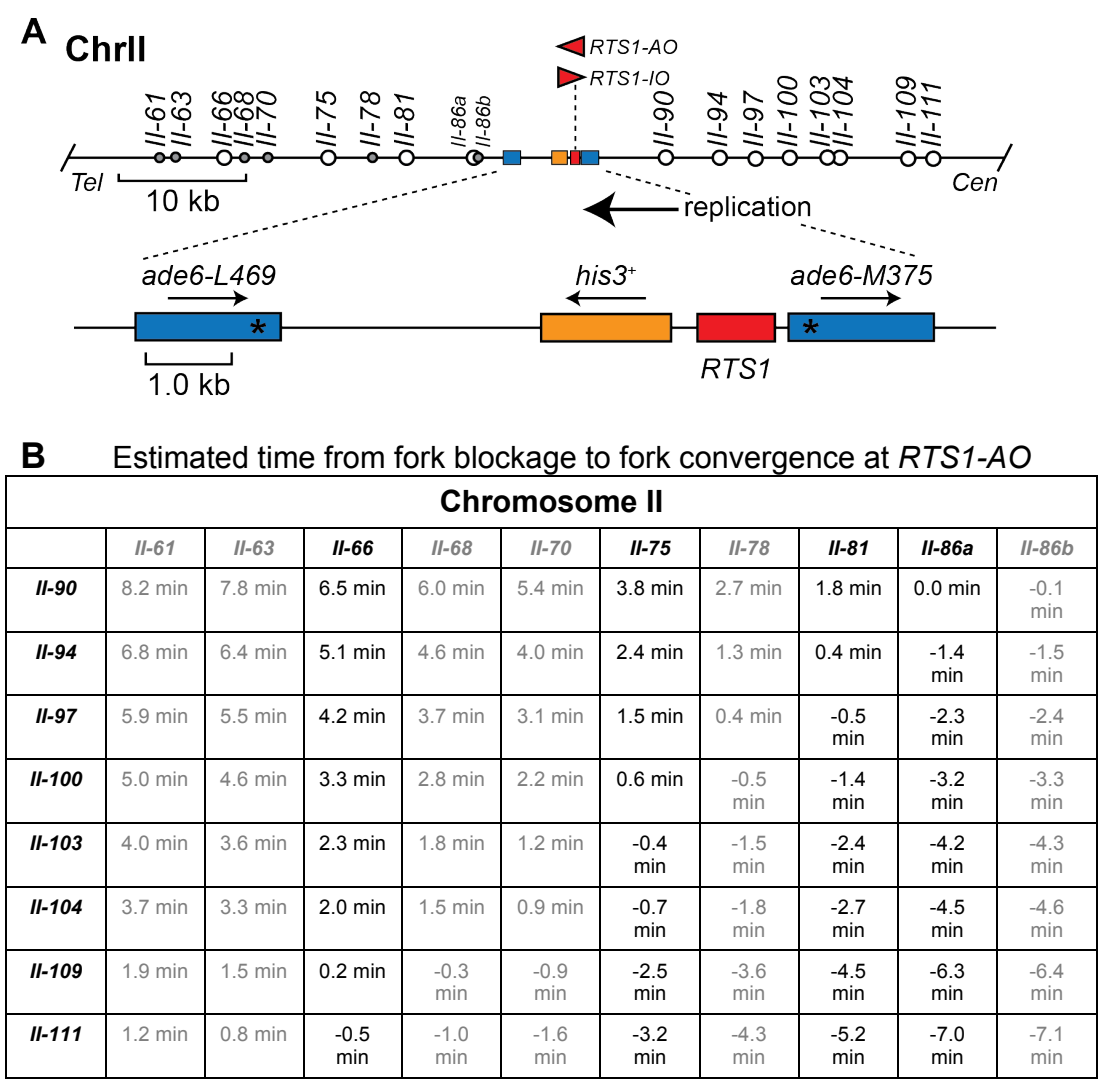


Figure 3.5 Experimental system for studying whether recombination can be strongly triggered at subtelomeric sites

(A) Schematic showing the location of the direct repeat recombination reporter in relation to nearby replication origins on chromosome II (ChrII). Likely origins are represented by white circles; dubious origins are represented by grey circles. Origin details are described in Table S3.1. On chromosome II, replication across the direct repeat recombination reporter occurs predominantly from the centromere towards the telomere. (B) Times are calculated from the distance between the midpoint of each origin (Siow et al. 2012) and a fork velocity of 3 kb/min, and assumes that each origin fires at the same time during S-phase. Timings involving only confirmed and/or likely origins are in black; timings involving dubious origins are in grey.

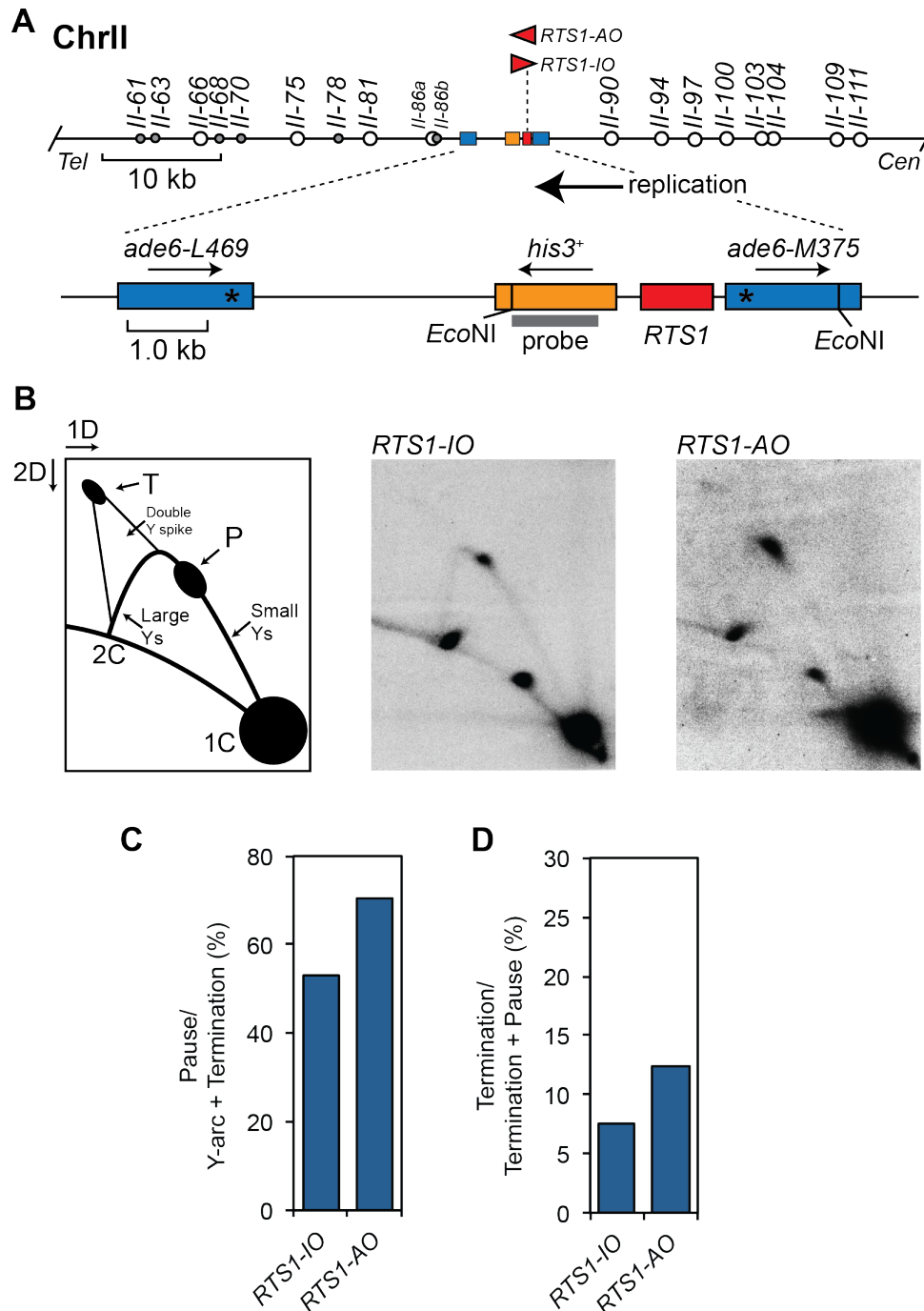


Figure 3.6 Replication across the direct repeat recombination reporter on Chromosome II occurs predominantly in the telomere-proximal direction

(A) Schematic showing the direct repeat recombination reporter on Chromosome II and the probe used for 2D gel analysis. (B) 2D gel analysis of replication intermediates in the *EcoNI* fragment shown in (A). The DNA is extracted from strains JN33 (*RTS1-IO*) and JN46 (*RTS1-AO*). (C) Amount of fork pausing at pause site (P) as a percentage of the total Y-arc and termination. (D) Amount of terminating replication forks (T) as a percentage of the total fork pausing and termination (P+T).

In the absence of *RTS1* at the chromosome II site, the frequency of conversion-type and deletion-type Ade⁺ recombinants is approximately 1.35 and 2.36 in every 10000 viable cells, respectively (Figure 3.7 and Table 3.3). Unlike at the chromosome I site where recombination induction is modest, in the chromosome II *RTS1-AO* strain, replication fork blockage results in a ~22-fold stimulation of gene conversion and a ~44-fold stimulation of deletion-types compared to the strain without *RTS1* (Figure 3.7 and Table 3.3). This stimulation of recombination is dependent on Rtf1 indicating that it is due to replication fork blockage (Figure 3.7 and Table 3.3). In contrast the *RTS1-IO* strain, exhibits only a small, albeit statistically significant, ~1.4-fold stimulation of gene conversion and ~1.5-fold stimulation of deletions compared to the strain with no *RTS1*. However, this slight increase is not dependent on Rtf1 indicating that it is not a consequence of replication fork stalling at *RTS1-IO* (Figure 3.7 and Table 3.3). Although the level of induction of recombination at the chromosome II site is considerably higher than at the chromosome I site, it is not as high as at the chromosome III site. One likely explanation for this is that the shorter inter-origin distances surrounding *RTS1* on chromosome II, compared to the *ade6* locus on chromosome III, on average result in less time between replication fork blockage and convergence, and therefore less time for RDR to occur (Figure 3.5B).

Together, these data show that recombination can be strongly triggered by replication fork blockage at subtelomeric sites. Therefore the relatively low level of recombination at the chromosome I site is unlikely to be due to some special feature of subtelomeric DNA that prevents recombinases acting at *RTS1*.

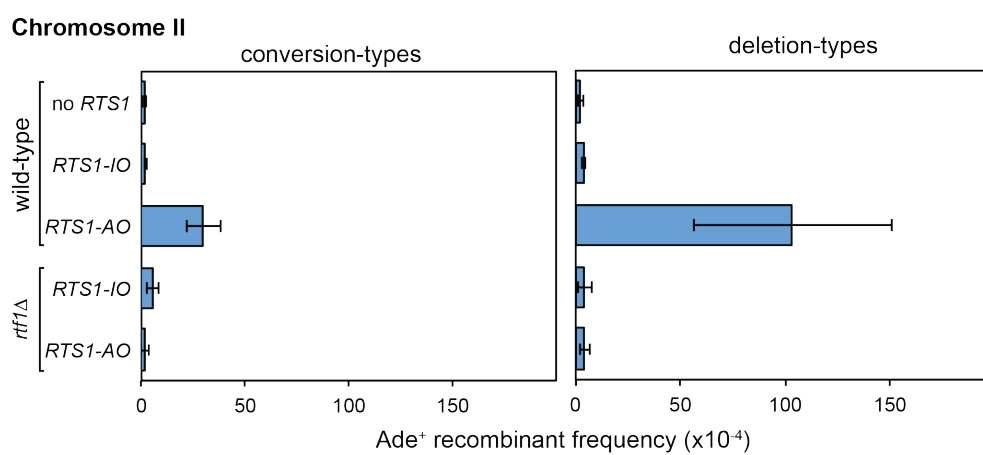


Figure 3.7 Direct repeat recombination on chromosome II

Bar chart showing the frequency of Ade⁺ recombinants in Chromosome II wild-type and *rtf1*Δ, no *RTS1*, *RTS1-IO* and *RTS1-AO* strains after 5 to 6 days at 30 °C. Error bars represent ± one standard deviation. In order of presentation, the strains are JN42, JN33, JN46, JN11 and JN18.

3.2.5 Does the disruption of replication fork termination affect *RTS1*-induced recombination at subtelomeric sites?

The data in sections 3.2.3 and 3.2.4 are consistent with the hypothesis that replication fork merging at *RTS1* is not normally a recombinogenic process. However, it potentially can become so if factors needed for its seamless progression are disrupted. One factor that has been implicated in promoting replication fork termination is the Pif1 DNA helicase, Pfh1. This helicase appears to promote both replication fork progression past DNA-protein barriers and fork merging at replication termination sites (Sabouri et al. 2012; Steinacher et al. 2012). However, at *RTS1* its primary function is thought to be to promote fork merging, and in its absence the impairment of merging correlates with an increase in recombination (Steinacher et al. 2012). If replication forks blocked at *RTS1-AO* on chromosome I are resolved primarily by convergence with the opposing replication fork, and Pfh1 is required to promote this process, loss of Pfh1 should result in a marked increase in recombination at this site. To investigate this I sought to measure recombination at the chromosome I site in strains deficient for Pfh1 activity. Since Pfh1 has an essential mitochondrial role, I employed the *pfh1-mt* mutant, which encodes proteins that are mostly excluded from the nucleus but localises normally to the mitochondria (Pinter et al. 2008).

The level of spontaneous conversion-type recombination remains unchanged in *pfh1-mt* cells, whereas, the deletion recombinant frequency increases slightly (~2-fold) compared to the wild-type (Figure 3.8A and Table 3.4). Remarkably, in the presence of *RTS1*, this increase is far more dramatic in

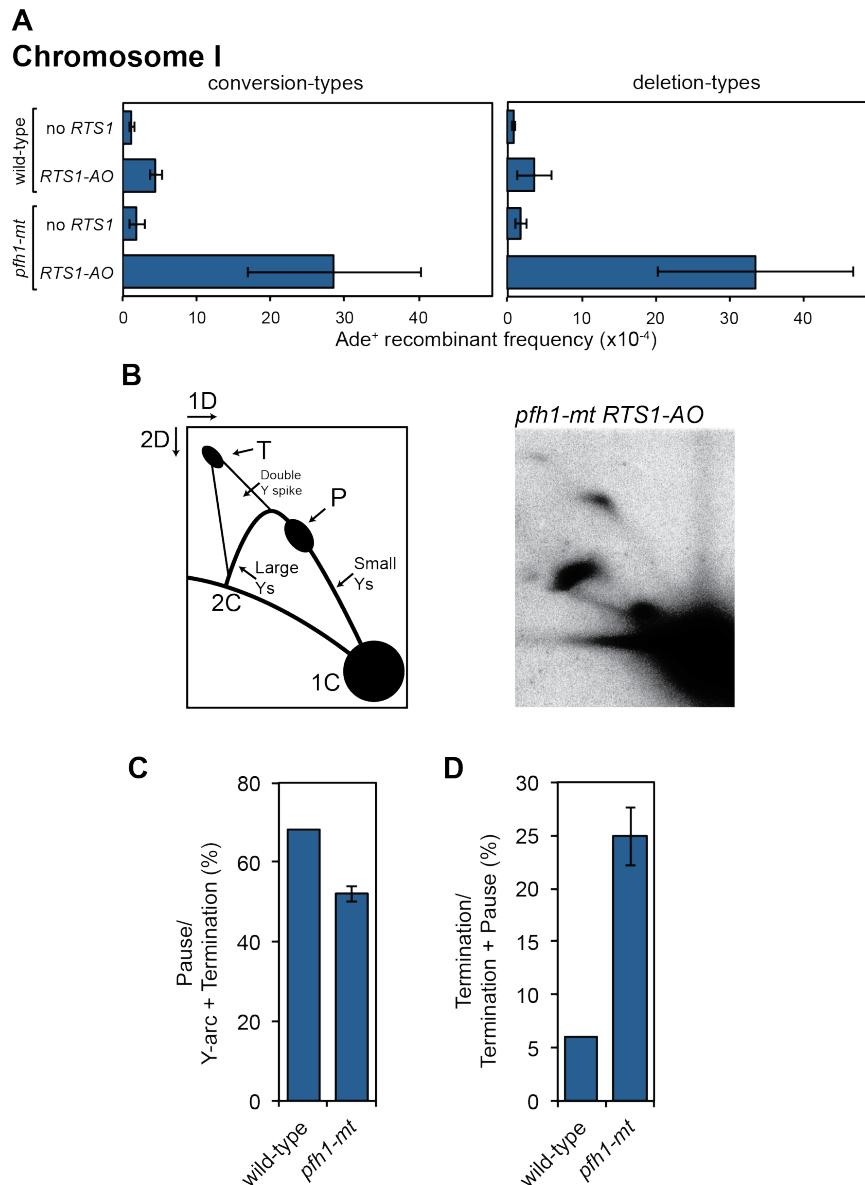


Figure 3.8 Pfh1 promotes replication fork termination at the *RTS1* site-specific replication fork barrier

(A) Bar chart showing the frequency of Ade⁺ recombinants in Chromosome I wild-type and *pfh1-mt*, no *RTS1* and *RTS1-AO* strains after 11 to 12 days at 30 °C. Error bars represent $\pm$ one standard deviation. In order of presentation, the strains are JN27, JN29, JN114 and JN116. Wild-type data shown here is the same as in Figure 3.4B. (B) 2D gel analysis of replication intermediates in the *Eco*NI fragment shown in Figure 3.6A. The DNA is extracted from strain JN116 (*pfh1-mt RTS1-AO*). (C) Amount of fork pausing at pause site (P) as a percentage of the total Y-arc and termination. Wild-type data is the same as in Figure 3.3C and Table 3.2. (D) Amount of terminating replication forks (T) as a percentage of the total fork pausing and termination (P+T). Wild-type data shown in this figure is the same as in Figure 3.3D and Table 3.2.

Table 3.4 Pfh1 promotes replication fork termination at the *RTS1* site-specific replication fork barrier

Direct repeat recombination data presented in the same order as Figure 3.8A. Wild-type data is the same as in Table 3.3.

Genotype	<i>RTS1</i> orientation	Position of direct repeat	Number of colonies analysed	Total Ade ⁺ recombinant frequency (x10 ⁻⁴)		% Ade ⁺ His ⁺ recombinants		Ade ⁺ His ⁺ recombinant frequency (x10 ⁻⁴)		Ade ⁺ His ⁺ recombinant frequency (x10 ⁻⁴)	
				Mean ^a	P-value ^b	Mean ^a	P-value ^b	Mean ^a	P-value ^b	Mean ^a	P-value ^b
wild-type	no <i>RTS1</i>	Chr I	9	1.98 (±0.28)	-	58.85 (±16.26)	-	1.19 (±0.42)	-	0.79 (±0.23)	-
wild-type	AO	Chr I	7	8.07 (±2.77)	0.001	58.25 (±14.59)	0.940	4.42 (±0.86)	<0.001	3.65 (±2.33)	0.017
<i>pfh1-mt</i>	no <i>RTS1</i>	Chr I	17	3.61 (±1.24)	< 0.001	50.30 (±17.22)	0.232	1.85 (±1.08)	0.037	1.76 (±0.80)	<0.001
<i>pfh1-mt</i>	AO	Chr I	16	62.20 (±18.30)	< 0.001	45.74 (±11.98)	0.043	28.60 (±11.83)	<0.001	33.59 (±13.22)	<0.001

^a The values in parentheses are the standard deviations about the mean.

^b *P*-values are derived from independent-sample t-tests comparing the mean values of the respective mutant to the corresponding mean value found in the control strain.

pfh1-mt cells compared to the wild-type – a ~6-fold increase in conversion-type recombinants and a ~9-fold increase in deletion-type recombinants.

Consistent with the wild-type data (Figure 3.3B), physical analysis of the replication intermediates in asynchronously growing *pfh1-mt RTS1-AO* strain reveal the same replication fork pause signal (P) and termination spot (T) (Figure 3.8B). In addition to these two spots, there is an additional spot close to where the 2C spot would normally lie. It is likely that the additional spot is an artifact, however a repeat of the experiment would be required for this to be verified. In *pfh1-mt* cells, a similar amount of pausing is observed as in wild-type cells (Figure 3.8C and Table 3.2), however there is a ~4-fold increase in terminating replication forks (Figure 3.8D and Table 3.2). Since the amount of paused replication forks is similar in both strains, Pfh1 does not act as a sweepase at the *RTS1* RFB on chromosome I. Therefore the greater accumulation of termination signal in the *pfh1-mt* strain is due to a failure in the process of replication fork termination and that Pfh1 is needed to facilitate replication fork merging at *RTS1* on chromosome I.

Together, these data indicate that replication fork termination is not normally a recombinogenic process and that the disruption of fork termination at *RTS1* on the chromosome I site strongly induces recombination.

3.2.6 Fml1 promotes gene conversion at a replication fork termination site

Fml1 has the ability to stimulate the regression of stalled replication forks through its branch migration activity, and this maybe how it promotes Rad51-

dependent gene conversion at *RTS1* (Sun et al. 2008). However, its branch migration activity could equally be used to reset regressed replication forks, which may be important during replication fork convergence as the positive supercoiling in DNA ahead of a converging fork may drive fork regression of the opposing fork (Postow et al. 2001). This regressed fork could be reset by Fml1 to facilitate fork termination. In the absence of Fml1, the regressed fork could extrude ssDNA at which RPA, and subsequently Rad52 could load to catalyse the formation of deletion-types through SSA (unpublished data). In support of this idea, a *fml1* Δ mutant exhibits high levels of Rad51-independent but Rad52-dependent *RTS1*-induced deletions (Sun et al. 2008 and unpublished data). If Fml1 is required to limit the loading of Rad52 at all sites of replication fork convergence then its loss should result in an increase in deletions at the chromosome I subtelomeric *RTS1* site. To test this, I deleted *fml1* in strains with the recombination reporter (both with and without *RTS1*-AO) at the subtelomeric chromosome I site.

Similar to the endogenous *ade6* locus on chromosome III (Sun et al. 2008), at the chromosome I site the absence of Fml1 results in a reduction in spontaneous conversion-type (~2-fold) compared to wild-type. In contrast, the deletion-type recombinant frequency increases significantly ($P < 0.001$) (~3-fold) compared to wild-type (Figure 3.9 and Table 3.5). The absence of Fml1 has an even bigger impact on the frequency of *RTS1*-induced conversion-types reducing them by ~8-fold ($P < 0.001$) compared to wild-type, but although the frequency of deletion-types rises slightly the increase is not highly significant ($P = 0.033$) (Figure 3.9 and Table 3.5). Taken together, these data reaffirm that Fml1 plays an important role in promoting both

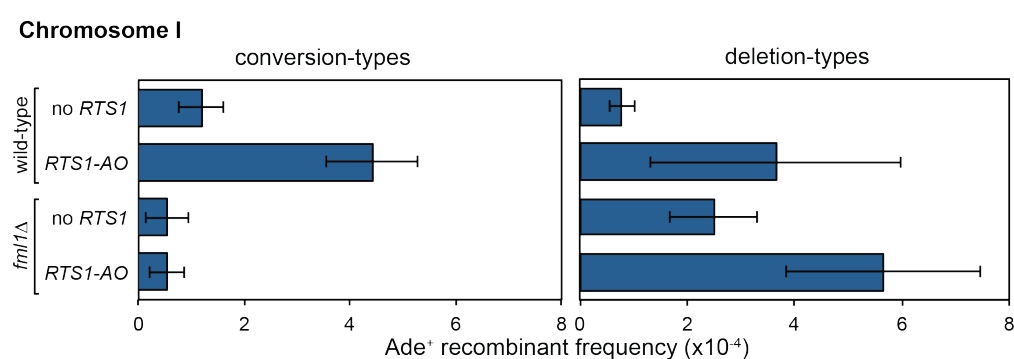


Figure 3.9 Effect of *fml1Δ* on direct repeat recombination

Bar chart showing the frequency of Ade⁺ recombinants in Chromosome I wild-type and *fml1Δ*, no *RTS1* and *RTS1-AO* strains after 11 to 12 days at 30 °C. Error bars represent ± one standard deviation. In order of presentation, the strains are JN27, JN29, JN118 and JN120. Wild-type data shown here is the same as shown in Figure 3.4B and Table 3.3.

Table 3.5 Effect of *fml1*Δ on direct repeat recombination frequencies

Direct repeat recombination data presented in the same order as Figure 3.9. Wild-type data is the same as shown in Table 3.3.

Genotype	<i>RTS1</i> orientation	Position of direct repeat	Number of colonies analysed	Total Ade ⁺ recombinant frequency (x10 ⁻⁴)		% Ade ⁺ His ⁺ recombinants		Ade ⁺ His ⁺ recombinant frequency (x10 ⁻⁴)		Ade ⁺ His ⁺ recombinant frequency (x10 ⁻⁴)	
				Mean ^a	P-value ^b	Mean ^a	P-value ^b	Mean ^a	P-value ^b	Mean ^a	P-value ^b
wild-type	no <i>RTS1</i>	Chr I	9	1.98 (±0.28)	-	58.85 (±16.26)	-	1.19 (±0.42)	-	0.79 (±0.23)	-
wild-type	AO	Chr I	7	8.07 (±2.77)	0.001	58.25 (±14.59)	0.940	4.42 (±0.86)	<0.001	3.65 (±2.33)	0.017
<i>fml1</i> Δ	no <i>RTS1</i>	Chr I	16	3.04 (±0.81)	< 0.001	18.12 (±12.86)	< 0.001	0.55 (±0.40)	<0.001	2.49 (±0.81)	<0.001
<i>fml1</i> Δ	AO	Chr I	17	6.20 (±1.72)	0.056	9.49 (±6.79)	< 0.001	0.53 (±0.33)	<0.001	5.66 (±1.81)	0.033

^a The values in parentheses are the standard deviations about the mean.

^b *P*-values are derived from independent-sample t-tests comparing the mean values of the respective mutant to the corresponding mean value found in the control strain.

spontaneous and RFB-induced gene conversion. They also show that Fml1 can play a role in suppressing the formation of deletions, however a failure to see a marked increase in deletions at *RTS1-AO* at the subtelomeric site on chromosome I suggests that Fml1 is not generally required to limit Rad52 activity during replication fork convergence.

3.3 Discussion

In this chapter, I have provided evidence that hyper-recombination induced by replication fork blockage at *RTS1* stems mainly (or possibly solely) from RDR, with relatively little or no input from the act of replication fork merging itself. When I positioned the direct repeat recombination reporter at a site on chromosome I where replication forks can be blocked and terminate in either orientation of *RTS1*, the induction of recombinant frequencies is much less than seen at the *ade6* locus on chromosome III where replication is unidirectional. At the *ade6* locus, because *RTS1* is located close to the origins on one side but far from origins on the other side, there is sufficient time between replication fork blockage and convergence for recombination to occur (Figure 3.1A). In contrast, when *RTS1* is positioned between equidistant and closely spaced origins on chromosome I, it is likely that when one fork arrives at *RTS1* and gets blocked, the opposing fork will follow shortly after and merge with it, constraining the time available for recombination to occur – hence the low levels of recombination (Figure 3.2, Figure 3.4 and Table 3.3). Based on origin efficiencies, as long as one of the origins between *I-51* to *I-79* (which have a total origin efficiency of 94.57 %) fire, and one of the origins between *I-85* to *I-113* (which have a total origin efficiency of 132.27 %) fire at

the same time, assuming a fork velocity of 3 kb/min, fork convergence will occur within 10 minutes of the first fork arriving at the barrier. The likelihood of this occurrence is very high since their total origin efficiencies are approximately 100 % (Figure 3.2B and Table S3.1). Nguyen *et al.*, has calculated that it takes about 10 minutes after a replication fork has been blocked at *RTS1* for Rad52 to be recruited (Nguyen et al. 2015). So the relative lack of *RTS1*-induced recombination at the chromosome I site is wholly consistent with the notion that fork merging tends to occur before recombinases have had a chance to load and act. The low level of recombination seen might result from the occasional cell in which recombinases have had time to load and act, or possibly the occasional aberrant fork termination event that gives rise to recombination. Based on my data I propose that the majority of recombinants seen at the *ade6* locus on chromosome III stem from RDR events at the blocked fork rather than by replication fork termination. This is contrary to speculation that fork termination may be a recombinogenic process (Louarn et al. 1991; Horiuchi et al. 1994; Fachinetti et al. 2010). In budding yeast, 67 out of 71 termination zones contain one or more elements that could potentially pause replication (Fachinetti et al. 2010). The strong correlation between termination and replication pausing, which often leads to recombination, suggests an association between the two events. A comparison of the frequency and temporal dynamics of Rad52 recruitment to *RTS1* at the chromosome I, II and III sites (by ChIP and/or live cell imaging) would provide a more direct way of determining whether recombination is activated at *RTS1*, and by altering the timings of replication fork arrival at the barrier (by deleting origins) one could

better test whether termination is recombinogenic or not. For example, unlike at the chromosome III site where Rad52 is rapidly recruited to *RTS1* in nearly all cells (Nguyen et al. 2015), I would expect to see very little recruitment at the chromosome I site. However, by deleting origins *I-62*, *I-70*, *I-74* and *I-79*, assuming that any one of the origins between *I-20* to *I-54* (which have a total origin efficiency of 97.06 %), and any one of the origins between *I-85* to *I-113* (which have a total origin efficiency of 132.27 %) fired at the same time, fork convergence at *RTS1-AO* would occur within 0.1 to 19.5 minutes (Daigaku et al. 2015) (Figure 3.2A and Table S3.1). This would provide more time for Rad52 recruitment, which should be apparent by ChIP or live cell imaging analysis, and correlate with an increase in Ade⁺ recombinant formation.

It is interesting to note that based on a fork velocity of 3 kb/min and assuming that each origin fires at the same time during S-phase, fork blockage to fork convergence at *RTS1-AO* would occur within 10 minutes on chromosomes I and II, providing insufficient time for recombination to occur (Figure 3.2B and Figure 3.5B). This clearly seems like the case on chromosome I but not on chromosome II. It is conceivable that on chromosome I, replication origins are positioned, and fire in a way that facilitates fork merging quite rapidly at *RTS1*. On the other hand, on the centromere-proximal side of chromosome II's *RTS1*, 8 of the origins (*II-90* to *II-111*) are likely origins with a total origin efficiency of 99.38 %. In contrast, on the telomere-proximal side of *RTS1*, 6 out of 10 of the origins shown in Figure 3.5A are but dubious origins which might have been wrongly predicted. The 4 likely origins *II-66* to *II-86a* have a total origin efficiency of 49.86 %, which mean that if none of the dubious origins fire, half the time, a fork blocked at *RTS1-AO* will not have a

converging fork and have to do RDR. (Figure 3.5A and Table S3.1). This explanation is supported by the fact that more forks are blocked by *RTS1-AO* than by *RTS1-IO* as seen during 2D gel analysis (Figures 3.6B and 3.6C). To verify this, future work could be done to determine if the dubious origins telomere-proximal to *RTS1* on chromosome II are indeed real origins. Additionally, the timing of origin firing of all of these origins (which would provide a clearer explanation of these data) by studying the appearance of bubble arcs on 2D gels using a centrifugal elutriation synchronised population of S-phase cells could be done.

So far, this analysis has been based on a fork velocity of 3.0 kb/min at 30 °C. However, it has been proposed that the fork travels at 1.5 kb/min instead at 27 °C (Daigaku et al. 2015). If the latter was right, and if any one of the origins between *I-51* to *I-79* and any one of the origins between *I-85* to *I-113* fire, fork convergence will occur between 0.0 to 18.8 minutes after the first fork gets blocked at *RTS1-AO*. Since some of these timings are greater than 10 minutes, recombination might have time to act in some cells. Instead, the level of recombination at this site is low. This could be due to origins between *I-70* to *I-79* and origins between *I-85* to *I-95* having higher origin efficiencies than what has been predicted, thus allowing forks to merge at *RTS1-AO* within 10 minutes (Figure 3.2B and Table S3.1). On the other hand, on chromosome II, if at least one of the indicated origins on each side of *RTS1* fires, fork convergence will occur within 16.5 minutes. This will provide sufficient time for RDR to occur, which is possibly giving rise to the recombination seen in at *RTS1* (Figure 3.7 and Table 3.3).

A caveat to this analysis is that the total origin efficiencies used, and the estimated times calculated from fork blockage to fork convergence at *RTS1-AO* assume mutually exclusive origin firing. Instead, it has been reported that origins fire in clusters stochastically (Kaykov and Nurse 2015). If this is true, in some cases, all the origins around *RTS1* on chromosomes I and II could fire together to allow forks to merge rapidly at *RTS1*, or not fire at all, in which case, a fork or two forks travelling from outside of this region would have to complete the replication of this region, probably assisted by RDR. If indeed origins fire in clusters, it is conceivable that origins around *RTS1* on chromosome I fire most of the time, allowing forks to merge rapidly, and the low level of recombination stimulated by *RTS1* can be accounted for by the rare occasions that none of the origins around *RTS1* fire (Figure 3.4 and Table 3.3). Contrarily, it is conceivable that the origins around *RTS1* on chromosome II do not fire as frequently, allowing replication to be polarised in the centromere towards telomere direction which account for the ~36-fold stimulation of recombination (Figure 3.7 and Table 3.3).

Whilst the normal process of fork merging at *RTS1* may not induce recombination, its impairment certainly can (Sommariva et al. 2005; Fachinetti et al. 2010; Sabouri et al. 2012; Steinacher et al. 2012). Consistent with the idea that Pfh1 plays an important role during fork merging, I have shown that its nuclear depletion results in a ~8-fold increase in Ade⁺ recombinants at the *RTS1-AO* chromosome I site. Although this ~8-fold increase at the chromosome I site is similar to the ~7-fold increase observed at the *ade6* locus on chromosome III, the actual frequency of recombination is ~32-fold lower (Steinacher et al 2012). This suggests that Pfh1 is less important for

constraining recombination during fork merging at the chromosome I site than at the chromosome III site. One possibility is that Pfh1 becomes more critical for fork merging if recombination proteins have already loaded at the blocked fork, i.e. fork merging may be more difficult if a Rad51 nucleofilament and its associated proteins have assembled onto one of the forks. Pfh1 may be necessary in such instances to help clear away this extra protein machinery via its so-called “sweepase” activity. As discussed above, unlike at chromosome III, at chromosome I the recombination proteins may not have time to load onto the blocked forks before fork merging, and therefore Pfh1 would only have to deal with Rtf1 and other replisome proteins. Future work to test this hypothesis would again include ChIP and live cell imaging to study the timing of Rad52 recruitment to the *RTS1* site.

Chapter 4: Investigating the biological significance of the MHF octamer

4.1 Introduction

In my other area of work, I was keen to investigate the actual mechanisms and proteins, in particular Mhf1 and Mhf2, which drive and influence recombination at a replication fork barrier. In order to gain a clearer understanding of the function of MHF, our laboratory approached collaborators, Stephen Carr and Simon Phillips at Harwell, to solve the fission yeast MHF crystal structure. Similar to the budding yeast, chicken and human MHFs, the fission yeast MHFs form a tetramer in the cell (Figure 4.1A). Intriguingly, our collaborators also obtained *in vitro* evidence that under high salt conditions, these proteins could form an octamer, which is reminiscent of the canonical histones H2A, H2B, H3 and H4 (Luger et al. 1997). This suggests that the MHFs could act as a novel nucleosome in the cell. Unlike the MHF octamer solved by Wang and colleagues (Wang et al. 2013) (Figure 1.14E), based on *in silico* modelling our collaborators hypothesised that the octamer comprises two MHF tetramers docked one above the other in a mirror image-like manner (Figure 4.1B). At the tetramer-tetramer interface, they identified three amino acids in Mhf2, serine (S) 59, glutamine (Q) 83 and aspartate (D) 87, which could play a key role in docking the two MHF tetramers together. S59 forms hydrogen bonds to D87 in a second copy of Mhf2, while Q83 forms hydrogen bonds to the main chain of N78 and E55 in a second copy of Mhf2 (Figure 4.1C). When individually mutated to alanine (A), these three point mutants (Mhf2^{S59A}, Mhf2^{Q83A} and Mhf2^{D87A}) disrupted the octamer formation *in vitro* (unpublished data). In order to explore the biological significance of the MHF octamer, I introduced these point mutations in fission yeast and analysed what impact they had on MHF function.

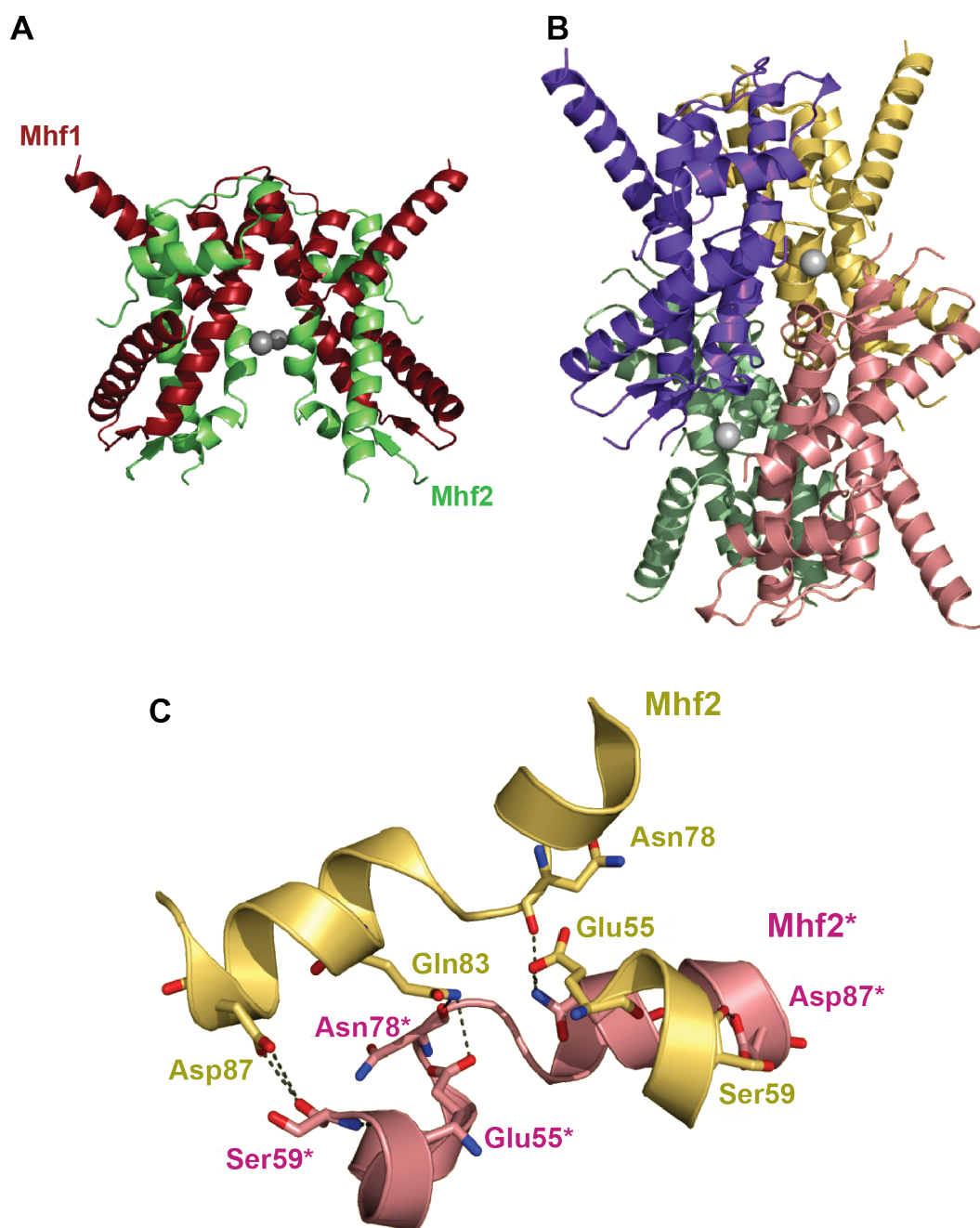


Figure 4.1 Fission yeast Mhf1 and Mhf2 complexes

(A) Ribbon diagram representation of the fission yeast Mhf1-Mhf2 tetramer. Mhf1 is coloured red; and Mhf2 is coloured in green (solved by Stephen Carr and Simon Phillips). (B) MHF octamer with each Mhf1-Mhf2 dimer coloured differently (constructed *in silico* by Stephen Carr and Simon Phillips). (C) In the MHF octamer interface, Ser59 of Mhf2 forms hydrogen bonds to Asp87 in a second copy of Mhf2. Gln83 of Mhf2 forms hydrogen bonds to the main chain of Asn78 and Glu55 in a second copy of Mhf2 (predicted based on *in silico* modelling by Stephen Carr and Simon Phillips).

4.2 Results

4.2.1 The *mhf2*^{S59A}, *mhf2*^{Q83A} and *mhf2*^{D87A} point mutants are hypersensitive to genotoxins

As described earlier, MHF has two known roles in the cell – a centromeric role and a DNA repair role (see section 1.4.1 and section 1.4.3). To test whether the MHF octamer is biologically important for either or both of its known roles, I generated the following mutated versions of *mhf2*: *mhf2*^{S59A}, *mhf2*^{Q83A} and *mhf2*^{D87A}, and analysed them for hypersensitivity to a range of toxins, including MMS, TBZ and cisplatin (Figure 4.2). In contrast to *mhf2*Δ, the three point mutants *mhf2*^{S59A}, *mhf2*^{Q83A} and *mhf2*^{D87A} did not confer a marked reduction in growth or viability compared to the wild-type strain, indicating that the octamer conformation of the MHF is not required to perform the most critical functions of MHF. The most obvious phenotype shared by the three strains carrying the different point mutations is hypersensitivity to the microtubule-destabilizing drug, TBZ (Figure 4.2). Unlike deletion of *mhf2*, neither *mhf2*^{S59A} nor *mhf2*^{Q83A} mutants exhibited hypersensitivity to cisplatin and only a modest hypersensitivity to MMS. However, in contrast the *mhf2*^{D87A} mutant exhibited marked hypersensitivity to both of these agents and in particular to MMS (Figure 4.2). To ascertain if there would be a more dramatic effect if the three mutations were combined, I generated a *mhf2*^{S59A, Q83A, D87A} triple mutant strain and found that *mhf2*^{S59A, Q83A, D87A} is epistatic with *mhf2*^{D87A} (Figure 4.3). In fact, the *mhf2*^{S59A, Q83A, D87A} triple mutant exhibits slightly less sensitivity to MMS than the *mhf2*^{D87A} mutant. The reason for this improvement in resistance to MMS is currently unclear. Since there is no indication of any

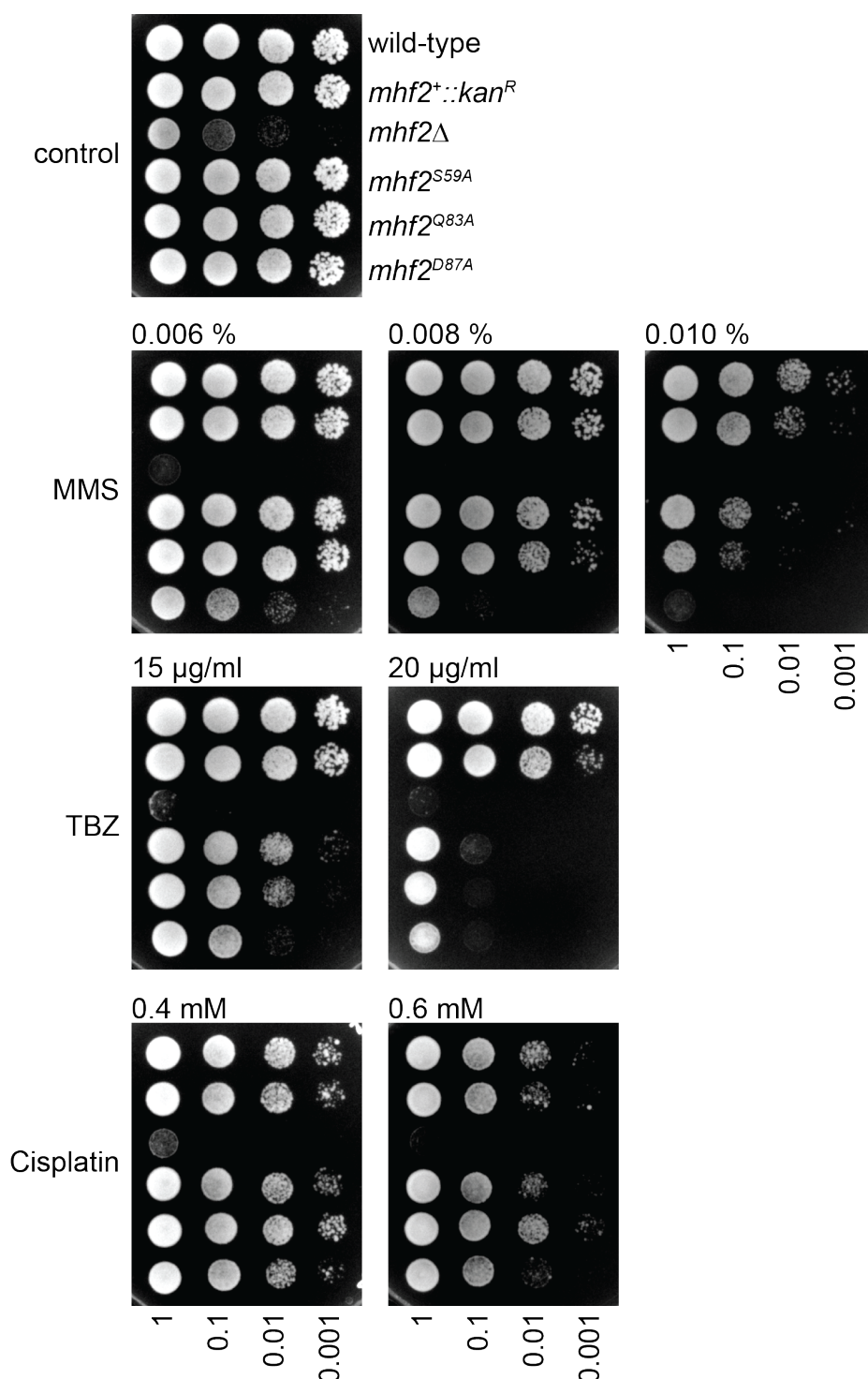


Figure 4.2 *mhf2^{S59A}*, *mhf2^{Q83A}* and *mhf2^{D87A}* point mutants are hypersensitive to the MMS, TBZ and cisplatin

Spot assays comparing the growth and genotoxin sensitivities of strains FO656, MCW5677, MCW5112, MCW6376, MCW6379 and MCW6367 after 4 days growth at 30 °C.

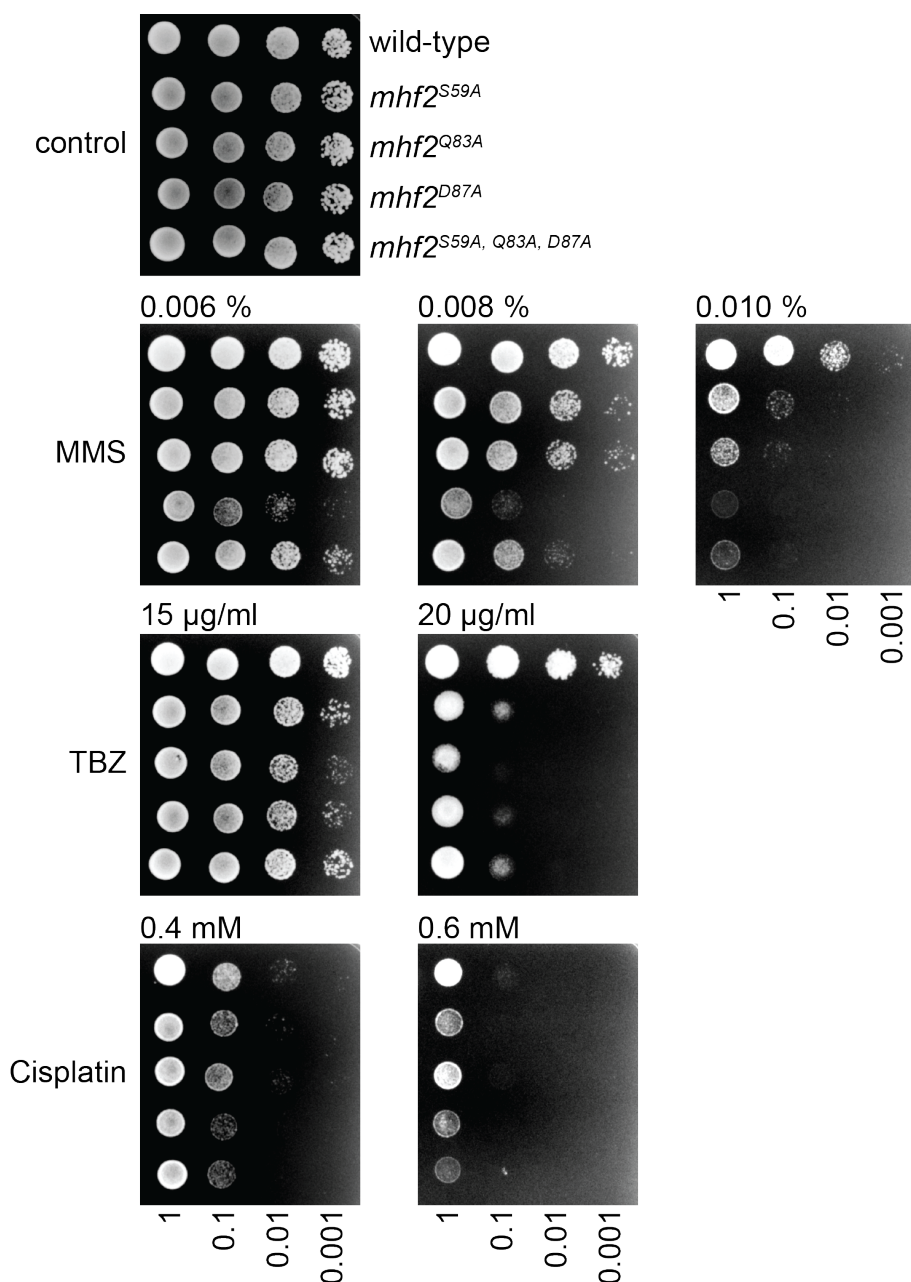


Figure 4.3 The *mhf2*^{S59A, Q83A, D87A} triple mutant has no synergistic effect compared to the *mhf2*^{S59A}, *mhf2*^{Q83A} and *mhf2*^{D87A} point mutants

Spot assays comparing the growth and genotoxin sensitivities of strains FO656, MCW6376, MCW6379, MCW6321 and JN109 after 4 days growth at 30 °C.

additive or synergistic effect when the mutations are combined in the *mhf2*^{S59A, Q83, D87A} triple mutant, it is possible that each single point mutation is sufficient to disrupt the octamer configuration of MHF *in vivo* as is the case *in vitro*. If true then the additional hypersensitivity of the *mhf2*^{D87A} mutant must result from some additional function of MHF that is specifically impaired by this mutation and not by either the S59A or Q83A mutations. What this function is will be explored in Chapter 5.

4.2.2 Do the *mhf2*^{S59A}, *mhf2*^{Q83A} and *mhf2*^{D87A} point mutants deficient in octamer formation exhibit genetic interactions with other mutants defective in centromere function?

The marked hypersensitivity of *mhf2*^{S59A}, *mhf2*^{Q83A} and *mhf2*^{D87A} strains to TBZ is reminiscent of mutants with impaired centromeric function. As MHF is in an integral component of the centromere that is needed for proper kinetochore assembly and function, the hypersensitivity of the *mhf2* point mutant strains suggests that the MHF octamer may be important for its centromeric role. If true, then combining these mutant alleles of *mhf2* with mutations in other key centromeric factors should result in additive or synergistic increases in TBZ sensitivity and even synthetic growth and viability effects. To investigate this I constructed a series of double mutant strains combining *mhf2*^{S59A}/*mhf2*^{Q83A}/*mhf2*^{D87A} with deletions of *sir2*, *dcr1* and *swi6*. Sir2 has been reported to be essential for the establishment, spreading and maintenance of centromeric heterochromatin (Buscaino et al. 2013). Dcr1 on the other hand generates short interfering RNAs (siRNAs) that guide and recruit histone H3K9 methyltransferase Clr4 to centromeric repeats (Verdel et

al. 2004; Zhang et al. 2008). Methylation of H3K9 by Clr4 provides binding sites for the Swi6 chromodomain protein, resulting in the formation of centromeric heterochromatin (Bannister et al. 2001; Zhang et al. 2008). Swi6 has also been implicated in the spreading and maintenance of centromeric heterochromatin (Buscaino et al. 2013).

In the case of Sir2, no reduction in viability or additional sensitivity to MMS, UV, HU, CPT or TBZ was observed in the *sir2Δ mhf2^{S59A}*, *sir2Δ mhf2^{Q83A}* or *sir2Δ mhf2^{D87A}*, double mutants relative to the single mutants (Figure S4.1 and Table 4.1). Similarly, in the case of Swi6, no reduction in viability or additional sensitivity to MMS, UV, HU, CPT or TBZ was observed in the *swi6Δ mhf2^{S59A}*, *swi6Δ mhf2^{Q83A}* or *swi6Δ mhf2^{D87A}*, double mutants relative to the single mutants (Figure S4.2 and Table 4.1). Likewise, in the case of Dcr1, no reduction in viability or additional sensitivity to MMS, UV, HU, CPT or TBZ was observed in the *dcr1Δ mhf2^{S59A}* or *dcr1Δ mhf2^{Q83A}*, double mutants relative to the single mutants (Figure S4.3 and Table 4.1). In contrast, the *dcr1Δ mhf2^{D87A}* double mutant, whilst exhibiting no reduction in viability, is considerably more sensitive to MMS, UV and cisplatin, compared to the single mutants (Figure 4.4 and Table 4.1). Since this epistasis analysis failed to reveal any consistent synergistic effects between the *mhf2* point mutants and the centromeric mutants, it suggests that the MHF octamer may not be important for MHF's centromeric role. The synergistic interaction between *dcr1Δ* and *mhf2^{D87A}* will be further explored in Chapter 7.

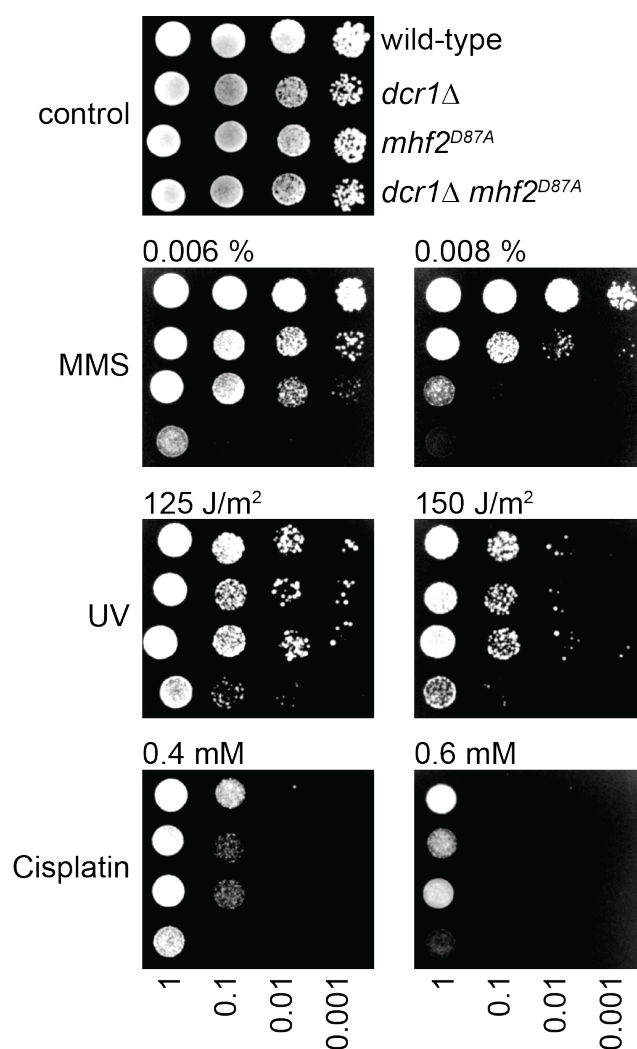


Figure 4.4 Genetic interaction between *mhf2*^{D87A} point mutant and *dcr1*Δ

Spot assays comparing the growth and genotoxin sensitivities of strains FO808, MCW6724, MCW6366 and JN70 after 4 days growth at 30 °C.

Table 4.1 Genetic interactions between *mhf2*^{S59A}, *mhf2*^{Q83A} and *mhf2*^{D87A} point mutants with other mutants defective in centromere function

Genetic interaction data between *mhf2*^{S59A}, *mhf2*^{Q83A} and *mhf2*^{D87A} point mutants and *sir2*Δ, *swi6*Δ and *dcr1*Δ centromeric mutants, presented in the same order as Figure S4.1, Figure S4.2, Figure S4.3 and Figure 4.4.

Genotoxins	Genotype		
	<i>sir2</i> Δ <i>mhf2</i> ^{S59A}	<i>sir2</i> Δ <i>mhf2</i> ^{Q83A}	<i>sir2</i> Δ <i>mhf2</i> ^{D87A}
MMS	Epistatic ^a effect	Epistatic effect	Epistatic effect
UV	Epistatic effect	Epistatic effect	Epistatic effect
HU	Epistatic effect	Epistatic effect	Epistatic effect
CPT	Epistatic effect	Epistatic effect	Epistatic effect
TBZ	Epistatic effect	Epistatic effect	Epistatic effect
Genotoxins	Genotype		
	<i>swi6</i> Δ <i>mhf2</i> ^{S59A}	<i>swi6</i> Δ <i>mhf2</i> ^{Q83A}	<i>swi6</i> Δ <i>mhf2</i> ^{D87A}
MMS	Epistatic effect	Epistatic effect	Epistatic effect
UV	Epistatic effect	Epistatic effect	Epistatic effect
HU	Epistatic effect	Epistatic effect	Epistatic effect
CPT	Epistatic effect	Epistatic effect	Epistatic effect
TBZ	Epistatic effect	Epistatic effect	Epistatic effect
Genotoxins	Genotype		
	<i>dcr1</i> Δ <i>mhf2</i> ^{S59A}	<i>dcr1</i> Δ <i>mhf2</i> ^{Q83A}	<i>dcr1</i> Δ <i>mhf2</i> ^{D87A}
MMS	Epistatic effect	Epistatic effect	Synergistic effect
UV	Epistatic effect	Epistatic effect	Synergistic effect
HU	Epistatic effect	Epistatic effect	Epistatic effect
CPT	Epistatic effect	Epistatic effect	Epistatic effect
TBZ	Epistatic effect	Epistatic effect	Epistatic effect
Cisplatin	Undetermined	Undetermined	Additive effect

^a No further reduction in growth, viability or survival observed in the double mutant compared to the single mutant with the strongest phenotype.

4.3 Discussion

In this chapter, I have shown that the *mhf2*^{S59A}, *mhf2*^{Q83A} and *mhf2*^{D87A} point mutants are hypersensitive to the microtubule poison TBZ, suggesting a centromeric function for the MHF octamer. In support of this, there has been a proposed role for recombination at the centromere (McFarlane and Humphrey 2010). Further epistasis analysis however failed to show that these point mutants have synergistic genetic interactions or synthetic growth defects with mutants defective in centromeric function. Since DNA recombination and repair mutants that are not known to have a critical role at the centromere can sometimes display sensitivity to high doses of TBZ (unpublished laboratory data), it is conceivable that the sensitivity of the *mhf2*^{S59A}, *mhf2*^{Q83A} and *mhf2*^{D87A} point mutants to TBZ does not relate to a defect in Mhf2's centromeric role, but rather a defect in its DNA repair/recombination role.

Besides exhibiting TBZ sensitivity, the three MHF point mutants also exhibited MMS hypersensitivity, suggesting that the MHF octamer may have a role in DNA repair. As a next step in determining the significance of the MHF octamer, *in vitro* studies will be needed to ascertain whether the octamer will form with DNA wrapped around it in a manner equivalent to nucleosome reconstitution.

Lastly, the greater genotoxin sensitivity of the *mhf2*^{D87A} mutant (compared to the *mhf2*^{S59A} and *mhf2*^{Q83A} mutants) will be followed up in the following chapter, while the synergistic effect between *dcr1*Δ and *mhf2*^{D87A} will be further explored in Chapter 7.

Chapter 5: The interaction between MHF and Fml1 is required for promoting genome stability

5.1 Introduction

As alluded to in section 4.2.1, unlike the *mhf2*^{S59A} and *mhf2*^{Q83A} octamer point mutants, the *mhf2*^{D87A} mutant had additional sensitivity to MMS and cisplatin compared to the wild-type. Since MHF is known to act cooperatively with FANCM in the FA DNA repair pathway (as mentioned in section 1.4.1), in this chapter, I investigate whether the additional sensitivity of the *mhf2*^{D87A} point mutant is due to a disruption of its DNA repair role as a result of a weakened interaction between MHF and Fml1.

5.2 Results

5.2.1 Does the D87A mutation in Mhf2 disrupt the interaction between Mhf2 and Fml1 *in vitro*?

To see whether the D87A mutation in Mhf2 disrupts the interaction between Mhf2 and Fml1 *in vitro*, I first purified the Mhf1 and His-Mhf2^{D87A} complex by nickel affinity and gel filtration chromatography. Analysis of the gel filtration profile of the Mhf1 and His-Mhf2^{D87A} complex relative to known standards showed that the peak of the protein (fraction 39) gel filtered with an estimated molecular weight of ~50 kDa, which would suggest that the complex purifies as a heterotetramer (Figures 5.1A and 5.1B). Analysis of the purified complex on a SDS-PAGE gel revealed two bands of the right size for Mhf1 (~12.8 kDa) and His-Mhf2^{D87A} (~13.1 kDa) (Figures 5.1B and 5.1C). It is noted that the His-Mhf2^{D87A} protein runs slightly faster than the His-Mhf2 protein, exhibiting essentially the same mobility on SDS-PAGE as Mhf1 (Figure 5.1C).

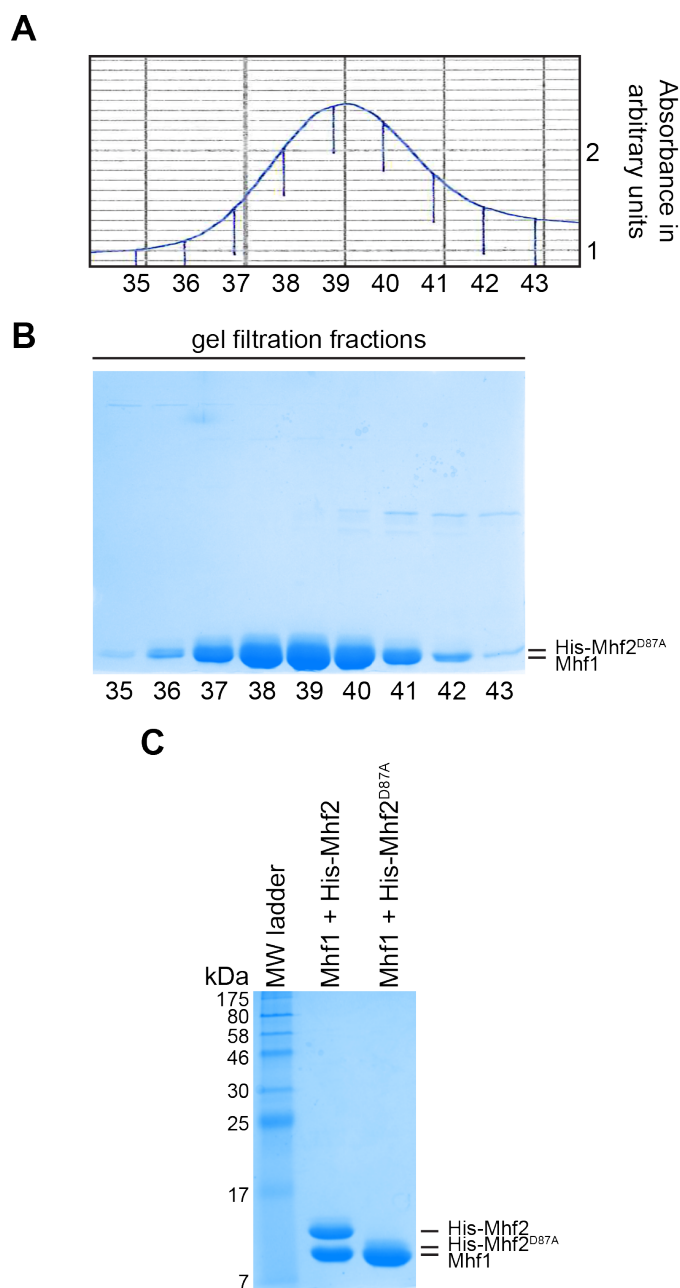


Figure 5.1 Purification and analysis of Mhf1 and His-Mhf2^{D87A} by gel filtration chromatography

(A) Absorbance at 280 nm of fractions eluting from the gel filtration column. (B) SDS-PAGE analysis of gel filtration fractions 35 to 43 from the purification of the Mhf1 and His-Mhf2^{D87A} complex. The gel was stained with Coomassie blue. (C) SDS-PAGE gel showing that the His-Mhf2^{D87A} protein runs slightly faster than the His-Mhf2 protein.

As full-length Fml1 has proved difficult to purify I made use of a C-terminal fragment (Fml1^{604–834}) to investigate the interaction with Mhf1-Mhf2 and Mhf1-Mhf2^{D87A} (Bhattacharjee et al. 2013). The Fml1^{604–834} fragment was fused to MBP and bound to amylose resin to test for its ability to retain Mhf1 and His-Mhf2^{D87A} on the resin at various NaCl concentrations (50, 200, 300 and 500 mM). As expected, under all NaCl concentrations tested, the MBP-Fml1^{604–834} fragment retained wild-type Mhf1 and His-Mhf2 on the resin (lanes c to f), whereas MBP alone did not (lane b) (Figure 5.2). I next tested the ability of the MBP-Fml1^{604–834} fragment to bind Mhf1 and His-Mhf2^{D87A}. Unlike with Mhf1-His-Mhf2 (lanes c to f), the Mhf1-His-Mhf2^{D87A} mutant protein complex had a reduced interaction with MBP-Fml1^{604–834} (lanes g to j) (Figure 5.2). The reduction was more apparent as the NaCl concentrations increased (lanes g to j in Figure 5.2). Since it was reported in Bhattacharjee *et al.*, that the mutation of tyrosine (Y) 672 together with arginines (R) 674 and 678 to alanine (A) of Fml1 (which is referred to as the Fml1^{AAA} mutant) reduced binding to MHF (Bhattacharjee et al. 2013), I wanted to test if combining the Mhf2^{D87A} and Fml1^{AAA} mutations would lead to a more dramatic disruption of interaction between MHF and Fml1. Interestingly, combining the Mhf2^{D87A} and Fml1^{AAA} mutations led to a greater disruption of interaction (lanes q to t) than when either proteins were mutated alone (lanes g to j and lanes m to p in Figure 5.2). Overall these data show that the Mhf2^{D87A} mutation does indeed disrupt the interaction between MHF and the C-terminal region of Fml1. They also suggest that the Mhf2^{D87A} mutation and Fml1^{AAA} mutation may disrupt different parts of the interaction interface.

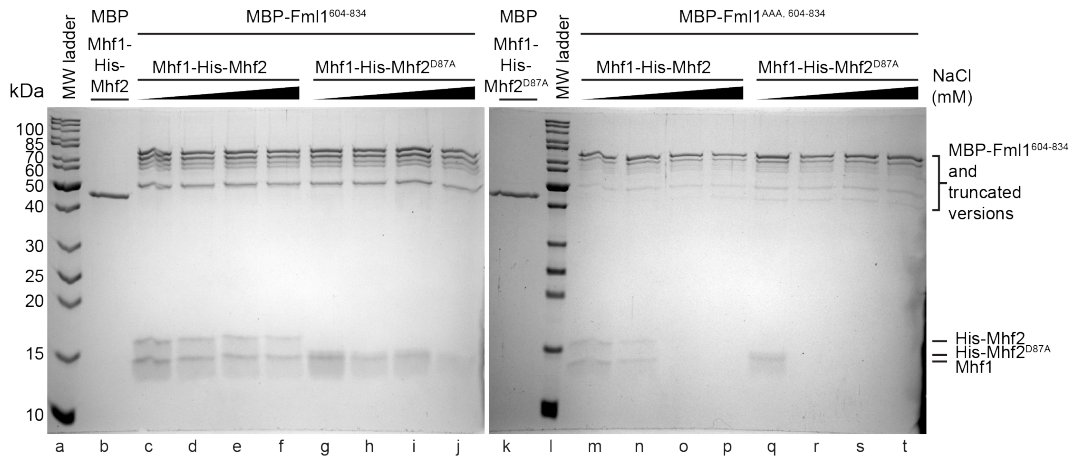
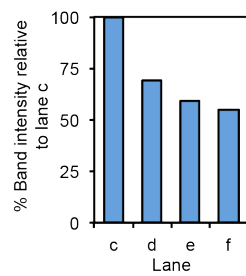
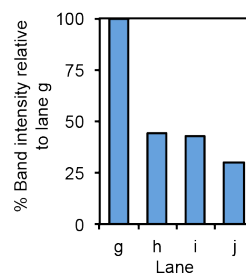
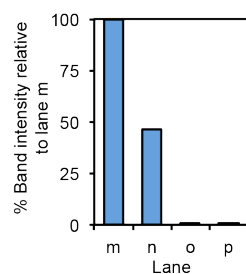
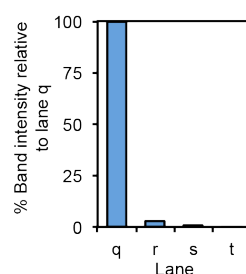
A**Coomassie Blue stained SDS-PAGE gels****B****C****D****E**

Figure 5.2 The Mhf2^{D87A} and Fml1^{AAA} mutations disrupt the interaction between Mhf2 and Fml1 *in vitro*

(A) Coomassie Blue stained SDS-PAGE gels showing the amount of Mhf1-His-Mhf2 and Mhf1-His-Mhf2^{D87A} retained on amylose resin that has been pre-incubated with MBP-Fml1⁶⁰⁴⁻⁸³⁴ or MBP-Fml1^{AAA, 604-834}. The concentrations of NaCl are 50 mM (lanes b, c, g, k, m and q), 200 mM (lanes d, h, n and r), 300 mM (lanes e, i, o and s) and 500 mM (lanes f, j, p and t). Quantification of Mhf1-His-Mhf2 and Mhf1-His-Mhf2^{D87A} retained on amylose resin with (B) % Band intensity of lanes d to f relative to lane c; (C) % Band intensity of lanes h to j relative to lane g; (D) % Band intensity of lanes n to p relative to lane m; and (E) % Band intensity of lanes r to t relative to lane q.

5.2.2 Does the D87A mutation in Mhf2 disrupt the DNA binding activity of MHF *in vitro*?

Having established that the D87A mutation affects the interaction between MHF and Fml1, I next investigated whether it impaired MHF's DNA binding activity. MHF has previously been shown to bind to linear dsDNA at high protein concentrations, but not to ssDNA (Bhattacharjee et al. 2013). I compared the DNA binding ability of the Mhf1-Mhf2 complex with the Mhf1-Mhf2^{D87A} complex using an electrophoretic mobility shift assay (EMSA) and the DNA substrate X-12 (Figure 5.3). X-12 (described in (Parsons et al. 1990)) is a synthetic X-junction made from four oligonucleotides of 49 to 51 nucleotides each. Its junction contains a homologous core of 12 bp, within which the junction point is free to branch-migrate. The heterologous arms flanking the core limit spontaneous unwinding of the substrate by branch migration of the junction points to the DNA ends. Mhf1-Mhf2 binds to X-12 to form a single retarded band at relatively high protein concentrations (8000 to 10000 nM) (lanes h and i in Figure 5.3). Likewise Mhf1-Mhf2^{D87A} binds at similar concentrations to form a protein-DNA complex with the same mobility as that formed by wild-type MHF (lanes p and q in Figure 5.3). Analysis of binding to a linear dsDNA substrate gave equivalent results (data not shown). These data show that the D87A mutation in Mhf2 does not impair DNA binding.

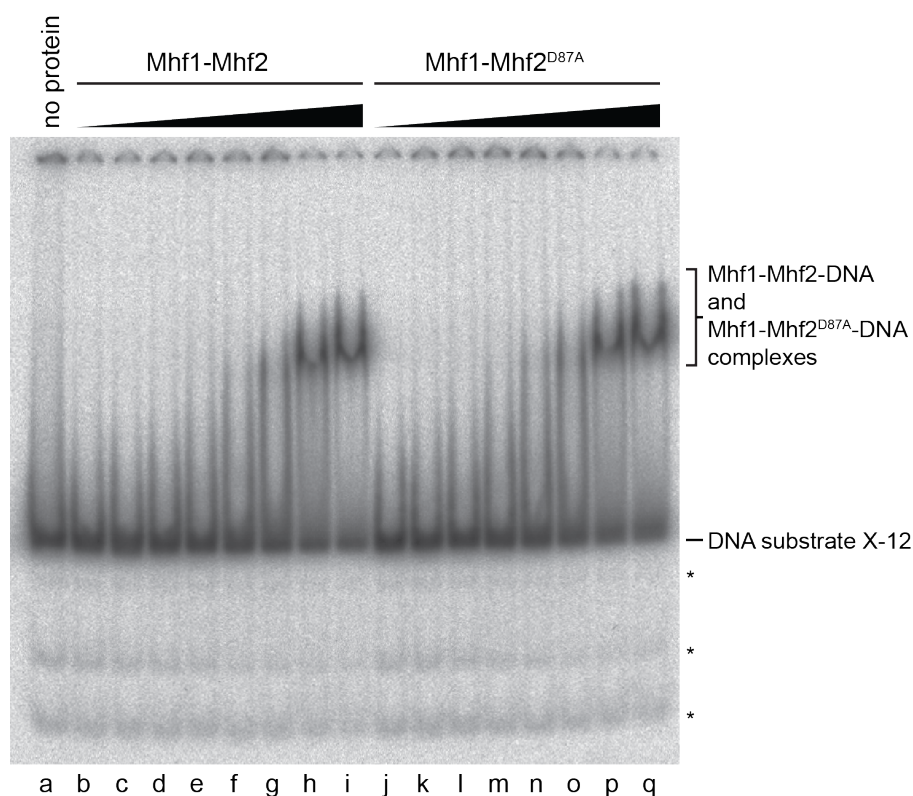


Figure 5.3 The Mhf2^{D87A} mutation does not disrupt the DNA binding activity of MHF *in vitro*

EMSA comparing the ability of Mhf1-Mhf2 and Mhf1-Mhf2^{D87A} binding to X-12. The amounts of protein are 100 nM (lanes b and j), 200 nM (lanes c and k), 500 nM (lanes d and l), 1000 nM (lanes e and m), 2000 nM (lanes f and n), 4000 nM (lanes g and o), 8000 nM (lanes h and p) and 10000 nM (lanes i and q). Reaction mixtures (20 μ l) contained the indicated concentration of Mhf1-Mhf2 or Mhf1-Mhf2^{D87A} with 0.1 nM ³²P-labeled X-12 in 1 X SSC buffer. Reactions were incubated on ice for 15 min before loading onto a 4 % polyacrylamide binding gel as described in section 2.5.3. Astericks refer to partially annealed DNA substrate.

5.2.3 Genetic interaction between *mhf2*^{D87A} and *fml1*Δ

To determine the effect of the D87A mutation of Mhf2 on *fml1*Δ, I studied the genetic interaction between *mhf2*^{D87A} and *fml1*Δ with respect to genotoxin sensitivity (Figure 5.4). The hypersensitivity of *fml1*Δ to MMS is suppressed when combined with *mhf2*^{D87A}, whereas its hypersensitivity to UV is epistatic when combined (Figure 5.4). In contrast the *fml1*Δ *mhf2*^{D87A} double mutant exhibits noticeably greater sensitivity to cisplatin than either of the single mutants. The epistatic relationship between *fml1*Δ and *mhf2*^{D87A} with respect to UV suggests that the D87A mutation specifically impairs MHF's ability to support Fml1's role in repairing UV-induced damage. Interestingly, the ability of *mhf2*^{D87A} to suppress the sensitivity of *fml1*Δ to MMS-induced lesions suggests that the MHF octamer could behave aberrantly in the absence of Fml1. On the other hand, the apparent synergistic increase in sensitivity to cisplatin suggests that MHF may have a separate role to Fml1 in repairing interstrand DNA crosslinks. Alternatively, the mutant MHF complex may harbour some deleterious activity that interferes with DNA ICL repair when Fml1 is absent.

5.2.4 Genetic interaction between *mhf2*^{D87A} and *fml1*^{AAA}

The *in vitro* data in section 5.2.1 suggest that the C-terminal domain of Fml1 (amino acids 604 to 834) is important for the interaction between MHF and Fml1, and that the AAA mutation in Fml1 along with the D87A mutation in Mhf2 disrupt this interaction. To further dissect the interaction between

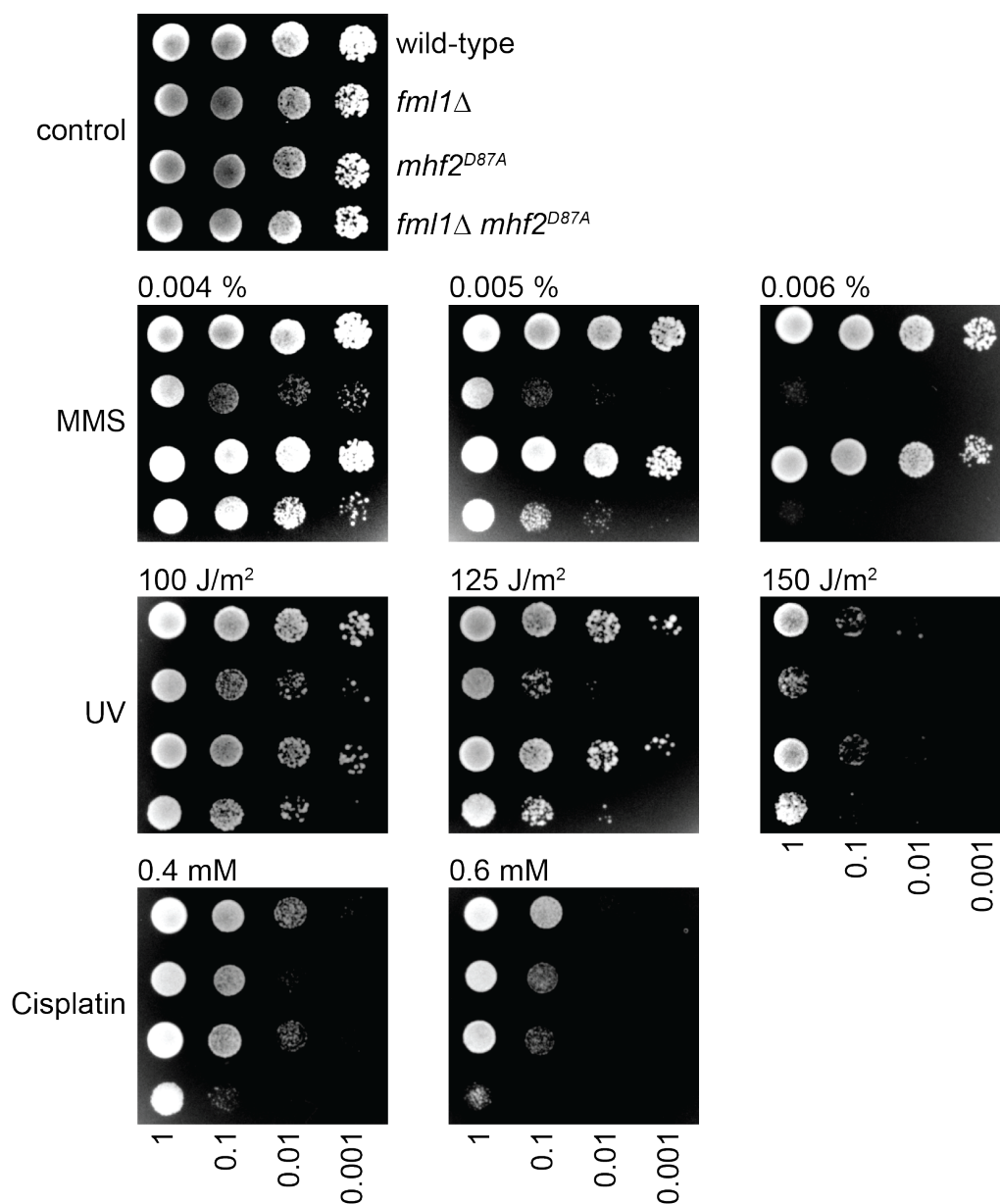


Figure 5.4 Genetic interaction between *fml1Δ* and *mhf2^{D87A}*

Spot assays comparing the growth and genotoxin sensitivities of strains FO808, MCW4708, JN21 and JN39 after 4 days growth at 30 °C.

Mhf2^{D87A} with Fml1^{AAA} and Fml1 Δ C¹⁻⁶⁰³ in promoting DNA repair, I generated a series of single and double *mhf2* and *fml1* mutant strains.

It has been published that the fission yeast *fml1*^{AAA} mutant, like the *fml1* Δ C¹⁻⁶⁰³ mutant, exhibits hypersensitivity to MMS and cisplatin that is intermediate between wild-type and *fml1* Δ (Bhattacharjee et al. 2013). However, re-analysis of the strains in our frozen collection revealed that they were diploid. Moreover, the *fml1*^{AAA} mutant carried additional mutations that had been missed in the original sequence analysis. I therefore reconstructed haploid *fml1*^{AAA} and *fml1* Δ C¹⁻⁶⁰³ mutants and retested their sensitivity to genotoxins.

Contrary to the published work, the *fml1*^{AAA} and *fml1* Δ C¹⁻⁶⁰³ mutants that I constructed did not exhibit the same sensitivities. Instead, the *fml1* Δ C¹⁻⁶⁰³ mutant that I constructed exhibits similar or even greater hypersensitivities than a *fml1* Δ strain, whereas the *fml1*^{AAA} strain is similar to wild-type (Figure 5.5). These data indicate that the C-terminal domain of Fml1, which is the region required for *in vitro* MHF-Fml1 interaction, is essential for Fml1's ability to confer resistance to genotoxins when *fml1* is expressed from its endogenous promoter. In contrast, the absence of hypersensitivity in the *fml1*^{AAA} mutant suggests that the AAA mutation is insufficient to fully impair the interaction between Fml1 and MHF *in vivo*. Since the AAA mutation only weakens but does not abolish the interaction between MHF and Fml1 *in vitro*, it is conceivable that the residual interaction that Fml1^{AAA} retains with MHF is sufficient for its role in DNA damage tolerance/repair *in vivo*.

Having established the correct phenotypes of the *fml1* Δ C¹⁻⁶⁰³ and *fml1*^{AAA} mutants I proceeded to determine their interaction with *mhf2*^{D87A}. The

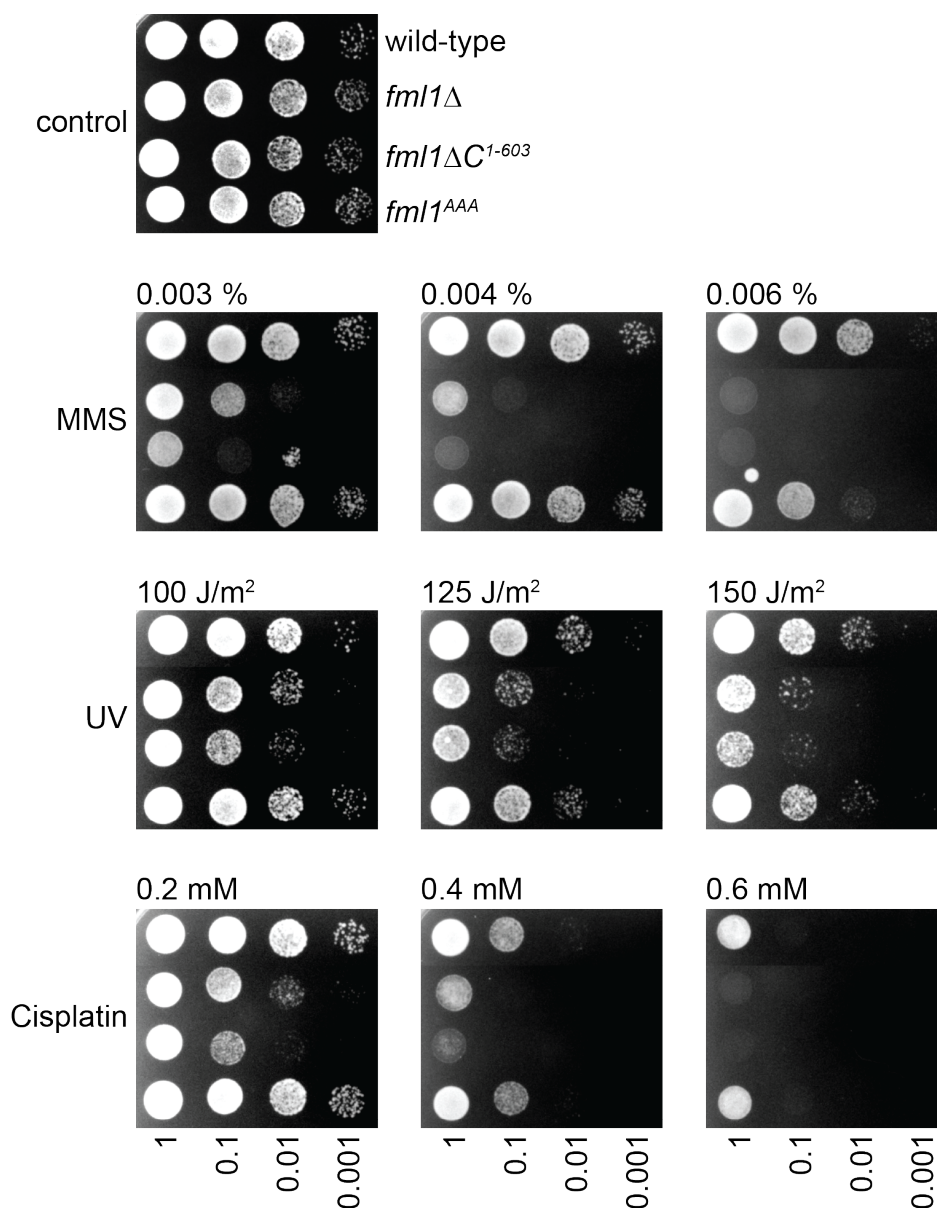


Figure 5.5 *fml1* ΔC^{1-603} is as sensitive to genotoxins as *fml1* Δ , while *fml1*^{AAA} is more genotoxin resistant than *fml1* Δ

Spot assays comparing the growth and genotoxin sensitivities of strains FO656, MCW2080, JN86 and MCW6976 after 4 days growth at 30 °C. These spot assays were done on the same plate but have been cropped and placed next to each other for ease of presentation.

*fml1*Δ^{C¹⁻⁶⁰³} *mhf2*^{D87A} double mutant exhibited similar sensitivity to MMS, UV and cisplatin as a *fml1*Δ mutant and slightly less sensitivity than a *fml1*Δ^{C¹⁻⁶⁰³} single mutant (Figure 5.6). The ability of *mhf2*^{D87A} to suppress the sensitivity of *fml1*Δ^{C¹⁻⁶⁰³} suggests that in the absence of a physical interaction between MHF and Fml1, the MHF octamer may be toxic to the cell. This is in contrast to the *fml1*Δ *mhf2*^{D87A} double mutant that exhibits a synergistic increase in cisplatin sensitivity. This suggests that in the absence of the MHF octamer, the N-terminal domain of Fml1 may be sufficient to counteract the deficiency in the loss of the MHF-Fml1 interaction during ICL repair.

Epistasis analysis of the *fml1*^{AAA} mutant with *mhf2*^{D87A} revealed a synergistic increase in MMS sensitivity in the double mutant compared to the single mutants (Figure 5.7). Indeed the sensitivity of the double mutant is almost as sensitive as a *fml1* deletion mutant. In contrast to its MMS sensitivity, the *fml1*^{AAA} *mhf2*^{D87A} double mutant was no more sensitive to UV and cisplatin than a *mhf2*^{D87A} single mutant, which is less sensitive than a *fml1*Δ mutant. In fact the double mutant might even be slightly more resistant to cisplatin than the *mhf2*^{D87A} single mutant, although the differences are small and would require a fully quantitative assay to substantiate.

5.2.5 Does the D87A mutation in Mhf2 disrupt the nuclear localisation of the MHF complex *in vivo*?

Given that MHF is a centromeric protein, in fission yeast, it co-localises with the CCAN component Mis6 (CENP-I). Additionally, it forms speckling of fluorescence throughout the rest of the nucleus, most likely representing its localisation to sites across the genome where Fml1 is actively engaged in

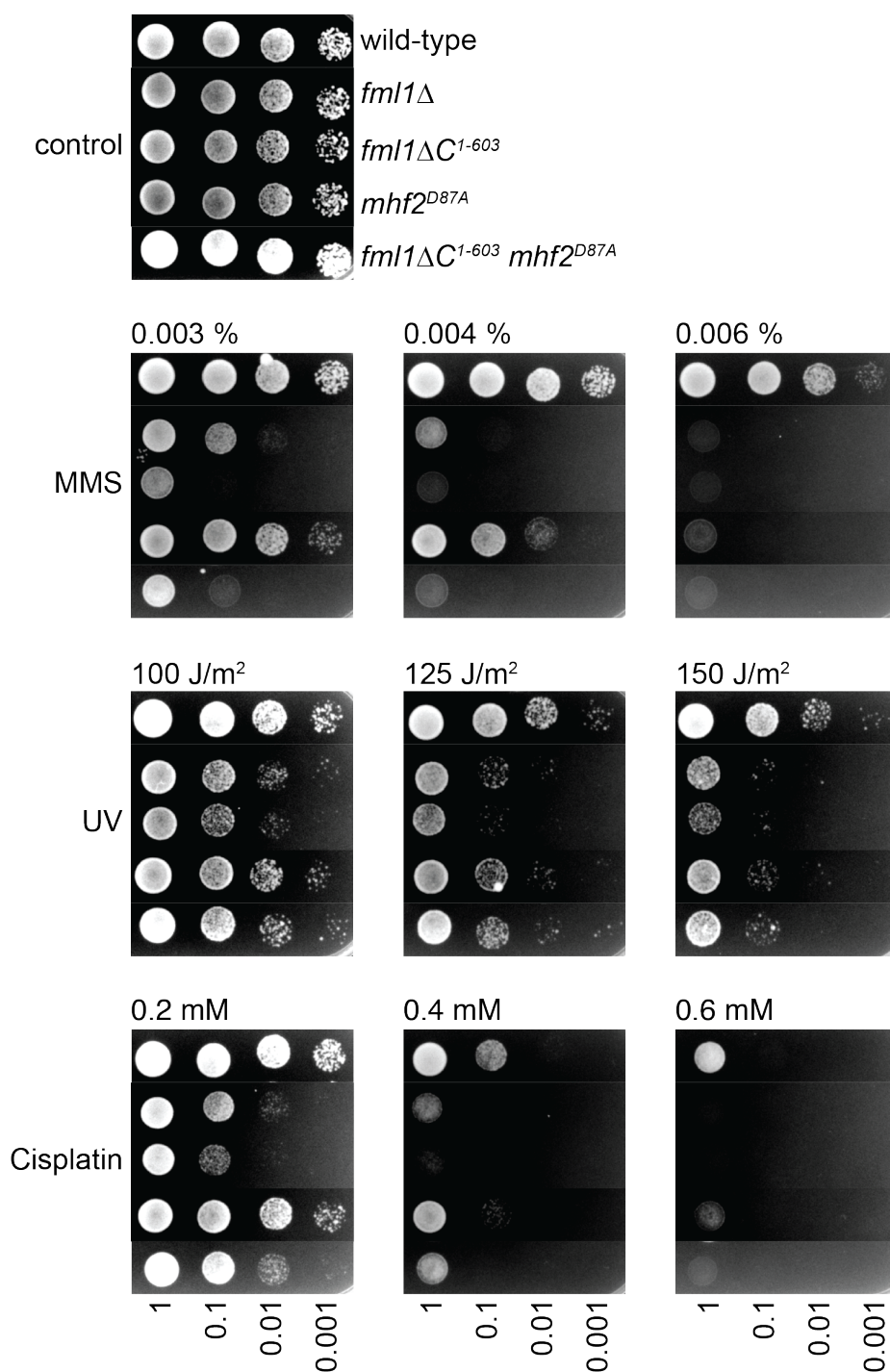


Figure 5.6 Genetic interaction between *fml* ΔC^{1-603} and *mhf2*^{D87A}

Spot assays comparing the growth and genotoxin sensitivities of strains FO656, MCW2080, JN86, MCW6321 and JN53 after 3 days growth at 30 °C. These spot assays were done on the same plate but have been cropped and placed next to each other for ease of presentation.

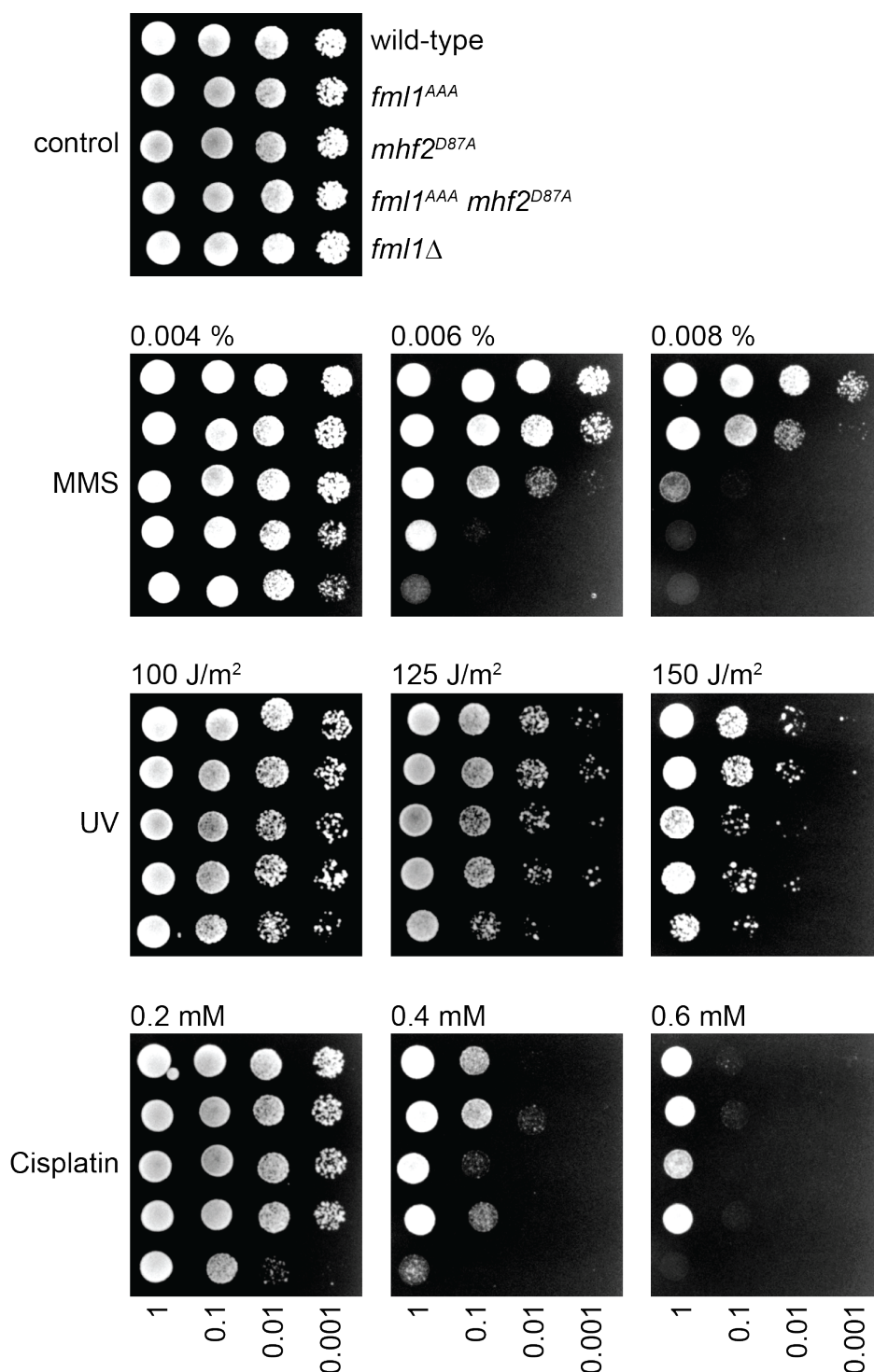


Figure 5.7 Genetic interaction between *fml1^{AAA}* and *mhf2^{D87A}*

Spot assays comparing the growth and genotoxin sensitivities of strains FO656, MCW6976, MCW6321, MCW7057 and MCW2080 after 4 days growth at 30 °C.

DNA repair and recombination (Bhattacharjee et al. 2013). To investigate whether the D87A mutation in Mhf2 disrupts the nuclear localisation of the MHF complex, I replaced the wild-type copy of *mhf2* with *mhf2*^{D87A} in a strain that expresses Mhf1-GFP from the endogenous *mhf1* promoter and analysed cells from an asynchronously growing culture by epifluorescence microscopy. In a *mhf2*^{D87A} strain, Mhf1-GFP centromeric localisation appears unaltered. In contrast, its detectable distribution throughout the wider nucleus is lost in all cells (Figure 5.8, Figure 5.9A and Figure 5.9B), reminiscent of the *fml1*^{AAA} and *fml1*Δ strains (Bhattacharjee et al. 2013). Furthermore, in a *mhf2*Δ strain, both the Mhf1-GFP centromeric and non-centromeric localisation are lost (Figure 5.8, Figure 5.9A and Figure 5.9B), indicating that the assembly of Mhf1 at centromeric and non-centromeric sites depends on the presence of Mhf2. Interestingly, besides the loss of Mhf1-GFP centromeric and non-centromeric localisation in the *mhf2*Δ strain, the *mhf2*Δ cells seem to have also lost Mis6-mCherry foci in ~50 % of its cells (Figure 5.9C). It is conceivable that in the absence of Mhf2, kinetochore assembly is disrupted, leading to the displacement of Mis6 from its centromeric localisation. Altogether, these data indicate that the disrupted interaction between Mhf2^{D87A} and Fml1 prevents the recruitment of MHF to non-centromeric chromosomal sites.

5.2.6 Does *mhf2*^{D87A} affect *RTS1*-induced direct repeat recombination?

To investigate the contribution of the D87 residue of Mhf2 towards processing blocked replication forks, I assessed the effect of *mhf2*^{D87A}, *fml1*^{AAA} (reconstructed), *fml1*^{AAA} *mhf2*^{D87A} and *fml1*ΔC^{1–603} (reconstructed) on direct repeat recombinant frequencies when the *RTS1* direct repeat recombination

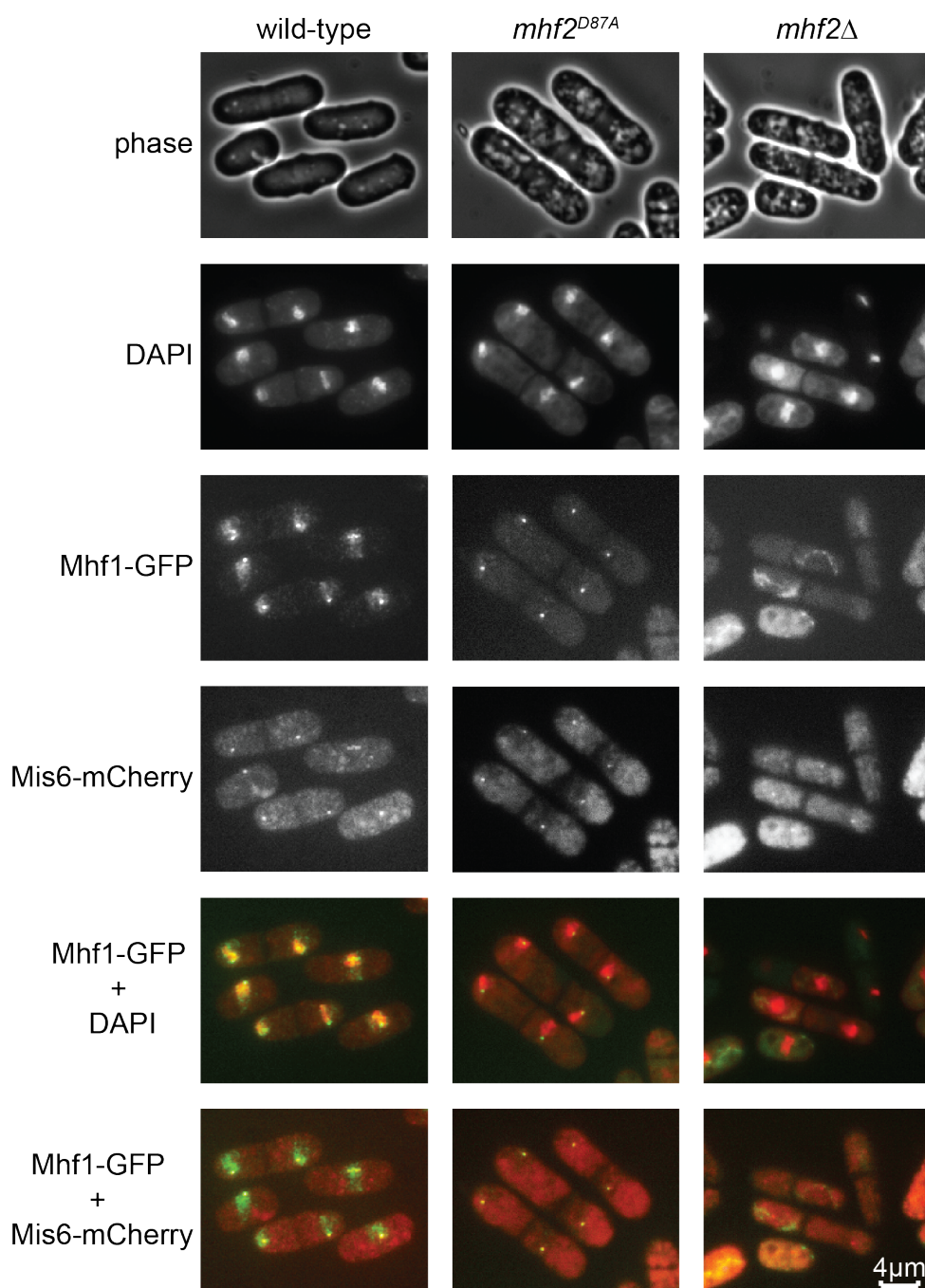


Figure 5.8 *mhf2^{D87A}* mutation causes a reduction in MHF's association with non-centromeric DNA *in vivo*

mhf2^{D87A} mutation does not disrupt Mhf1-GFP's localisation at centromeric DNA, as judged by its colocalisation with centromeric protein Mis6, but does reduce Mhf1-GFP fluorescence in the rest of the nucleus. The strains are MCW5846, JN84 and JN81.

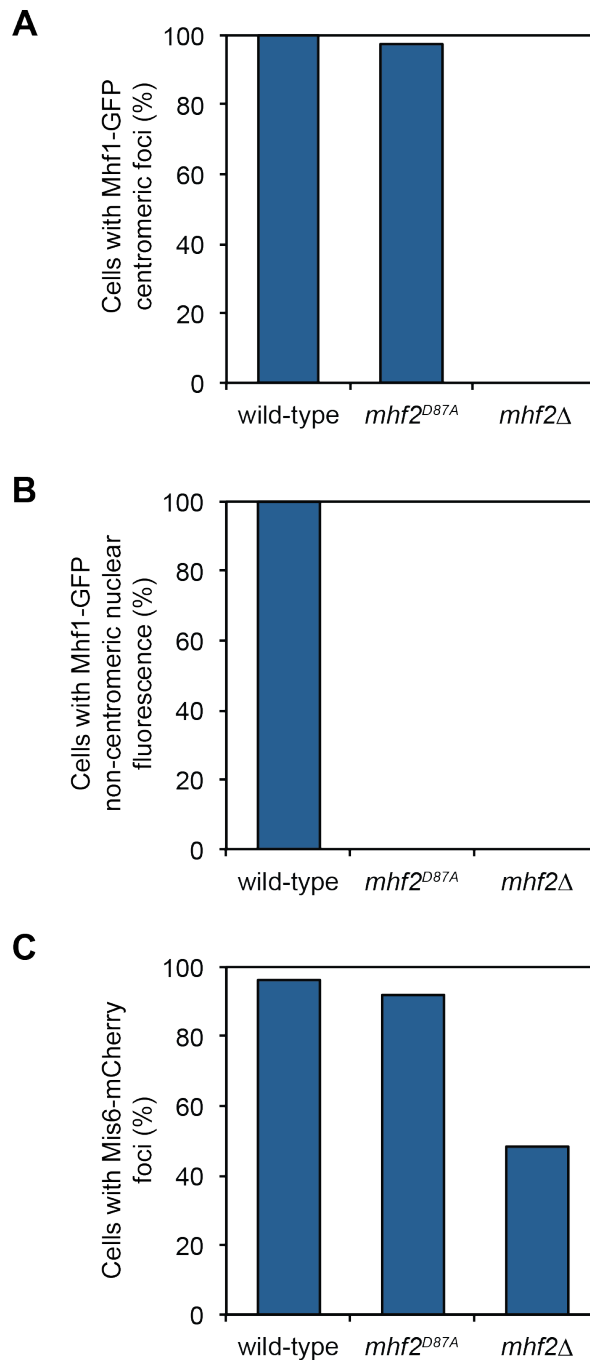


Figure 5.9 *mhf2*^{D87A} mutation causes a reduction in MHF's association with non-centromeric DNA *in vivo*

(A) Frequency of cells with Mhf1-GFP centromeric foci, (B) frequency of cells with Mhf1-GFP non-centromeric nuclear fluorescence and (C) frequency of cells with Mis6-mCherry foci in strains MCW5846, JN84 and JN81. In (A) and (B), only cells with clear Mis6-mCherry foci are scored. Values are based on the analysis of more than 100 cells.

reporter mentioned above (in section 3.1) is positioned at the endogenous *ade6* locus on chromosome III. The wild-type *RTS1-AO* data shown here is the same data as shown in Figure 3.1B, Table 3.1.

When *RTS1* is in the active orientation, the reconstructed *fml1^{AAA}* mutant phenotype resembles that of the wild-type control strain in terms of deletion-type frequency, and has slightly more conversion-types than the wild-type ($P = 0.025$) (Figure 5.10A and Table 5.1). This is contrary to the reduction of ~2-fold in conversion-types seen previously (Bhattacharjee et al. 2013).

Consistent with published data, when *fml1* is deleted in the *RTS1-AO* strain, the frequency of conversion-types is significantly reduced by ~6-fold compared to wild-type ($P < 0.001$) (Sun et al. 2008) (Figure 5.10A and Table 5.1). By contrast, there is a significant increase in the frequency of deletion-types (~2-fold relative to wild-type) ($P < 0.001$) (Figure 5.10A and Table 5.1). Similar to what was seen in the genotoxin spot assay (in section 5.2.4), the *fml1 Δ C¹⁻⁶⁰³* mutant resembles *fml1 Δ* in terms of conversion-type frequency. However, unlike the *fml1 Δ* mutant there is no significant increase in the deletion-type frequency (Figure 5.10A and Table 5.1).

When *RTS1* is in the active orientation, *mhf2^{D87A}* exhibits a similar effect on recombination as either *mhf1 Δ* or *mhf2 Δ* mutants, with the conversion-type frequency decreasing by ~2-fold ($P = 0.001$) and the deletion-type frequency increasing by ~2-fold ($P < 0.001$) compared to the wild-type (Figure 5.10A and Table 5.1) (Bhattacharjee et al. 2013). Intriguingly the *fml1^{AAA}* mutation further reduces the conversion-type frequency of *mhf2^{D87A}* by another ~2-fold, and at

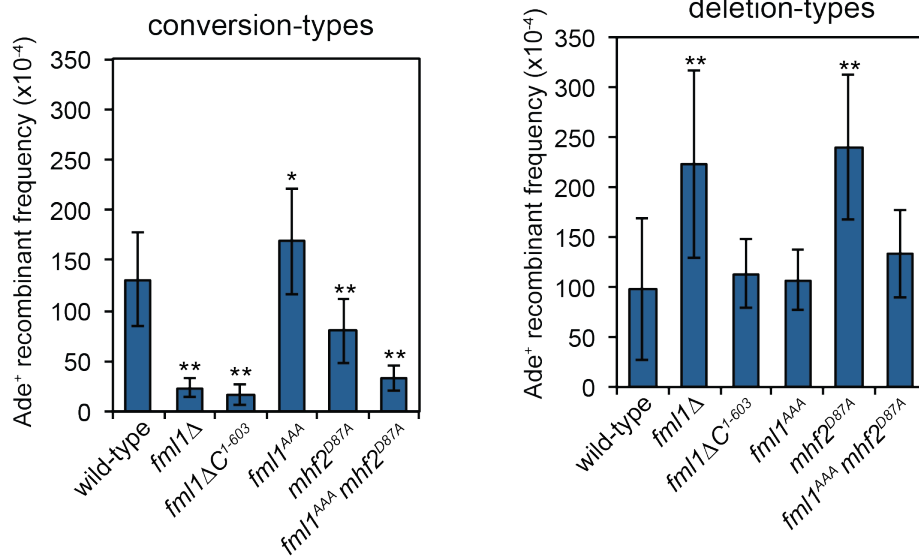
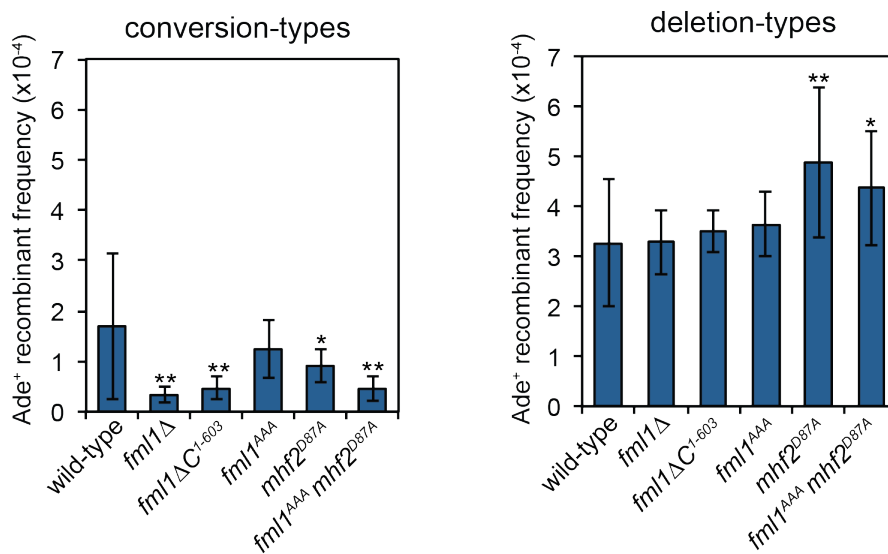
A *RTS1-AO***B** *RTS1-IO*

Figure 5.10 Effect of *fml1Δ*, *fml1ΔC¹⁻⁶⁰³*, *fml1^{AAA}*, *mhf2^{D87A}* and *fml1^{AAA} mhf2^{D87A}* on direct repeat recombination

Bar chart showing the frequency of Ade⁺ recombinants in the wild-type, *fml1Δ*, *fml1ΔC¹⁻⁶⁰³*, *fml1^{AAA}*, *mhf2^{D87A}* and *fml1^{AAA} mhf2^{D87A}* (A) *RTS1-AO* and (B) *RTS1-IO* strains after 5 to 6 days at 30 °C. Error bars represent ± one standard deviation. In order of presentation, the strains are MCW4713, MCW3061, MCW5932, MCW7062, MCW7136, MCW7138, MCW4712, MCW3059, MCW7189, MCW7059, MCW7106 and MCW7108. The wild-type *RTS1-AO* data shown in this figure is the same as shown in Figure 3.1B and Table 3.1. Statistical significance in comparison with wild-type is indicated as **P* < 0.05 and ***P* < 0.01 (for *P* values, refer to Table 5.1).

Table 5.1 Direct repeat recombination frequencies in wild-type, *fml1*Δ, *fml1*Δ*C*¹⁻⁶⁰³, *fml1*^{AAA}, *mhf2*^{D87A} and *fml1*^{AAA} *mhf2*^{D87A} strains

Direct repeat recombination data presented in the same order as Figure 5.10. Wild-type *RTS1*-AO data shown here is the same as shown in Table 3.1.

Genotype	<i>RTS1</i> orientation	Position of direct repeat	Number of colonies analysed	Total Ade ⁺ recombinant frequency (x10 ⁻⁴)		% Ade ⁺ His ⁺ recombinants		Ade ⁺ His ⁺ recombinant frequency (x10 ⁻⁴)		Ade ⁺ His recombinant frequency (x10 ⁻⁴)	
				Mean ^a	<i>P</i> -value ^b	Mean ^a	<i>P</i> -value ^b	Mean ^a	<i>P</i> -value ^b	Mean ^a	<i>P</i> -value ^b
wild-type	AO	Chr III	24	229.0 (±85.6)	-	59.8 (±15.0)	-	130.9 (±46.8)	-	98.1 (±70.8)	-
<i>fml1</i> Δ	AO	Chr III	23	246.4 (±94.0)	0.510	10.6 (±5.2)	< 0.001	23.5 (±10.0)	<0.001	222.9 (±94.0)	<0.001
<i>fml1</i> Δ <i>C</i> ¹⁻⁶⁰³	AO	Chr III	14	129.7 (±35.2)	< 0.001	12.9 (±6.9)	< 0.001	16.4 (±9.8)	<0.001	113.3 (±34.4)	0.381
<i>fml1</i> ^{AAA}	AO	Chr III	15	275.7 (±69.8)	0.085	60.8 (±6.9)	0.785	168.6 (±52.2)	0.025	107.1 (±30.4)	0.590
<i>mhf2</i> ^{D87A}	AO	Chr III	13	320.2 (±86.2)	0.004	25.0 (±8.1)	< 0.001	80.2 (±32.2)	0.001	239.9 (±72.2)	<0.001
<i>fml1</i> ^{AAA} <i>mhf2</i> ^{D87A}	AO	Chr III	14	166.1 (±44.7)	0.005	20.4 (±8.2)	< 0.001	32.5 (±12.4)	<0.001	133.7 (±44.3)	0.099
wild-type	IO	Chr III	17	4.97 (±2.03)	-	31.93 (±12.84)	-	1.70 (±1.46)	-	3.27 (±1.28)	-
<i>fml1</i> Δ	IO	Chr III	12	3.63 (±0.72)	0.021	9.51 (±3.79)	< 0.001	0.35 (±0.16)	0.002	3.28 (±0.64)	0.974
<i>fml1</i> Δ <i>C</i> ¹⁻⁶⁰³	IO	Chr III	15	3.96 (±0.42)	0.061	11.65 (±5.06)	< 0.001	0.46 (±0.22)	0.003	3.50 (±0.41)	0.503
<i>fml1</i> ^{AAA}	IO	Chr III	16	4.88 (±0.89)	0.874	24.87 (±9.04)	0.079	1.24 (±0.57)	0.244	3.64 (±0.65)	0.302
<i>mhf2</i> ^{D87A}	IO	Chr III	14	5.80 (±1.57)	0.222	16.21 (±5.19)	< 0.001	0.92 (±0.33)	0.046	4.88 (±1.51)	0.003
<i>fml1</i> ^{AAA} <i>mhf2</i> ^{D87A}	IO	Chr III	16	4.82 (±1.16)	0.802	9.82 (±4.74)	< 0.001	0.47 (±0.25)	0.003	4.36 (±1.13)	0.015

^a The values in parentheses are the standard deviations about the mean.

^b *P*-values are derived from independent-sample t-tests comparing the mean values of the respective mutant to the corresponding mean value found in the control strain.

the same time reduces the frequency of deletion-types back down to approximately wild-type levels (Figure 5.10A and Table 5.1).

As Fml1 is also required to promote the formation of “spontaneous” conversion-types, I determined recombinant frequencies in strains with *RTS1* in the inactive orientation (Figure 5.10B and Table 5.1). Wild-type and *fml1*^{AAA} strains each exhibit similar Ade⁺ recombinant frequencies, which are considerably lower than the strains with *RTS1*-AO. Similar to what is observed in the *RTS1*-AO strains the deletion or truncation of *fml1* reduces conversion-type formation (in this case ~4-fold compared to the wild-type) ($P = 0.002$ and $P = 0.003$ respectively), however no increases in deletion-types are observed in *fml1* Δ ($P = 0.974$) and *fml1* Δ C¹⁻⁶⁰³ ($P = 0.503$) (Figure 5.10B and Table 5.1). Comparable to when *RTS1* is in the active orientation, *mhf2*^{D87A} exhibits a slight but significant decrease in conversion-type frequency ($P = 0.046$) and a slight but significant increase in deletion-type frequency ($P = 0.003$) compared to the wild-type (Figure 5.10B and Table 5.1). A further ~2-fold reduction in conversion-types is observed in the *fml1*^{AAA} *mhf2*^{D87A} strain ($P < 0.001$), which also exhibits slightly fewer deletion-types than a *mhf2*^{D87A} single mutant, although this latter difference is not statistically significant ($P = 0.288$).

Taken together, these data indicate that Fml1 plays an important role in promoting both spontaneous and *RTS1*-induced gene conversion. In addition it appears to be needed to help limit the formation of deletion-types at blocked replication forks. The C-terminal domain of Fml1, which is also the region required for *in vitro* MHF-Fml1 interaction (see section 5.2.1), is needed along with its N-terminus for the promotion of gene conversion. However, seemingly

only the N-terminal helicase domain of Fml1 is needed for suppressing the formation of deletion-types. *mhf2*^{D87A} exhibits a similar effect on recombination as *fml1*Δ in reducing the formation of conversion-types and increasing the formation of deletion-types in the presence and absence of *RTS1* fork blockage, albeit not to a similar extent as *fml1*Δ in the reduction of conversion-types.

5.2.7 Does *mhf2*^{D87A} influence the crossover versus non-crossover ratio during mitotic DSB repair?

Having established that the interaction between Fml1 and Mhf2 is required for the efficient promotion of gene conversion, I next investigated whether it is required for DSB repair. For this purpose, I employed a plasmid gap repair assay, which measures the repair of a linearised plasmid through recombination with homologous DNA on the chromosome (Orr-Weaver and Szostak 1983) (Figure 5.11A). The plasmid is cut within a copy of *ade6*, which carries a 153 bp interstitial deletion at the cut site. Cut plasmids are then transformed into strains where they can be repaired by gene conversion with mutant allele *ade6-M26* on chromosome III, resulting in integration of the plasmid into the chromosome (crossover) or re-circularisation of the plasmid (non-crossover) (Figure 5.11A and Figure S5.1 in the appendix).

To determine the efficiency of DSB repair, I carried out parallel transformations with the uncut plasmid to derive a ratio for the number of cut plasmid transformants to uncut plasmid transformants ($TE_{\text{cut}}/TE_{\text{uncut}}$). This ratio is essentially the same in the wild-type, *fml1* and *mhf2* mutant strains tested here, indicating that the *fml1* and *mhf2* mutant strains tested here are

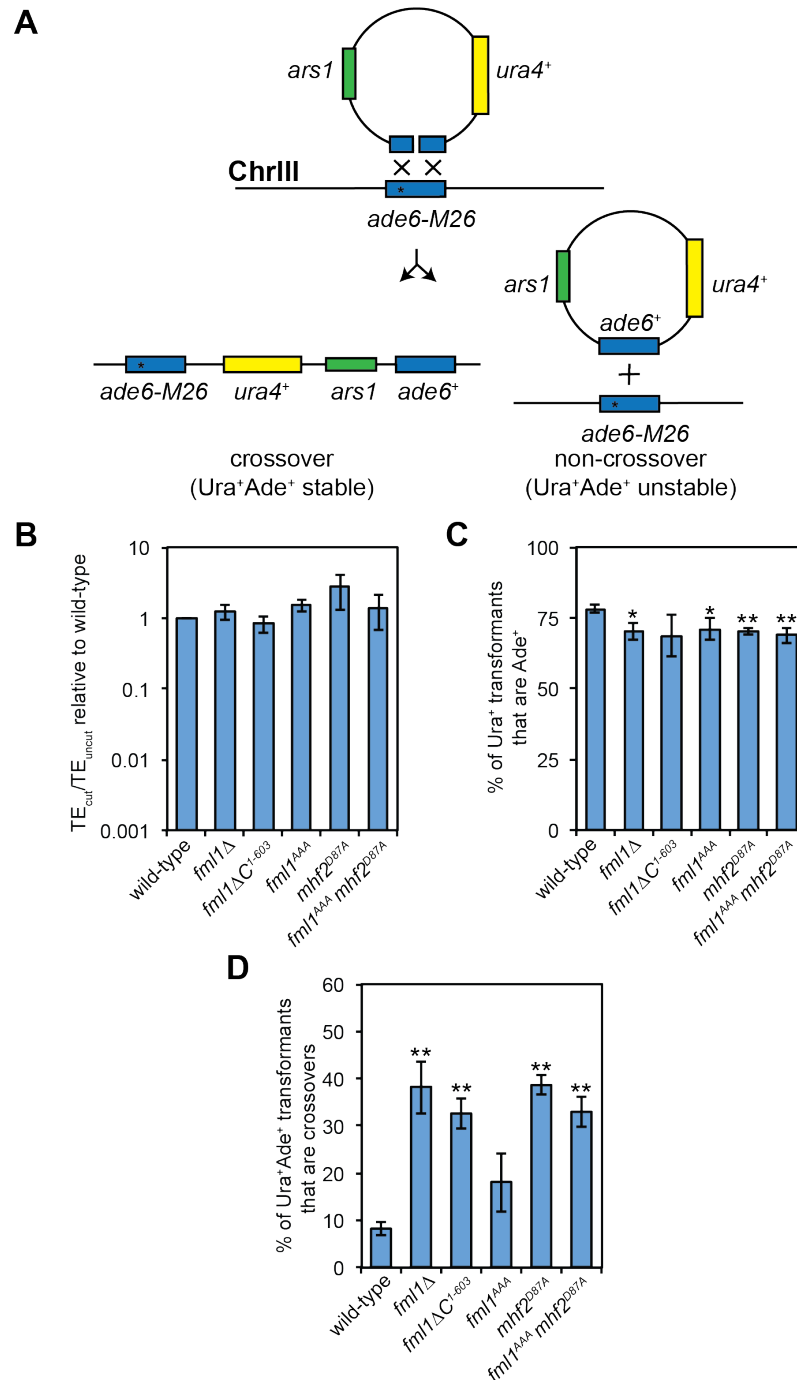


Figure 5.11 Effect of *mhf2*^{D87A} on crossover recombination during mitotic DSB repair

(A) Schematic of the plasmid gap repair assay. Astericks indicate the position of mutations in *ade6-M26*. (B) Relative efficiency of plasmid gap repair, (C) relative efficiency of gene conversion and (D) percentage of Ade⁺ transformants that are crossovers in strains MCW1193, MCW2096, MCW6001, MCW7055, MCW7174 and MCW7176. Values are means from three experiments $\pm$ s.d. Statistical significance in comparison with wild-type is indicated as * $P < 0.05$ and ** $P < 0.01$ (for P values, refer to Table 5.2).

Table 5.2 Mitotic DSB repair frequencies

Mitotic DSB repair data presented in the same order as Figure 5.11.

Genotype	TE _{cut} / TE _{uncut} relative to wild-type	% of Ura ⁺ transformants that are Ade ⁺		% of Ura ⁺ Ade ⁺ transformants that are crossovers	
	Mean ^a	Mean ^a	<i>P</i> -value ^b	Mean ^a	<i>P</i> -value ^b
wild-type	1.00 (±0.00)	78.2 (±1.3)	-	8.3 (±1.4)	-
<i>fml1</i> Δ	1.24 (±0.32)	70.2 (±3.1)	0.015	38.2 (±5.6)	<0.001
<i>fml1</i> ΔC ¹⁻⁶⁰³	0.83 (±0.21)	68.6 (±7.4)	0.093	32.6 (±3.2)	<0.001
<i>fml1</i> ^{AAA}	1.53 (±0.30)	71.1 (±3.8)	0.038	18.0 (±6.1)	0.055
<i>mhf2</i> ^{D87A}	2.73 (±1.42)	70.4 (±1.3)	0.002	38.8 (±2.1)	<0.001
<i>fml1</i> ^{AAA} <i>mhf2</i> ^{D87A}	1.38 (±0.71)	68.8 (±2.7)	0.006	33.0 (±3.2)	<0.001

^a The values in parentheses are the standard deviations about the mean.

^b *P*-values are derived from independent-sample t-tests comparing the mean values of the respective mutant to the corresponding mean value found in the control strain.

proficient for DSB repair (Figure 5.11B and Table 5.2). In the wild-type strain and all the mutant strains tested here, typically ~75 % of the repaired plasmids contain a fully restored copy of *ade6*⁺ resulting from a gene conversion event (Figure 5.11C and Table 5.2).

Consistent with published data, in wild-type cells, only ~10 % of Ade⁺ recombinants are crossovers, whereas ~40 % are crossovers in a *fml1*Δ mutant (Sun et al. 2008; Bhattacharjee et al. 2013) (Figure 5.11D and Table 5.2). Similar to previous data the *fml1*^{AAA} mutant exhibits a slight increase in the proportion of crossovers compared to wild-type, however in this case the difference is not statistically significant (*P* = 0.055). Both *fml1*Δ*C*¹⁻⁶⁰³ and *mhf2*^{D87A} mutants exhibit ~4-fold increase in the percentage of crossovers, similar to *fml1*Δ, and in the case of *mhf2*^{D87A} this is not altered significantly by the *fml1*^{AAA} mutation (Figure 5.11D and Table 5.1). These data show that the C-terminal domain of Fml1 is essential for its ability to suppress crossover formation during mitotic DSB repair. Interestingly the D87A mutation in Mhf2 appears to render the MHF complex completely incapable of promoting Fml1-dependent crossover avoidance, even though it remains partially active for promoting gene conversions at the *RTS1* RFB.

5.2.8 Does *mhf2*^{D87A} impair meiotic crossover avoidance?

MHF and Fml1 are required for directing non-crossover recombination during meiosis (Lorenz et al. 2012). To test whether the interaction between MHF and Fml1 is important for this, I employed a genetic recombination assay that consists of a meiotic recombination hotspot allele, *ade6-3083* and two flanking scorable markers (Figure 5.12A). I compared the percentage of crossovers

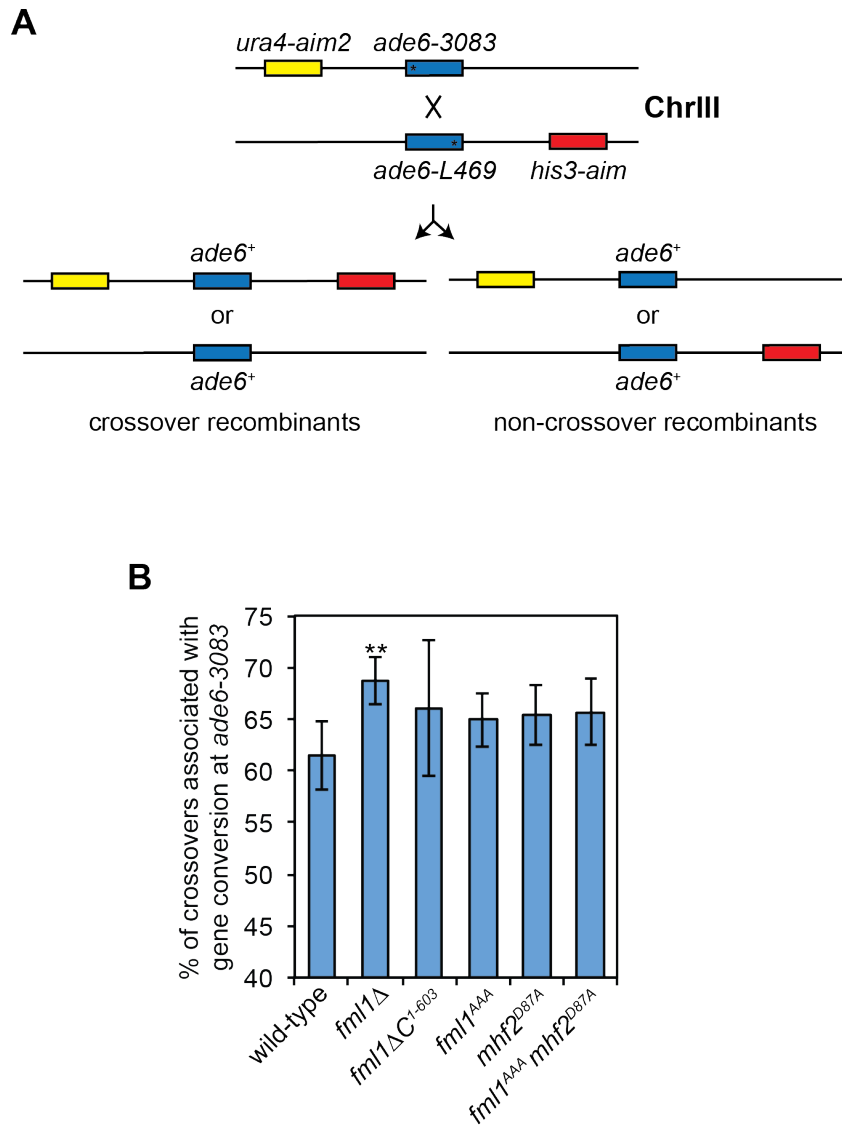


Figure 5.12 Effect of *mhf2*^{D87A} on crossover recombination during meiotic DSB repair

(A) Schematic of the meiotic recombination assay showing the different types of crossover and non-crossover recombinants that can be associated with an *ade6*⁺ convertant. Asterisks indicate the position of mutations in *ade6-3083* and *ade6-L469*. (B) Percentage of crossovers associated with gene conversion events at *ade6-3083* from wild-type and mutant crosses. Values are means from six crosses $\pm$ s.d. Statistical significance in comparison with wild-type is indicated as * $P < 0.05$ and ** $P < 0.01$ (for P values, refer to Table 5.3).

Table 5.3 Frequency of gene conversion and crossing over in the *ura4⁺-aim2 – ade6 – his3⁺-aim* interval

Meiotic DSB repair data presented in the same order as Figure 5.12. The values are the means from *n* independent crosses. The number of Ade⁺ recombinants tested is indicated, as is the total number of viable spores analysed for crossing over between *ura4⁺-aim2* and *his3⁺-aim*. *ade6-3083* is a known hot spot for recombination and therefore acts predominantly as a recipient of genetic information, this and the order of markers explains the disparity between P1/R1 and P2/R2 classes. CentiMorgan (cM) are calculated from the accumulated data of the independent crosses, not from the mean values, using the mapping function of Haldane. P values are calculated using a two-tailed Mann-Whitney U test in G* Power 3.1 against the data from the wild-type cross (MCW3202 x MCW3200) assuming a power of 1- β = 0.8.

Cross		n	Freq. of Ade ⁺ in % ^a	Ade ⁺ tested	% Ade ⁺				% crossovers associated with gene conversion		Tested	Crossovers (CO)	
strain	genotype				Ura ⁻ His ⁺ (P1) ^a	Ura ⁺ His ⁻ (P2) ^a	Ura ⁻ His ⁻ (R1) ^a	Ura ⁺ His ⁺ (R2) ^a	Mean ^a	P-value ^b		Freq. of CO in % ^a	cM
MCW3202 x MCW3200	wild-type	6	0.989 (0.120)	1119	5.86 (1.45)	32.68 (4.12)	58.11 (5.61)	3.35 (2.35)	61.46 (3.37)	-	1132	12.80 (5.01)	14.78
MCW4174 x MCW4173	<i>fml1</i> Δ	6	1.315 (0.116)	1172	9.54 (1.32)	21.74 (2.20)	65.53 (2.93)	3.19 (1.36)	68.72 (2.31)	0.001	1085	16.56 (3.28)	20.11
MCW6142 x MCW6141	<i>fml1</i> ΔC ^{T-603}	6	1.002 (0.113)	1197	12.80 (6.27)	21.15 (1.71)	62.03 (6.95)	4.02 (0.87)	66.05 (6.54)	0.158	1142	14.14 (4.10)	16.62
MCW7178 x MCW7052	<i>fml1</i> ^{AAA}	6	1.277 (0.130)	1232	7.08 (2.27)	28.03 (2.71)	60.92 (3.06)	3.97 (0.87)	64.89 (2.56)	0.075	972	16.70 (3.60)	20.33
MCW7180 x MCW7151	<i>mhf2</i> ^{D87A}	6	1.098 (0.167)	1163	8.22 (1.71)	26.37 (3.72)	63.11 (2.46)	2.31 (1.01)	65.42 (2.93)	0.056	1190	14.22 (5.16)	16.73
MCW7182 x MCW7153	<i>fml1</i> ^{AAA} <i>mhf2</i> ^{D87A}	6	1.175 (0.105)	1151	10.50 (2.79)	23.80 (2.40)	61.21 (2.93)	4.48 (1.58)	65.70 (3.20)	0.050	1064	13.08 (4.57)	15.16

^a The values in parentheses are the standard deviations about the mean.

^b P-values are derived from independent-sample t-tests comparing the mean values of the respective mutant to the corresponding mean value found in the control strain.

associated with gene conversion at the *ade6-3083* hotspot in wild-type, *fml1* and *mhf2* mutant strains (Figure 5.12B and Table 5.3). As our laboratory has shown, there is a small albeit significant increase ($P = 0.001$) in the percentage of crossovers associated with gene conversion in the *fml1* Δ mutants compared to the wild-type (Lorenz et al. 2012; Bhattacharjee et al. 2013) (Figure 5.12B and Table 5.3). Small increases were also observed in *fml1* Δ C^{1–603}, *fml1*^{AAA}, *mhf2*^{D87A} and *fml1*^{AAA}*mhf2*^{D87A} strains when compared to wild-type, however none of these were statistically significant (Figure 5.12B and Table 5.3). Based on these data it remains unclear whether the interaction between the C-terminal domain of Fml1 and MHF is crucial for Fml1's role in suppressing crossover formation during meiosis. The differences between wild-type and *fml1* Δ are quite small, and therefore it is conceivable that subtle increases in crossover levels are lost within the variability of data in this assay. As Fml1 is required for the markedly improved spore viability of a *mus81* Δ mutant in the absence of Sfr1 (Lorenz et al. 2012), this could be used in future as a more sensitive assay to probe the importance of Fml1's C-terminal domain (and its interaction with MHF) for the processing of meiotic recombination intermediates.

5.3 Discussion

In an effort to understand the interaction between Fml1 and MHF in fission yeast, I have characterised the *fml1*^{AAA} and *mhf2*^{D87A} mutants, which alone partially disrupt, but together appear to completely disrupt the interaction between Fml1 and MHF *in vitro*. To have a quantitative measure of how much the MHF-Fml1 interaction is impaired in these mutants, further work will be

needed using biophysical approaches such as surface plasmon resonance (SPR).

Based on previous work from our laboratory it was concluded that the interaction between Fml1 and MHF is mediated by the C-terminal domain of Fml1 (Bhattacharjee et al. 2013). Indeed my data confirm that amino acids 604 to 834 in Fml1 form a strong enough interaction with MHF *in vitro* to resist salt concentrations of at least 500 mM. Similar studies using the N-terminal helicase domain of Fml1 (amino acids 1 to 603) reveal no interaction with MHF (Io Nam Wong, unpublished data). Consistent with MHF playing an important role in supporting Fml1's role in DNA repair, I have shown that a *fml1*ΔC¹⁻⁶⁰³ strain exhibits approximately the same hypersensitivities to MMS, UV and cisplatin as a *fml1*Δ mutant. This contrasts with the earlier study from our laboratory, which revealed levels of sensitivity that were intermediate between those of wild-type and *fml1*Δ. The difference is most likely explained by the earlier study inadvertently using a diploid strain, as it is known that diploid yeast strains are more resistant to genotoxins than haploids (Heude and Fabre 1993; Gangloff et al. 2000; Barbour and Xiao 2006). Interestingly overexpression of the Fml1ΔC¹⁻⁶⁰³ N-terminus helicase domain of Fml1 is sufficient to rescue the genotoxin hypersensitivity of a *fml1*Δ mutant (Sun et al. 2008; Nandi and Whitby 2012). Hence, the interaction that Fml1's C-terminal domain mediates with MHF is only vital under normal levels of Fml1 expression. This is consistent with the notion that MHF is an accessory factor that simply augments Fml1 activity. As with genotoxin sensitivity, a *fml1*ΔC¹⁻⁶⁰³ mutant exhibits similar deficiencies in crossover avoidance during DSB repair and gene conversion at the *RTS1* RFB as a *fml1*Δ mutant. However, it

does appear to differ from a *fml1*Δ mutant with respect to suppression of deletion-type recombinants that are induced by replication fork blockage at *RTS1*. As alluded to in section 3.2.6, unpublished work from our lab suggests that Fml1 is required to suppress the formation of Rad52-dependent deletions that can occur during replication fork merging at *RTS1*. This putative function of Fml1 appears to be less reliant on its C-terminal than its N-terminal domain.

Mutation of tyrosine (Y) 672 together with arginines (R) 674 and 678 to alanine (A) of Fml1 (Fml1^{AAA}) was previously shown to disrupt Fml1 interaction with MHF *in vitro* and to confer a phenotype *in vivo* that was intermediate between that of wild-type and *fml1*Δ with respect to genotoxin sensitivity, direct repeat recombination and crossover avoidance (Bhattacharjee et al. 2013). However, whilst I have confirmed that the AAA mutation does reduce the interaction between Fml1's C-terminal domain and MHF *in vitro*, the analysis of a newly constructed *fml1*^{AAA} mutant revealed essentially the same phenotypes as the wild-type control strain. It appears that the original strain carries additional mutations that account for its greater genotoxin sensitivity and effects on direct repeat recombination and crossover avoidance. Whilst the single *fml1*^{AAA} mutant does not exhibit a phenotype, it appeared to manifest a synergistic interaction with *mhf2*^{D87A}, consistent with the near total loss of interaction between Fml1^{AAA, 604–834} and Mhf1-His-Mhf2^{D87A} observed *in vitro*.

An intriguing finding from my work is that a *mhf2*^{D87A} mutation affects *RTS1*-induced gene conversion relatively less than it affects crossover avoidance during mitotic DSB repair. Given that Fml1 is proposed to promote gene

conversion at *RTS1* by catalysing fork reversal and crossover avoidance by unwinding D-loops, my data suggest that D-loop unwinding might be more critically dependent on its interaction with MHF than fork reversal. This difference might be apparent *in vitro* using model replication fork and D-loop substrates, however these experiments have yet to be done. I suspect that the true importance of MHF to Fml1 will only be apparent under *in vivo* conditions, where its likely role in helping to recruit Fml1 to its target DNA becomes more critical in the face of other competing DNA binding proteins. Previous studies have proposed that MHF is required to augment FANCM/Fml1 DNA binding (Yan et al. 2010), but it may also be needed to promote interaction with other proteins, which could be quite different at sites of replication fork blockage compared to DSBs. It is also possible that the putative octameric configuration of MHF plays a more important role for crossover avoidance than *RTS1*-induced gene conversion. Future studies will be needed to determine whether the other “octamer mutants” identified in chapter 4 have any effect on crossover levels in the gap repair assay.

Chapter 6: The role of MHF in inhibiting the septation initiation network

6.1 Introduction

Chicken DT40 cells deficient in Mhf1 and Mhf2 are viable but display mitotic defects, for example, delayed prophase to anaphase transition, and greater prevalence of anaphase bridges (Amano et al. 2009). In human cells, the MHF complex is seemingly redundant for the assembly of CCAN components at the centromere, nevertheless, the knockdown of Mhf1 in HeLa cells leads to a more pronounced effect with most of the mitotic cells displaying misaligned chromosomes at the metaphase plate. Besides, the loss of MHF changes the structure of the outer kinetochore, reducing the amount of Ndc80 and KNL1, and increasing the intra-kinetochore distance between Ndc80 and CENP-T (Schleiffer et al. 2012). I was intrigued to know whether the centromeric function of the MHF complex was conserved in fission yeast, therefore, I set about investigating whether the *mhf1* Δ and *mhf2* Δ mutants exhibited phenotypes consistent with a defect in the kinetochore. Further investigation led me to uncover a previously unknown role of the MHF complex, which is in inhibiting the septation initiation network (SIN).

6.2 Results

6.2.1 Chromosome segregation is impaired in strains that are deficient in *mhf1*, *mhf2*, *cenp-t* or *cenp-w*

Cytological examination with staining with the DNA-specific dye, SYBR green, and the septum staining dye, calcofluor white, revealed that in both the *mhf1* Δ and *mhf2* Δ mutants, chromosome segregation is impaired (Figure 6.1). Many cells showed a septum with failed chromosome segregation in small cells.

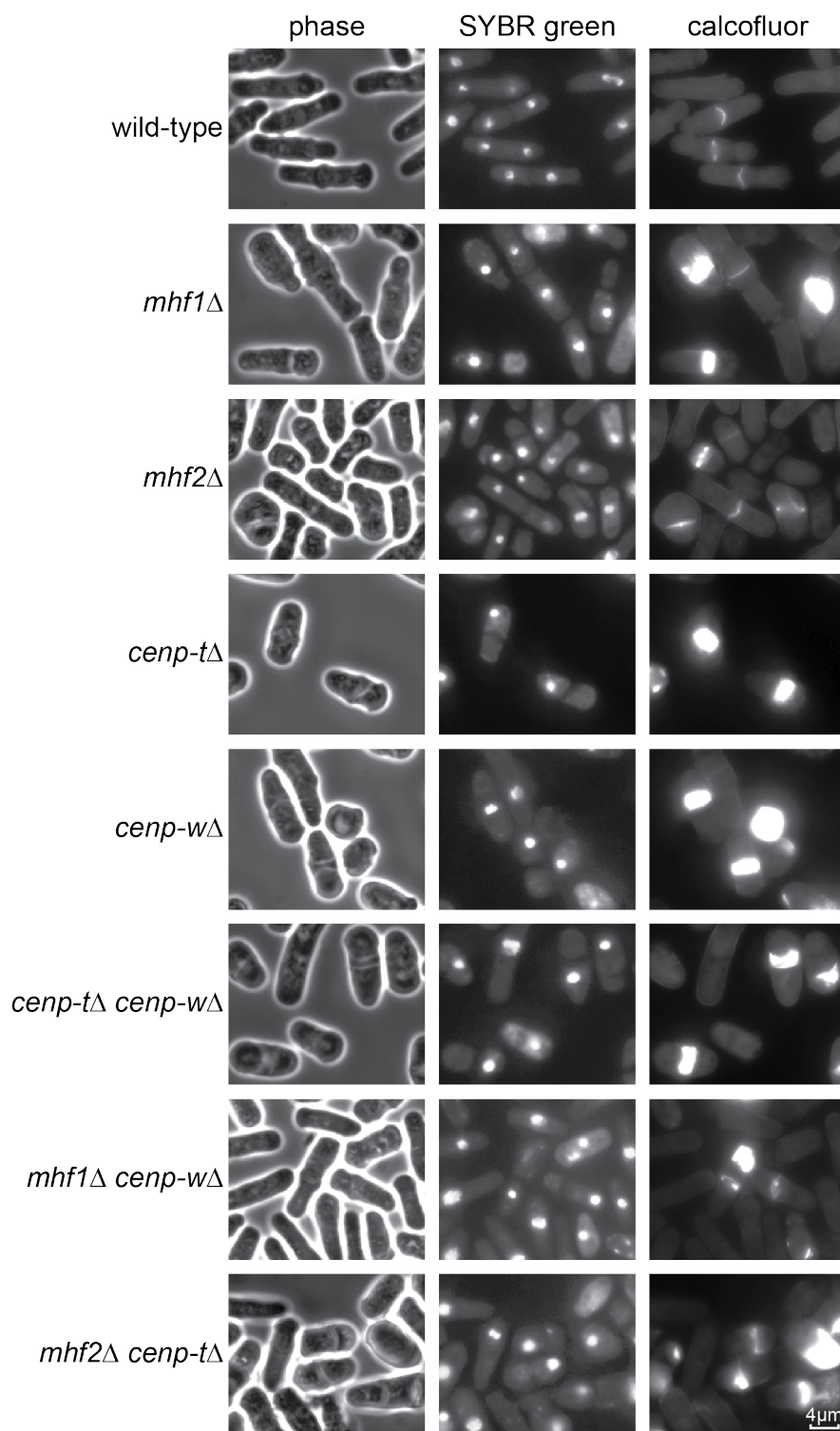


Figure 6.1 Chromosome segregation is impaired in strains that are deficient in *mhf1*, *mhf2*, *cenp-t* or *cenp-w*

Aberrant chromosome segregation in strains FO656, MCW4639, MCW5112, MCW6450, MCW6455, MCW6908, MCW7026 and MCW6899. Example epifluorescent images of SYBR green stained and calcofluor white stained cells taken from asynchronously growing cultures are shown.

The division septum has been laid down in these dividing cells, but the entire DNA is in one daughter, indicating a total failure of chromosome segregation. To investigate that further, I looked to see whether the *cenp-tΔ*, *cenp-wΔ* and double mutant combinations exhibited a similar phenotype (Figure 6.1). ~40 % of septated cells had failed chromosome segregation (Figure 6.2). Interestingly, a large number of cells in these mutant strains exhibit abnormally high levels of aberrant calcofluor white staining, suggestive of over-active septum formation. Altogether these data indicate that the Mhf1-Mhf2-CENP-T-CENP-W complex is needed for normal chromosome segregation as might be expected for an integral component of the CCAN. However, the prevalence of small septated cells with failed segregation, and abnormally high levels of calcofluor white staining suggested that there might also be a problem with the proper timing and control of septum formation.

6.2.2 Generation of a conditional *mhf1/2* mutant

As shown in Figure 6.5 and Figure 4.2 respectively, the *mhf1* and *mhf2* null mutants although viable, are very sick and grow very poorly compared to the wild-type at standard growth temperature, reflecting a high rate of stochastic cell death. Moreover, these sick strains readily acquire suppressor mutations that restore near normal levels of growth and viability (data not shown). These properties of *mhf1/2* deletion mutants complicates their analysis by hampering disentanglement of cause and effect of MHF loss. Consequently, in order to better study the immediate and first consequence of MHF ablation, I set about making a conditional or temperature sensitive *mhf1/2* mutant (Figure 6.3).

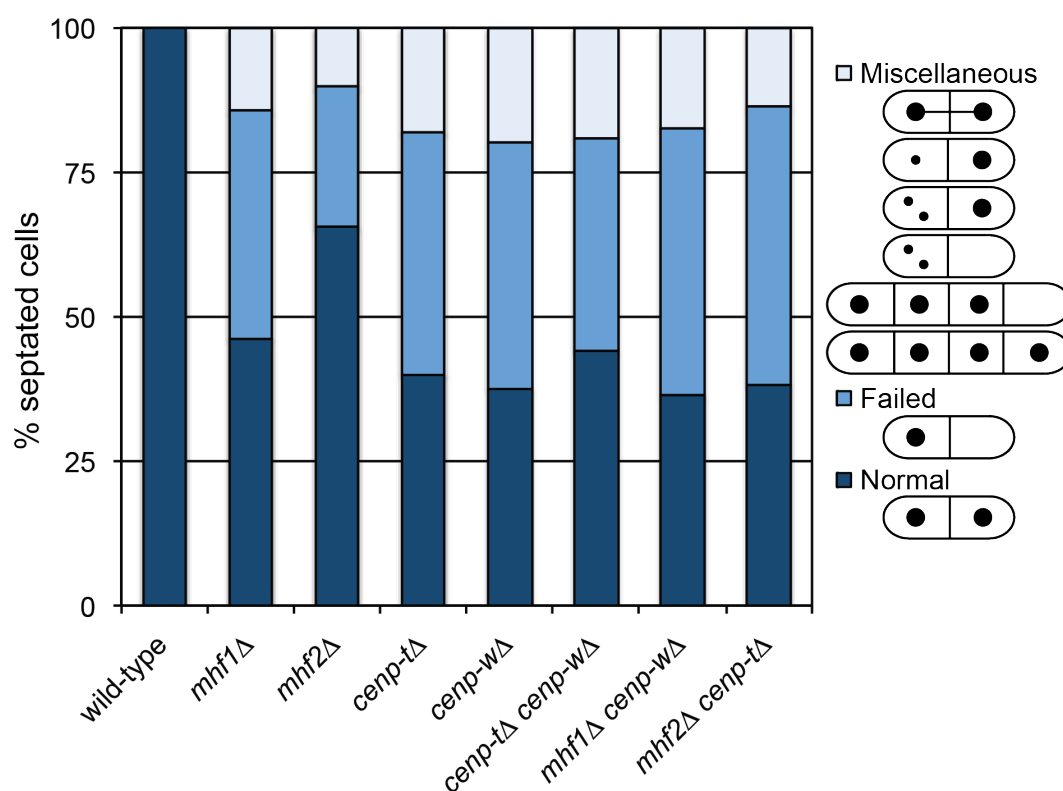


Figure 6.2 Chromosome segregation is impaired in strains that are deficient in *mhf1*, *mhf2*, *cenp-t* or *cenp-w*

Frequency of aberrant chromosome segregation among septated cells in strains FO656, MCW4639, MCW5112, MCW6450, MCW6455, MCW6908, MCW7026 and MCW6899. Values are based on the analysis of at least 100 septated cells from asynchronously growing cultures at 25 °C.

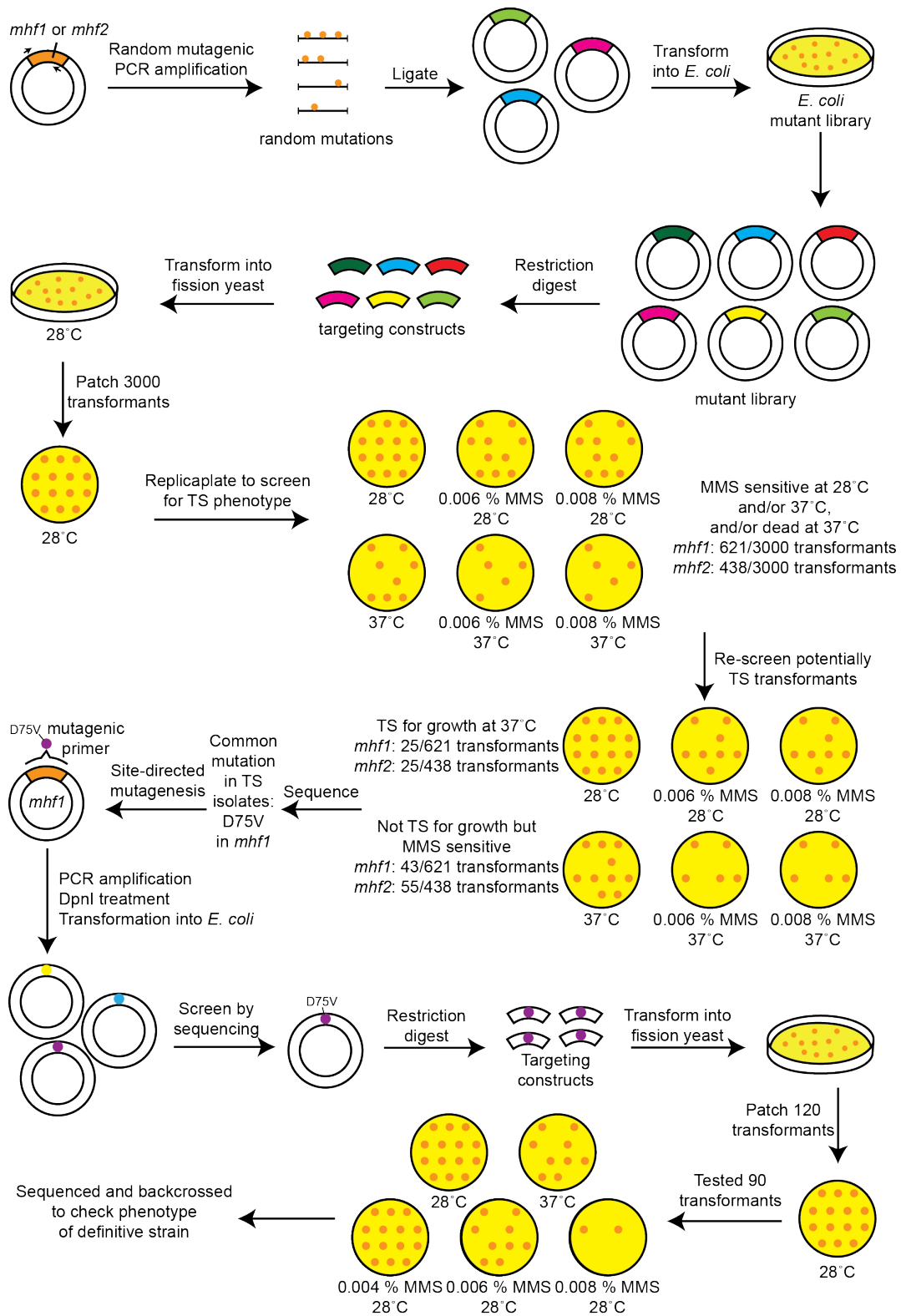


Figure 6.3 Schematic for the generation of a temperature sensitive *mhf1/2* mutant

TS denotes temperature sensitive. Refer to text for more details.

To isolate temperature sensitive *mhf1/2* mutants, I generated a library of *mhf1* and *mhf2* random mutations using random mutagenic PCR amplification (Figure 6.3). The mutagenized PCR product was digested and ligated back into backbones. The resultant plasmids were next transformed into *E. coli* to create mutant libraries each consisting of ~5000 transformants (Figure 6.3). The plasmid mutant libraries were isolated from the *E. coli* transformants following amplification and subject to restriction digest to produce fission yeast targeting constructs. The targeting constructs were transformed into fission yeast at 28 °C, and 3000 transformants for *mhf1* and *mhf2* each were patched onto YES plates at 28 °C (Figure 6.3). The patched plates were then replicated onto YES, 0.006 % MMS and 0.008 % MMS plates, and incubated at 28 °C (the permissive temperature) and 37 °C (the restrictive temperature) to identify potentially temperature sensitive transformants (Figure 6.3). The transformants were tested for MMS hypersensitivity in order to determine if temperature sensitivity of the transformants related to impairment of function. From the first screen, 621 out of 3000 *mhf1* transformants and 438 out of 3000 *mhf2* transformants were MMS sensitive at 28 °C and/or 37 °C, and/or dead at 37 °C (Figure 6.3).

The putative temperature sensitive transformants were re-screened by spotting onto YES, 0.006 % MMS and 0.008 % MMS plates, at 28 °C and 37 °C (Figure 6.3). Out of the 621 *mhf1* transformants, 25 of them were temperature sensitive for growth at 37 °C (refer to Table S6.1 in the appendix), while 43 of them were not temperature sensitive for growth at 37 °C, but MMS sensitive (Figure 6.3 and Table S6.2 in the appendix). The *mhf1* genomic region was sequenced in six temperature sensitive *mhf1* transformants (# 138, 153, 255,

260, 343 and 506) to identify candidate mutations (refer to Table S6.1 and Table S6.5 in the appendix). Similarly, out of the 438 *mhf2* transformants, 25 of them were temperature sensitive for growth at 37 °C (refer to Table S6.3 in the appendix), while 55 of them were not temperature sensitive for growth at 37 °C, but MMS sensitive (Figure 6.3 and Table S6.4 in the appendix). Two temperature sensitive *mhf2* transformants (# 9 and 67) were sequenced to identify candidate mutations (refer to Table S6.3 and Table S6.5 in the appendix). All of the temperature sensitive isolates that were sequenced carried at least one mutation that would result in a coding change (refer to Table S6.5 in the appendix). Intriguingly two of the *mhf1* isolates (#138 and #255) carried the same mutation, which results in aspartate (D) 75 changing to valine (V) (refer to Table S6.5 in the appendix).

To confirm that a D75V mutation in Mhf1 causes a temperature sensitive phenotype, I constructed a strain containing just this mutation. To do this I first used site-directed mutagenesis to introduce the required mutation into *mhf1* on the gene targeting plasmid (Figure 6.3), excised the targeting DNA fragment by restriction digestion and transformed this into the wild-type strain FO656. Geneticin resistant transformants were patched onto YES plates at 28 °C and 90 of these were tested by spotting onto YES, 0.004 % MMS, 0.006 % MMS and 0.008 % MMS at 28 °C, and YES at 37 °C (Figure 6.3 and Figure 6.4). Isolate # 6, exhibited the expected temperature sensitivity and MMS hypersensitivity and was therefore subjected to a diagnostic colony PCR to confirm that the *mhf1*^{D75V}::*kan*^R cassette had integrated at the correct site, followed by DNA sequencing to confirm the presence of the D75V mutation. The strain was then backcrossed to a wild-type strain to confirm that its

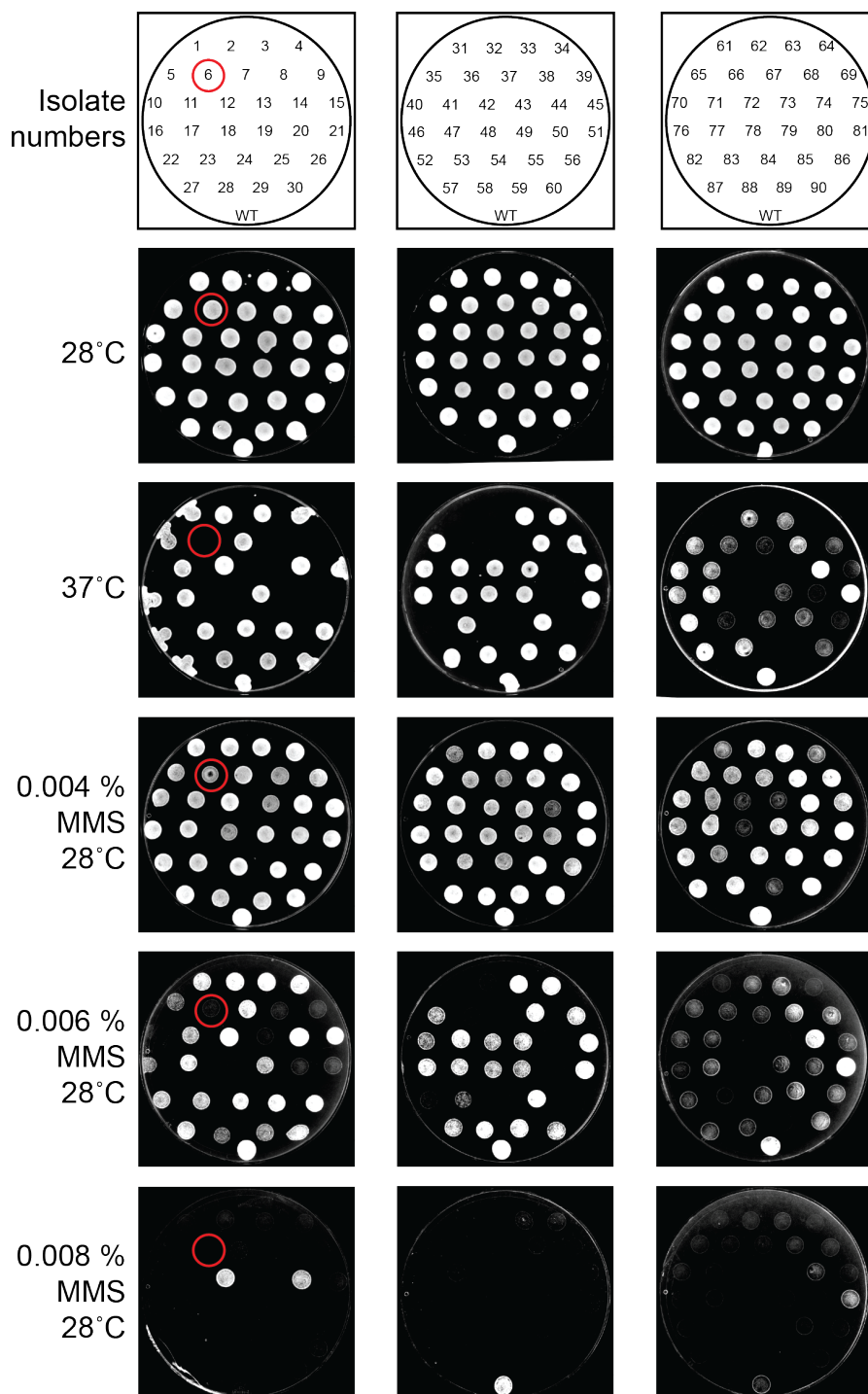


Figure 6.4 Screening putative *mhf1*^{D75V} transformants for temperature sensitivity and MMS hypersensitivity

Transformants were resuspended in sterile water and spotted onto the indicated plates so that growth and MMS sensitivities could be compared. The plates were photographed after 3 days growth at the indicated temperatures. Isolate 6 (circled in red) was sequenced, backcrossed, and subsequently selected to be the definitive *mhf1*^{TS} strain.

mutant phenotypes were indeed linked to the D75V mutation. The resultant definitive temperature sensitive *mhf1* strain will be referred to as *mhf1*^{TS} hereafter for simplicity.

6.2.3 The *mhf1*^{TS} mutant is temperature sensitive

To determine more precisely the temperature at which the *mhf1*^{TS} mutant is temperature sensitive, I analysed its growth at temperatures ranging from 25 °C to 37 °C. MMS sensitivity at 28 °C was also analysed. Unlike a *mhf1*Δ mutant, the *mhf1*^{TS} mutant grows as well as wild-type at temperatures ranging from 25 °C to 30 °C (Figure 6.5). However, at temperatures above 30 °C the *mhf1*^{TS} mutant starts to show impaired growth and at 36 °C and 37 °C virtually no growth is detected (Figure 6.5). The *mhf1*^{TS} mutant also exhibits hypersensitivity to MMS at 28 °C, but this is significantly less than observed with a *fml1*Δ mutant (Figure 6.5). Structural data from our collaborators Stephen Carr and Simon Phillips at the Research Complex at Harwell have shown that the D75V mutation in *mhf1*^{TS} is likely to disrupt a salt bridge in the tetramerisation interface of the MHF tetramer, and at 37 °C, the MHF tetramer falls apart resulting in Mhf1-Mhf2 heterodimers (unpublished data).

Since *mhf1*^{TS} is hypersensitive to MMS at 28 °C (its permissive temperature), under the strict definition of what a temperature sensitive mutant is, *mhf1*^{TS} is not perfectly temperature sensitive; instead, it is better classified as a hypomorphic mutant. Nevertheless, *mhf1*^{TS} grows a lot better than the null mutant, which is very sick, and therefore is a more robust strain to work with. However, like *mhf1*Δ, *mhf1*^{TS} readily acquires suppressor mutations that enable it to grow at its restrictive temperature as can be seen from the few

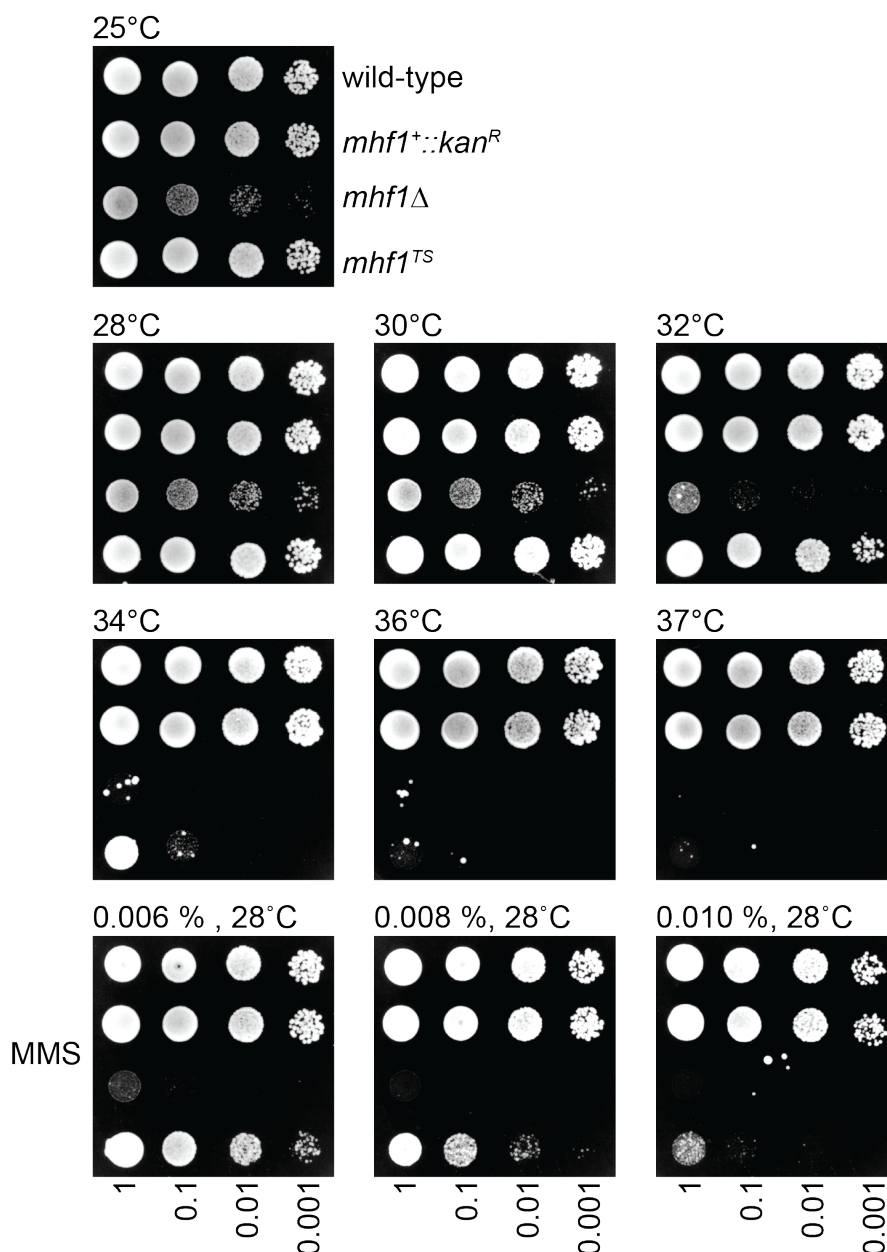


Figure 6.5 *mhf1^{TS}* grows normally at 25 °C, 28 °C and 30 °C, but is dead at 37 °C

Spot assays comparing the growth and MMS sensitivities of strains FO656, MCW5991, MCW4639 and JN13 after 4 days growth at the respective temperatures.

colonies appearing at 37 °C (Figure 6.5). For this reason, precautions have to be taken when handling these strains, including culturing them in liquid culture at 25 °C for only a limited period so that they go through relatively few generations before testing them.

6.2.4 The *mhf1*^{TS} mutant exhibits phenotypes consistent with premature activation of the septation initiation network at its restrictive temperature

To determine the terminal phenotype of the *mhf1*^{TS} mutant at its restrictive temperature, *mhf1*^{TS} was grown alongside the wild-type at 25 °C and 37 °C for 24 hours. The wild-type cells segregated DNA normally. However, with the *mhf1*^{TS} mutant, there were a lot of short septated cells with failed chromosome segregation – something characteristic of the deletion mutant (Figure 6.1 and Figure 6.6A). To evaluate the extent to which the temperature sensitive mutation was causing this phenotype, I compared the proportion of cells failing to segregate DNA in the *mhf1*^{TS} mutant and the wild-type. Additionally, I sought to establish after how many cell cycles at the restrictive condition would this phenotype appear.

At 25 °C, virtually all of the wild-type and *mhf1*^{TS} cells segregated DNA normally. Interestingly, after 9 hours of shifting to the restrictive temperature, there was a 60 % accumulation of cells with failed DNA segregation in *mhf1*^{TS} (Figure 6.6B). Clearly, failed chromosome segregation is one of the pathologies that occurs early on when MHF is attenuated since at 9 hours (i.e. within ~3 generations) the majority of septated cells have accumulated this problem. Similar to *mhf1*Δ and *mhf2*Δ mutants, the *mhf1*^{TS} mutant, after

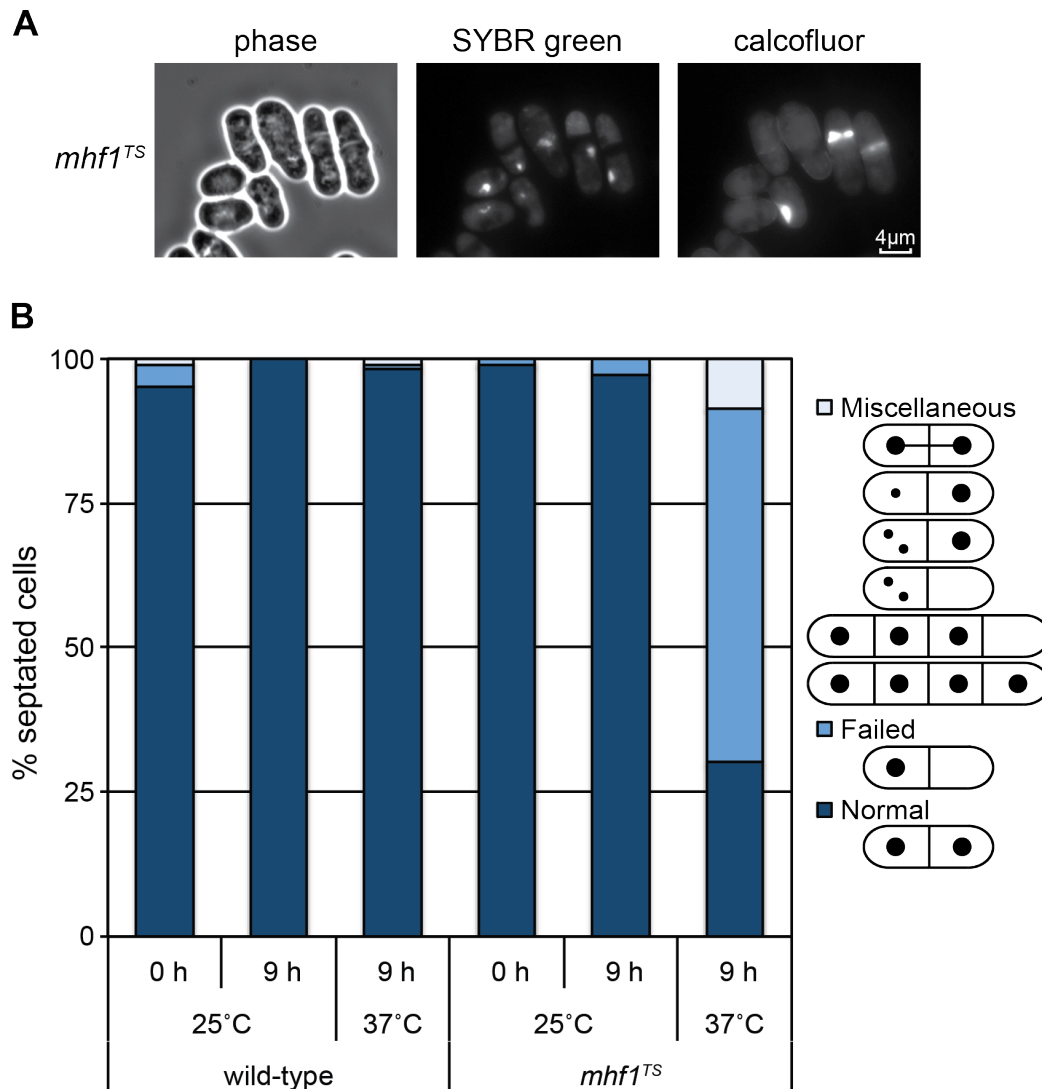


Figure 6.6 The *mhf1^{TS}* mutant exhibits impaired chromosome segregation at its restrictive temperature

(A) Aberrant chromosome segregation in strain JN13. Example epifluorescent images of SYBR green stained and calcofluor white stained cells taken from an asynchronously growing culture after 9 hours at 37 °C is shown. (B) Frequency of aberrant chromosome segregation among septated cells in strains FO656 and JN13. Values are based on the analysis of at least 100 septated cells from asynchronously growing cultures after the respective number of hours at the respective temperatures.

9 hours at 37 °C, exhibits a large proportion of cells (~25 %) with abnormally high levels of aberrant calcofluor white staining (Figure 6.7).

A comparison of the mean septated cell length of wild-type and *mhf1^{TS}* cells after 9 hours at 37 °C showed that they both have similar means of ~11 µm (Figure 6.8A). However, within *mhf1^{TS}*, the mean length of *mhf1^{TS}* cells that had normal DNA segregation was ~13 µm, significantly longer ($P = 0.017$) than the mean wild-type septated cell length. The mean length of *mhf1^{TS}* cells with failed DNA segregation was ~10 µm, significantly shorter ($P = 0.001$) than the mean wild-type septated cell length. Also, *mhf1^{TS}* cells that had failed DNA segregation were significantly shorter ($P < 0.001$) than *mhf1^{TS}* cells that segregated DNA normally (Figure 6.8A). Further, *mhf1^{TS}* cells with aberrant calcofluor white staining had a mean length of ~9 µm, and was also significantly shorter ($P < 0.001$) than *mhf1^{TS}* cells that segregated DNA normally (Figure 6.8A). Importantly, there was a broader distribution of septated cell lengths in *mhf1^{TS}*, ranging 6 to 22 µm, compared to the wild-type septated cell lengths ranging from 6 to 16 µm ($P < 0.001$) (Figures 6.8B and 6.8C). These data suggest that following attenuation of the MHF complex, cells undergo septation prematurely. The septum is laid down in interphase, before the cells have completed a normal amount of growth and undergone mitosis, hence the shorter mean cell length and the frequent appearance of septated cells containing DNA in just one daughter.

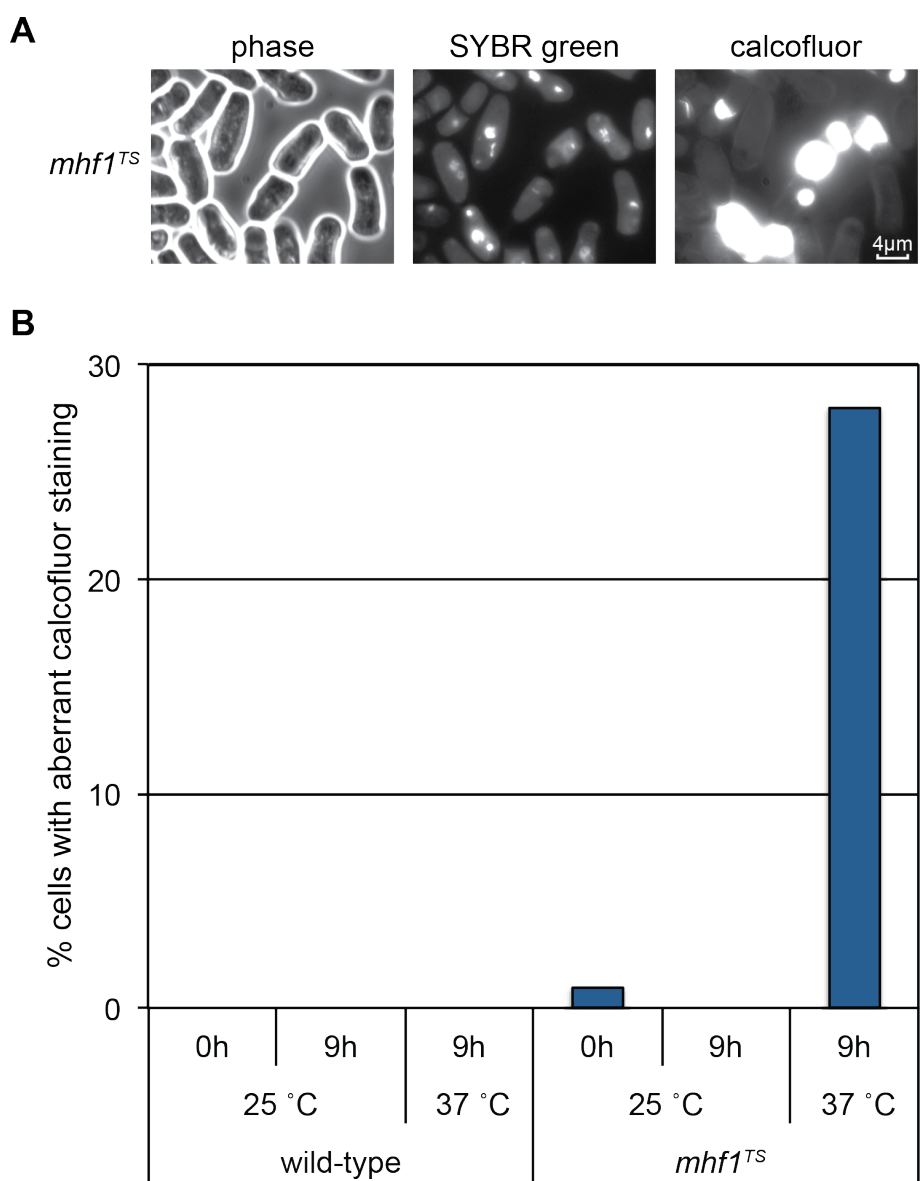


Figure 6.7 The *mhf1^{TS}* mutant exhibits aberrant calcofluor white staining at its restrictive temperature

(A) Aberrant calcofluor white staining in strain JN13. Example epifluorescent images of SYBR green stained and calcofluor white stained cells taken from an asynchronously growing culture after 9 hours at 37 °C is shown. (B) Percentage of cells with aberrant calcofluor white staining in strains FO656 and JN13. Values are based on the analysis of at least 100 septated cells from asynchronously growing cultures after the respective number of hours at the respective temperatures.

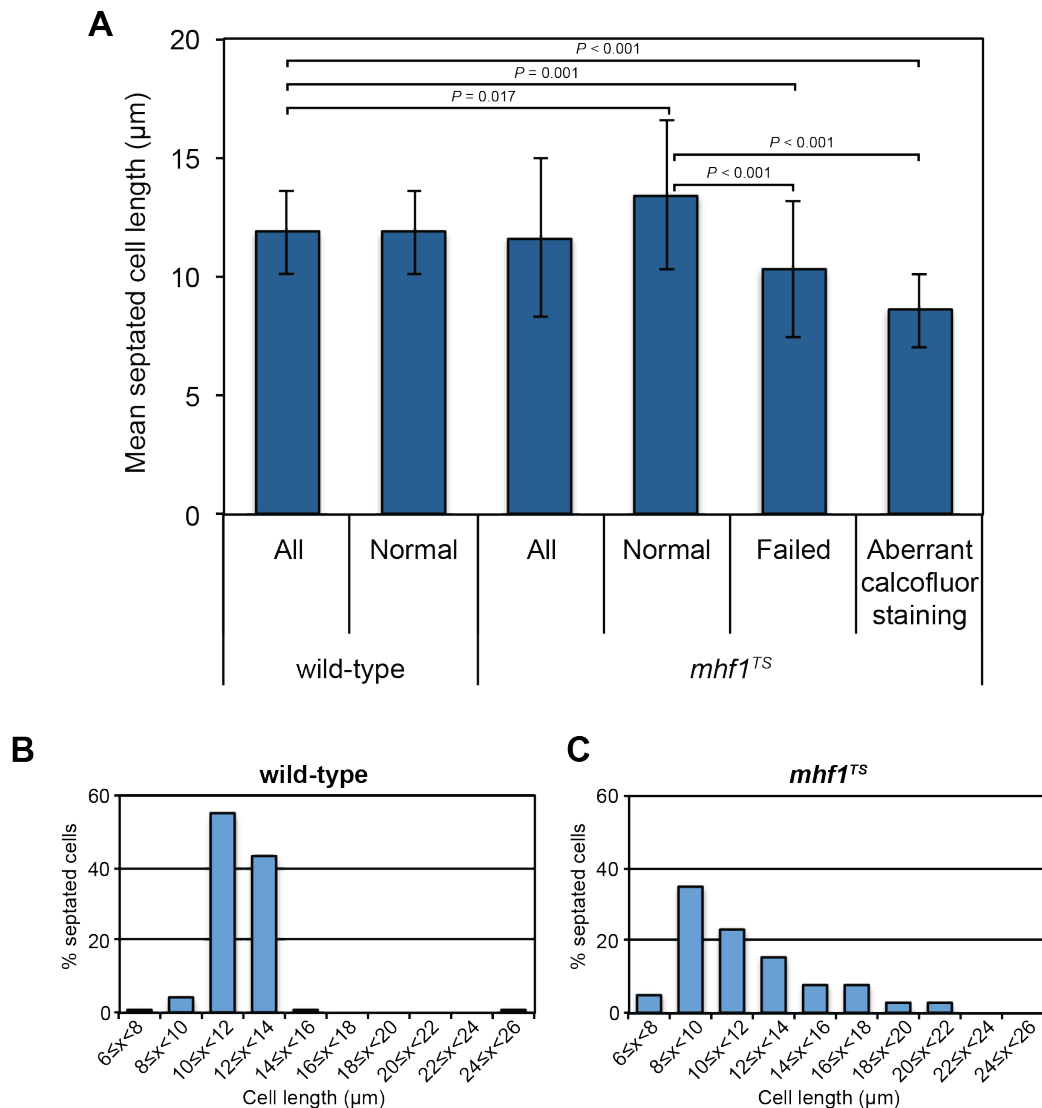


Figure 6.8 Mean septated cell lengths of the wild-type and the *mhf1*^{TS} mutant strains at the restrictive temperature

(A) Mean septated cell lengths in strains FO656 and JN13 from asynchronously growing cultures after 9 hours at 37 °C. Values are based on the analysis of at least 100 septated cells. Error bars represent $\pm$ one standard deviation. Statistical significance in comparison with “all wild-type” and “normal *mhf1*^{TS}” cells is indicated. (B and C) The distribution of lengths of septated cells in strains (B) wild-type, FO656 ($n = 105$) and (C) *mhf1*^{TS}, JN13 ($n = 151$) from asynchronously growing cultures after 9 hours at 37 °C. Values are based on the analysis of at least 100 septated cells.

6.2.5 The effect of *wee1* Δ on the aberrant chromosome segregation phenotype of the *mhf1*^{TS} mutant

As seen in Figure 6.8A, a comparison of the *mhf1*^{TS} mutant that had normal and failed DNA segregation revealed a significant reduction in the mean septated cell length from ~13 μ m to ~10 μ m (Figure 6.8A). This short cell length of the *mhf1*^{TS} mutant is reminiscent of the *wee1* Δ mutant, which exhibits a reduced cell length at septation (Nurse 1975). Wee1 is a key protein kinase that negatively regulates the cyclin-dependent kinase Cdk1. Cdk1 drives cells from G2 to M phase. In the absence of Wee1, Cdk1 is prematurely activated and cells enter mitosis early, before completing a normal amount of growth prior to division (Lundgren et al. 1991). If attenuation of MHF results in premature activation of the SIN prior to mitosis, it is conceivable that deletion of *wee1* would re-establish a correct order of events allowing mitosis to occur prior to septation in the absence of MHF.

To see whether the *wee1* Δ mutation would have an effect on the aberrant chromosome segregation phenotype of the *mhf1*^{TS} mutant, the *mhf1*^{TS} mutation was introduced into the *wee1* Δ strain. Unfortunately, at the restrictive temperature of 37 °C, the *wee1* Δ single mutant exhibited high rates of aberrant chromosome segregation, and rather than improving chromosome segregation in a *mhf1*^{TS}, it made it worse (Figure 6.9). However, in studies performed by another member of my laboratory, it was found that a *mhf1* Δ *wee1* Δ double mutant at 25 °C exhibits far fewer septated cells with failed DNA segregation than a *mhf1* Δ single mutant. This is consistent with the idea

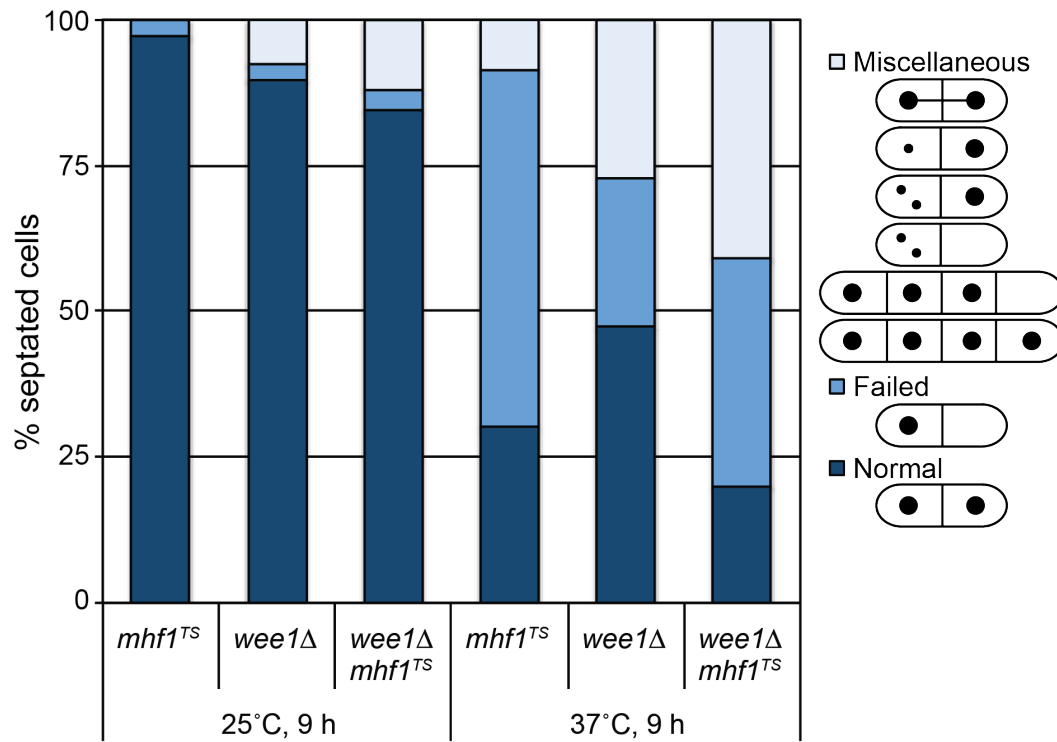


Figure 6.9 The effect of *wee1Δ* on the aberrant chromosome segregation phenotype of the *mhf1^{TS}* mutant

Frequency of aberrant chromosome segregation among septated cells in strains JN13, MCW5390 and JN79. Values are based on the analysis of at least 100 septated cells from asynchronously growing cultures after the respective 9 hours at the respective temperatures. *mhf1^{TS}* data shown in this figure the same as in Figure 6.6B.

that allowing mitosis to occur earlier can restore the proper temporal order of events that is thrown out of kilter by loss of MHF.

6.2.6 Does the impairment of MHF alter localisation of the Cdc7 protein?

Premature septation in the absence of MHF suggests a third role of the MHF, which is in controlling or influencing the activation of the SIN. The SIN is the pathway that controls the formation of the division septum in fission yeast. In the SIN cascade, Spg1 acts as the key GTPase. When it gets activated, Spg1-GTP binds to its effector kinase Cdc7 to elicit the activation of downstream SIN kinases, which translocate to the cytokinetic ring to promote cytokinetic ring assembly and constriction (Johnson et al. 2012). To investigate this proposed third role of the MHF, I monitored the activation of Spg1 through analysing the recruitment of Cdc7-GFP to the spindle pole body to see whether the presence or absence of Mhf1 would disrupt the pattern of localisation in a way that is indicative of premature activation of the SIN. Ideally, this analysis would have been conducted with the *mhf1^{TS}* conditional mutant, however since Cdc7-GFP foci were undetectable at 37 °C, the *mhf1Δ* was used instead.

The pattern of Cdc7 localisation to the spindle pole body at set phases of the cell cycle has been well described in a wild-type cell (Figures 6.10A and 6.10B). At metaphase, Spg1 in its GTP-bound form recruits Cdc7 to both spindle pole bodies (Mehta and Gould 2006) (Figures 6.10A and 6.10B). Upon the onset of anaphase B, whereas the Cdc7 signal fades away at the old spindle pole body, it grows brighter at the new spindle pole body, peaking

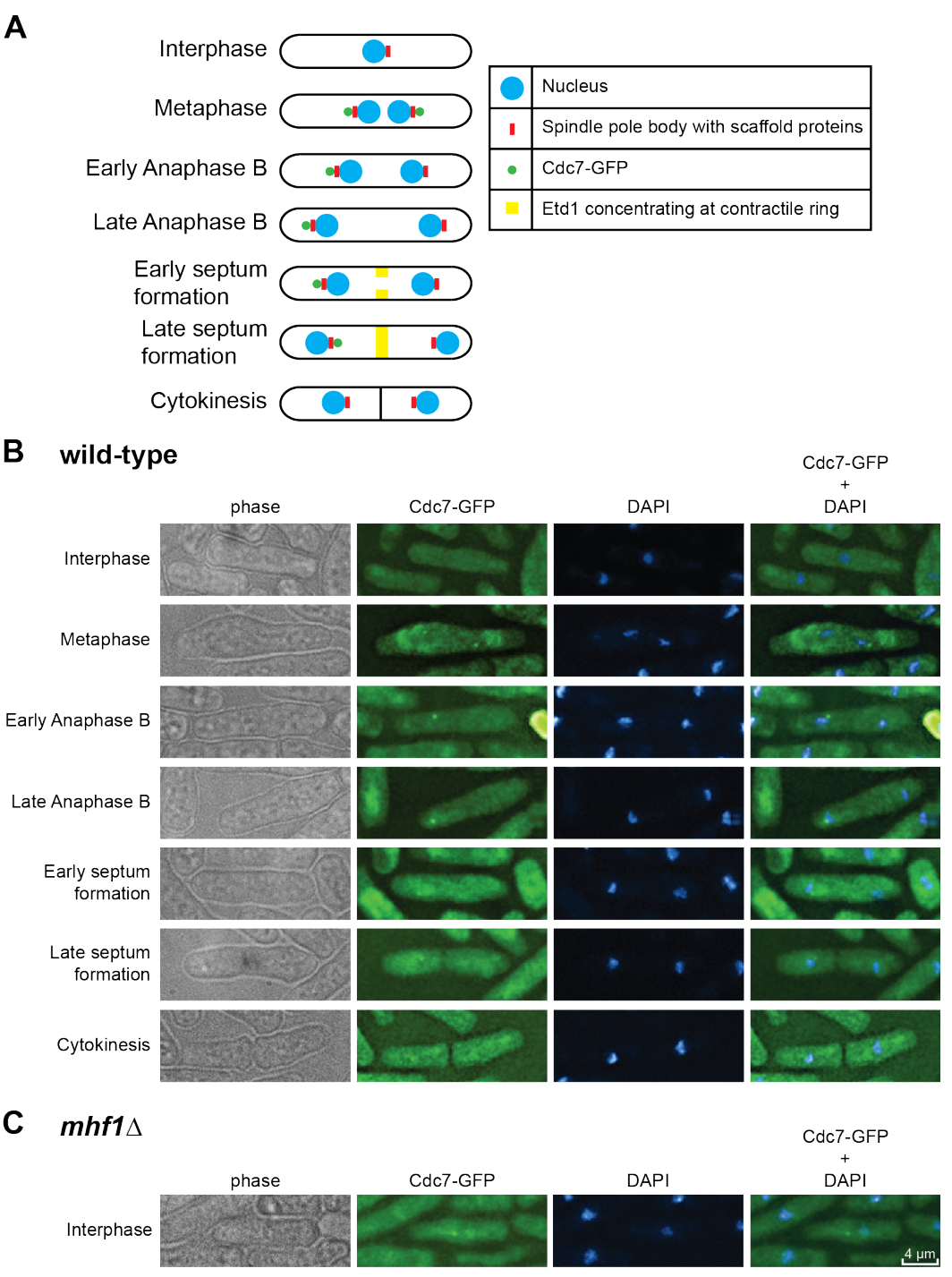


Figure 6.10 Localisation of Cdc7-GFP during interphase and mitosis

(A) The cartoon depicts the localisation of Cdc7-GFP during interphase and mitosis in a wild-type cell. (B) Example epifluorescent images of DAPI stained cells depicting the localisation of Cdc7-GFP during interphase and mitosis from an asynchronously growing culture. The strain is MCW6864. (C) Example epifluorescent images of DAPI stained cells depicting the localisation of Cdc7-GFP during interphase in the absence of Mhf1 from an asynchronously growing culture. The strain is MCW6809.

as the spindle pole bodies approach the cell tips (Sohrmann et al. 1998; Garcia-Cortes and McCollum 2009) (Figures 6.10A and 6.10B). During cytokinesis, the disappearance of Cdc7 from the new spindle pole body is associated with the cytokinetic ring constriction (Garcia-Cortes and McCollum 2009) (Figures 6.10A and 6.10B).

In the wild-type and the *mhf1* Δ strains, there is a similar percentage of cells that are mononucleates, binucleates and septated binucleates, albeit the wild-type has no septated mononucleates (i.e. septated cells with failed chromosome segregation) (Figure 6.11A). In the *mhf1* Δ *cdc7-GFP* culture, ~34 % of septated cells exhibit the failed DNA segregation phenotype which is comparable to ~40 % in *mhf1* Δ (Figure 6.2 and Figure 6.11A). In comparing the mononucleates, there are noticeably more *mhf1* Δ mononucleates that have a Cdc7-GFP focus than the wild-type (~15 % of *mhf1* Δ compared to ~2 % wild-type) (Figure 6.11B). Where septated mononucleates are present in *mhf1* Δ , ~18 % had the Cdc7-GFP focus on the DNA side, whereas ~2 % of the cells had a Cdc7-GFP focus on the non-DNA side (Figure 6.11C). There is a general trend of more binucleates with one focus as opposed to two in the wild-type and *mhf1* Δ , with ~48 % and ~35 % having one focus respectively, and ~11 % having two foci in both strains (Figure 6.11D). This is expected due to the asymmetric nature of the SIN. When the two DNA masses are very close together in a binucleate, they are expected to each have a Cdc7-GFP focus but as the nuclei move apart, only one focus remains (Figures 6.10A and 6.10B). As for the septated binucleates, only a minority (less than 22 %) of wild-type and *mhf1* Δ cells have a Cdc7-GFP focus, and out of this relatively small population only one focus tends to be seen. However, ~7 % of *mhf1* Δ

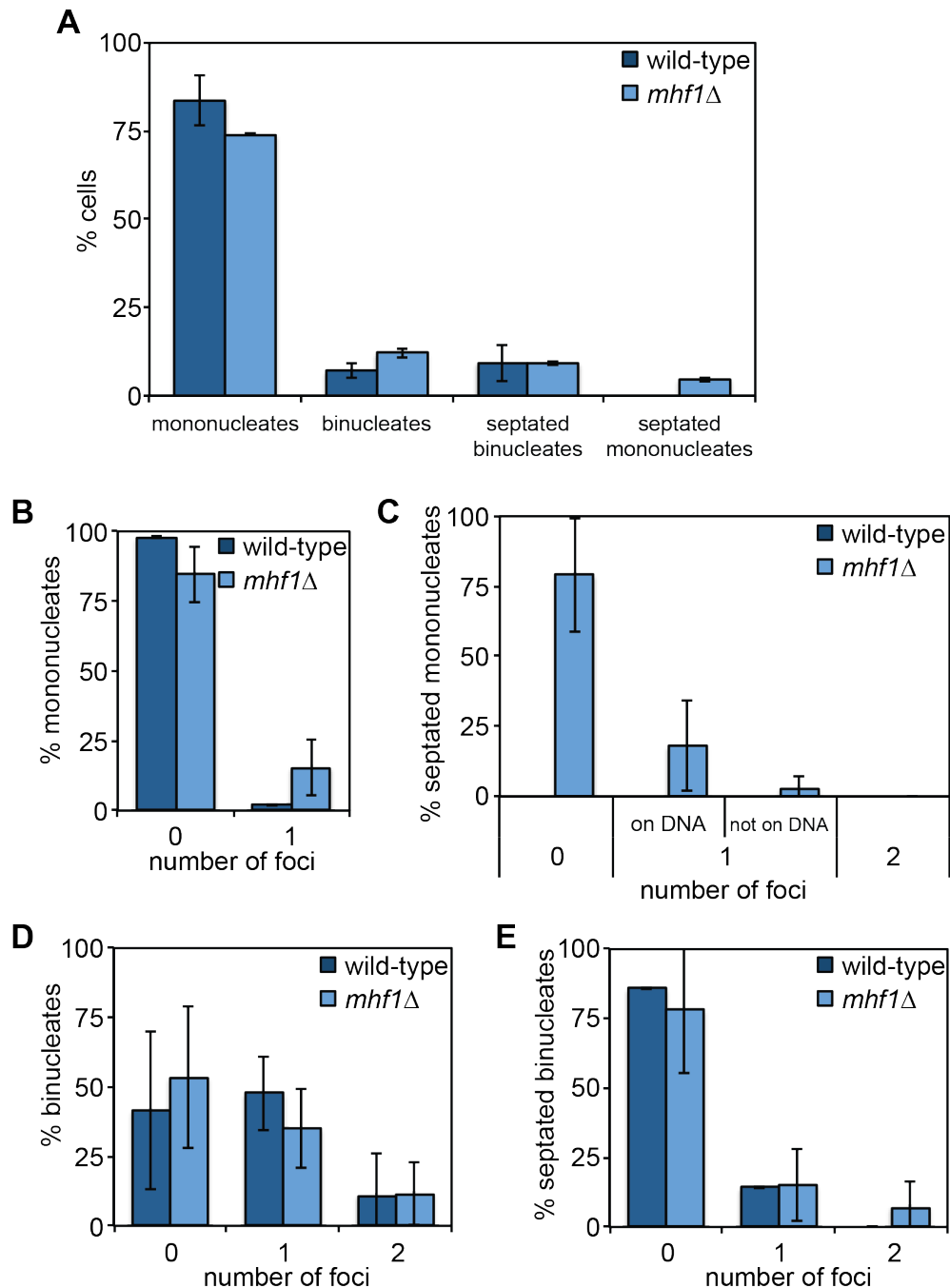


Figure 6.11 Does the impairment of MHF alter the localisation of the Cdc7-GFP protein?

(A) Percentage of cells that are mononucleates, binucleates, septated binucleates and septated mononucleates. (B) Percentage of mononucleates that have 0 or 1 Cdc7-GFP foci. (C) Percentage of septated mononucleates that have 0, 1 or 2 Cdc7-GFP foci. (D) Percentage of binucleates that have 0, 1 or 2 Cdc7-GFP foci. (E) Percentage of septated binucleates that have 0, 1 or 2 Cdc7-GFP foci. Strains featured are MCW6864 and MCW6809 for wild-type and *mhfl* Δ respectively. Values are based on the analysis of the mean of two independent experiments with at least 300 cells from asynchronously growing cultures. Error bars represent one standard deviation.

septated binucleates exhibit two foci (Figure 6.11E). Overall these data suggest that the normal pattern of localisation of Cdc7-GFP foci is partially disrupted in the absence of Mhf1. In particular the appearance of Cdc7-GFP foci in a greater proportion of interphase cells is consistent with the idea that MHF is required to prevent the premature activation of the SIN.

Whilst analysing Cdc7-GFP foci I observed that there was a tendency for foci to be further from the DAPI stained DNA in *mhf1* Δ cells compared to wild-type cells (Figure 6.10C). Measurement of the distance between Cdc7-GFP and DAPI stained DNA revealed that there was indeed a slight increase in septated and unseptated binucleate cells (from a mean of 0.17 μm in wild-type to 0.56 μm in *mhf1* Δ) ($P < 0.001$) (Figures 6.12A and 6.12B). However, the distance between Cdc7-GFP and the DAPI stained DNA was much greater in the few mononucleate cells that exhibited foci (Figures 6.12C and 6.12D). This was true for both wild-type (mean distance of 0.56 μm) ($P < 0.001$) and *mhf1* Δ (mean distance of 0.99 μm) ($P < 0.001$). Although the mean distance between Cdc7-GFP foci and DAPI stained DNA seems greater in *mhf1* Δ mononucleates, the rarity of such cells in the wild-type makes this comparison questionable. Altogether these data show that Cdc7-GFP foci tend to be further away from the DNA when Mhf1 is missing, which suggests that the spindle pole body may be less securely anchored to the nuclear membrane.

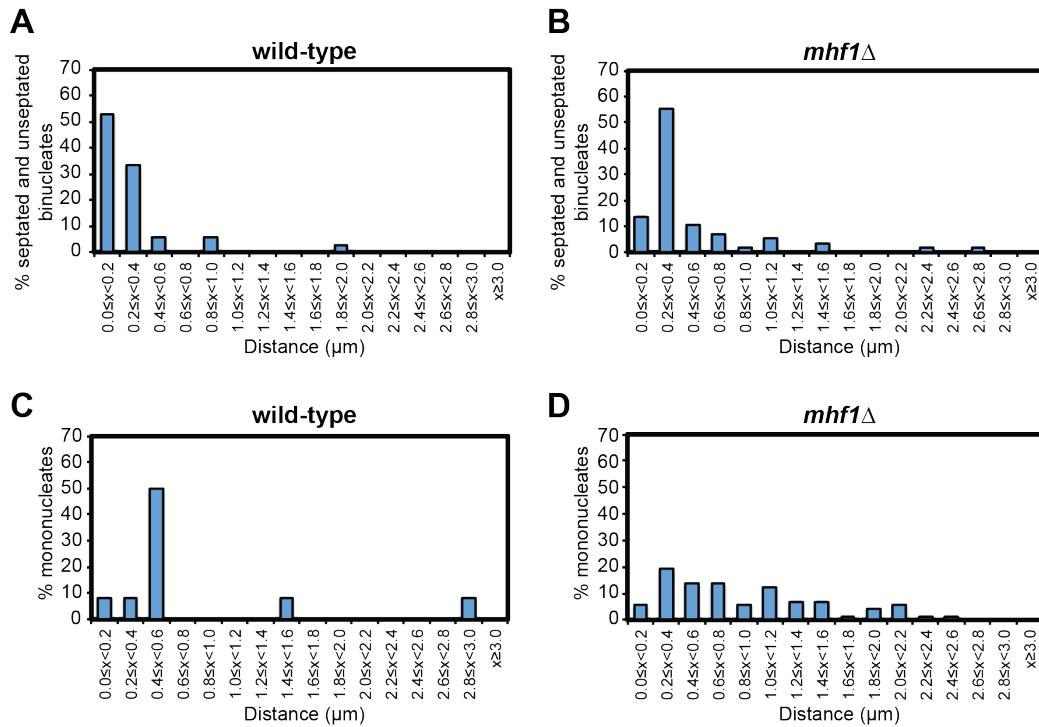


Figure 6.12 Distance from DAPI stained DNA to Cdc7-GFP foci

The distribution of distances from DAPI stained DNA to Cdc7-GFP foci in (A) wild-type septated and unseptated binucleates ($n = 36$) and (B) *mhf1* Δ septated and unseptated binucleates ($n = 58$), (C) wild-type mononucleates ($n = 12$) and (D) *mhf1* Δ mononucleates ($n = 73$) in strains MCW6864 and MCW6809 from two independent asynchronously growing cultures.

6.2.7 Does the impairment of MHF alter localisation of the Spg1 protein?

To further investigate MHF's putative role in inhibiting SIN activation during interphase, I compared the localisation of Spg1-GFP at the spindle pole body in wild-type and *mhf1* Δ cells.

The pattern of Spg1 localisation to the spindle pole body at set phases of the cell cycle has been well described in a wild-type cell (Figures 6.1A and 6.13B). At metaphase, Spg1 in its GTP-bound form recruits Cdc7 to both spindle pole bodies (Sohrmann et al. 1998; Mehta and Gould 2006) (Figures 6.13A and 6.13B). Upon the onset of anaphase B, Spg1 remains anchored to both spindle pole bodies. Its signal grows brighter at both spindle pole bodies, peaking as they approach the cell tips (Sohrmann et al. 1998) (Figures 6.13A and 6.13B). During cytokinesis, Spg1 remains associated to both spindle pole bodies in their daughter nuclei (Garcia-Cortes and McCollum 2009) (Figures 6.13A and 6.13B).

As observed for the strains carrying Cdc7-GFP, both wild-type and *mhf1* Δ strains harbouring Spg1-GFP exhibit similar percentages of cells that are mononucleates, binucleates and septated binucleates (Figure 6.14A). As before the main difference between the strains is the presence of septated mononucleates in the *mhf1* Δ mutant (Figure 6.14A). In the *mhf1* Δ *spg1*-GFP culture, ~50 % of septated cells exhibit the failed DNA segregation phenotype which is comparable to ~40 % in *mhf1* Δ (Figure 6.2 and Figure 6.14A). There is no major difference in the ability to detect Spg1-GFP foci in the wild-type

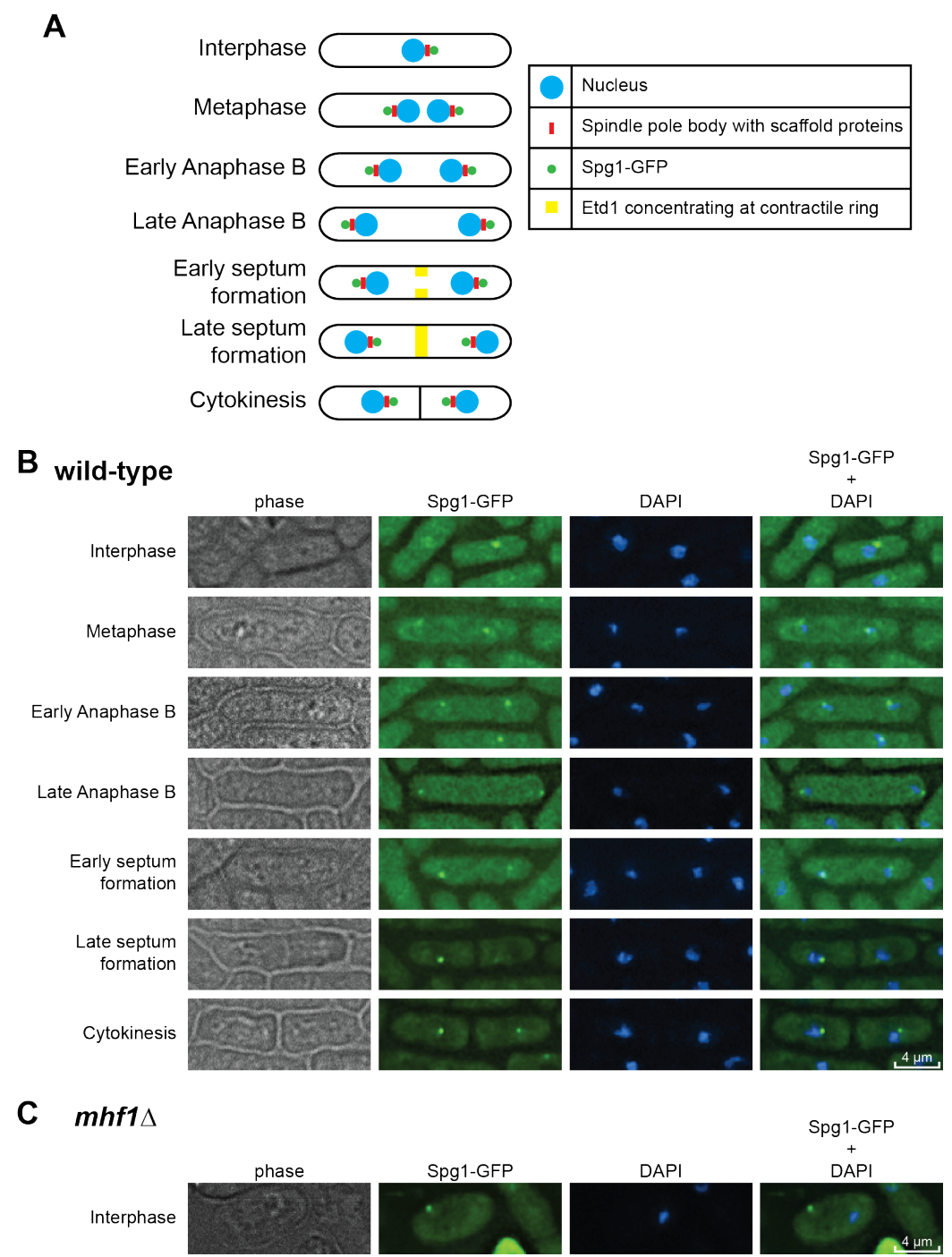


Figure 6.13 Localisation of Spg1-GFP during interphase and mitosis

(A) The cartoon depicts the localisation of Spg1-GFP during interphase and mitosis in a wild-type cell. (B) Example epifluorescent images of DAPI stained cells depicting the localisation of Spg1-GFP during interphase and mitosis from an asynchronously growing culture. The strain is MCW6675. (C) Example epifluorescent images of DAPI stained cells depicting the localisation of Spg1-GFP during interphase in the absence of Mhf1 from an asynchronously growing culture. The strain is MCW6717.

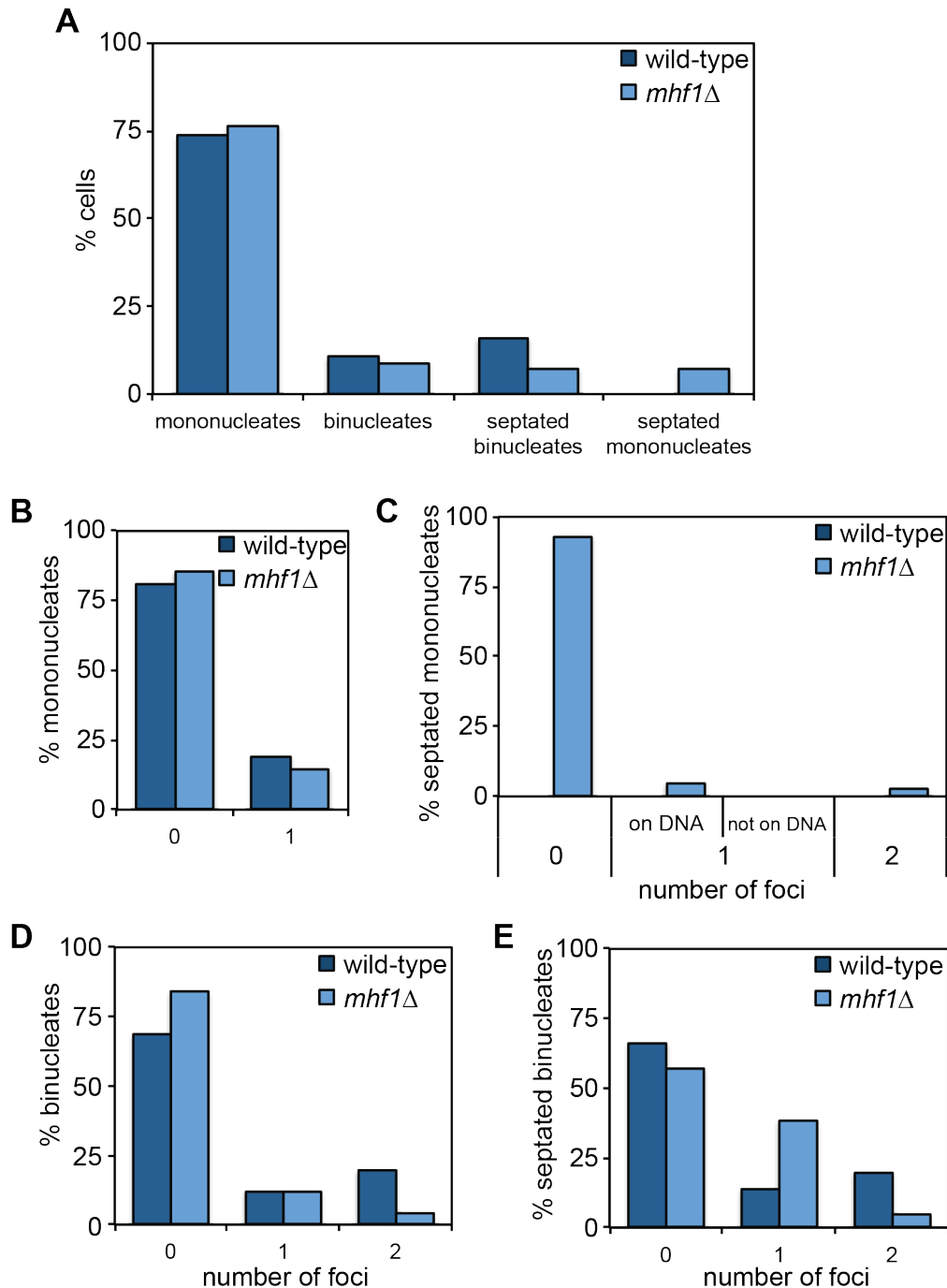


Figure 6.14 Does the impairment of MHF alter the localisation of the Spg1-GFP protein?

(A) Percentage of cells that are mononucleates, binucleates, septated binucleates and septated mononucleates. (B) Percentage of mononucleates that have 0 or 1 Spg1-GFP foci. (C) Percentage of septated mononucleates that have 0, 1 or 2 Spg1-GFP foci. (D) Percentage of binucleates that have 0, 1 or 2 Spg1-GFP foci. (E) Percentage of septated binucleates that have 0, 1 or 2 Spg1-GFP foci. Strains featured are MCW6675 and MCW6717 for wild-type and *mhfl*Δ respectively. Values are based on the analysis of at least 500 cells from an asynchronously growing culture.

and *mhf1* Δ . Contrary to published data I failed to detect Spg1-GFP foci in the majority of wild-type cells (Figures 6.14B, 6.14D and 6.14E) (Sohrmann et al. 1998), which may be a consequence of the method of cell fixation that I used. This failure to detect Spg1-GFP foci make a quantitative comparison of foci between wild-type and *mhf1* Δ unreliable, however Spg1-GFP foci were detected in the *mhf1* Δ mutant and at a similar frequency as in wild-type.

Analysis of the distance from DAPI stained DNA to Spg1-GFP foci showed a tendency for Spg1-GFP foci to be further away from the DNA staining material in *mhf1* Δ compared to the wild-type (Figure 6.15 and Figure 6.13C). Although the majority of wild-type and *mhf1* Δ mononucleate cells exhibit a similar distribution of distances ($P = 0.605$), a small percentage of *mhf1* Δ cells (~6 %) exhibit an abnormally large distance between the Spg1-GFP focus and nuclear DNA ($\geq 3.0 \mu\text{m}$) (Figures 6.15C and 6.15D). Such distances are not observed in septated and unseptated binucleates, however in these cells the distances between Spg1-GFP foci and the DNA within the nucleus is on average greater in the *mhf1* Δ (~0.41 μm) mutant than in wild-type (~0.27 μm) ($P = 0.109$) (Figures 6.15A and 6.15B). Altogether these data, similar to the data for Cdc7-GFP foci, suggest that the spindle pole body may be more loosely adhered to the nuclear membrane when MHF is absent.

6.3 Discussion

The phenotype of the *mhf1*^{TS} mutant at its restrictive temperature, and those of *mhf1* Δ , *mhf2* Δ , *cenp-t* Δ and *cenp-w* Δ mutants growing at 25 °C are consistent with the idea that the Mhf1-Mhf2-CENP-T-CENP-W tetramer is needed to prevent precocious activation of the SIN. Further evidence in

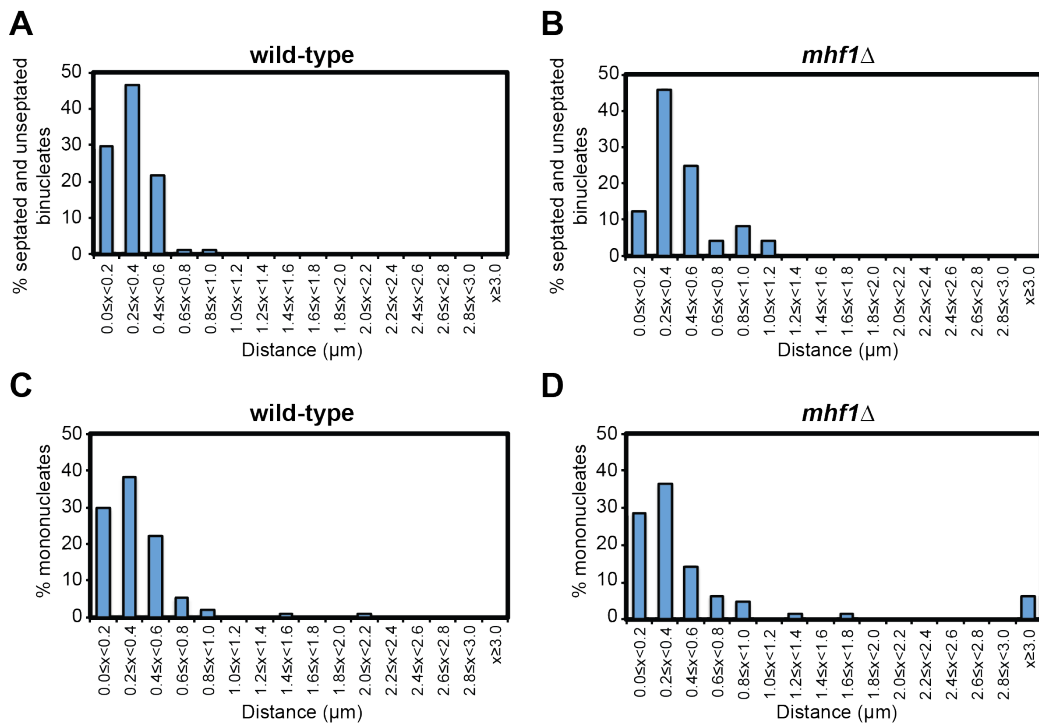


Figure 6.15 Distance from DAPI stained DNA to Spg1-GFP foci

The distribution of distances from DAPI stained DNA to Spg1-GFP foci in (A) wild-type septated and unseptated binucleates (n = 88), (B) *mhf1Δ* septated and unseptated binucleates (n = 24), (C) wild-type mononucleates (n = 94) and (D) *mhf1Δ* mononucleates (n = 63), in strains MCW6675 and MCW6717 from asynchronously growing cultures.

support of this idea comes from the observation that in the absence of Mhf1, there is more recruitment of Cdc7-GFP to the spindle pole body in mononucleate cells, leading to premature septation.

How might Mhf1-Mhf2-CENP-T-CENP-W prevent SIN activation? In fission yeast, the spindle pole body connects cytoplasmic components of the SIN through the nuclear envelope, down to the kinetochore and the centromere in the nucleus. Spg1 is constitutively held at the spindle pole body and during anaphase, the elongation of the mitotic spindle brings Spg1 in contact with Etd1 which is found at the cell cortex (Daga et al. 2005) (Figure 6.16A). Contact between Etd1 and Spg1 is thought to activate the SIN (Garcia-Cortes and McCollum 2009; Johnson et al. 2012). Conceivably, besides anchoring the kinetochore to centromeric DNA (Amano et al. 2009), Mhf1-Mhf2-CENP-T-CENP-W is also needed for anchorage of the spindle pole body to the chromatin to restrict its movement and prevent interaction of Spg1 with Etd1 (Figure 6.16B). In the absence of Mhf1, there might be a tendency for the tethering of the spindle pole body to the chromatin to be looser, increasing the likelihood for it to migrate around the cytoplasm (Figure 6.16B). G2 cells are fairly small and the loss of tethering of Spg1 could allow its contact with Etd1 (Figure 6.16B). This hypothesis is supported by the observation that Cdc7-GFP and Spg1-GFP foci tend to be further from the DNA staining material in *mhf1* Δ cells compared to the wild-type (Figure 6.12 and Figure 6.15). In the wild-type cells, there are occasions where the distance between the DNA staining material and the Cdc7-GFP or Spg1-GFP foci is large, highlighting the dynamic behaviour of the spindle pole body, however such instances are far less common than in *mhf1* Δ cells.

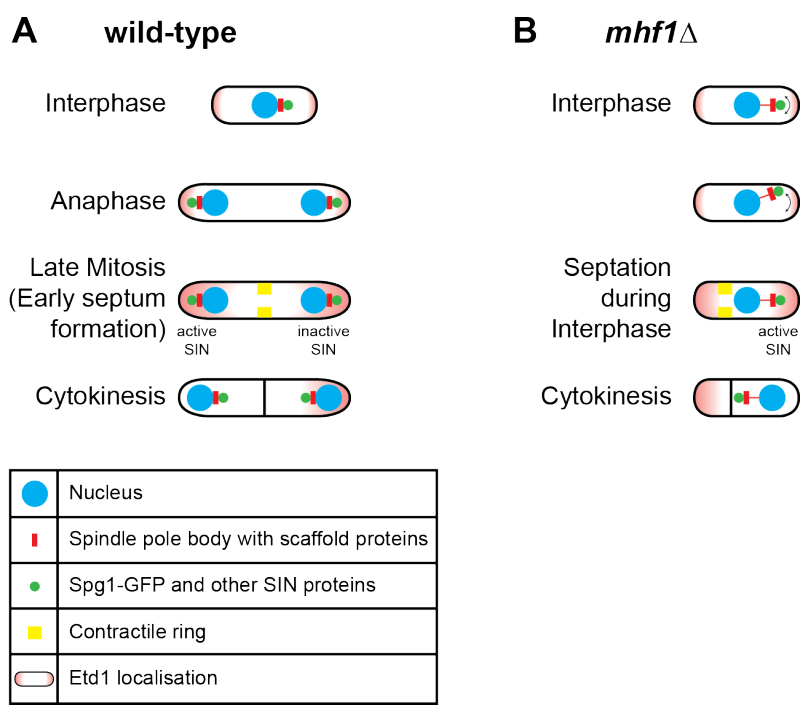


Figure 6.16 Model for how MHF prevents precocious SIN activation

(A) Spg1 is constitutively held at the spindle pole body. During anaphase, the elongation of the mitotic spindle brings Spg1 in contact with Etd1, which is found at the cell cortex (Daga et al. 2005). Contact between Etd1 and Spg1 activates the SIN (Garcia-Cortes and McCollum 2009; Johnson et al. 2012).

(B) In the absence of Mhf1, there might be a tendency for the tethering of the spindle pole body to the chromatin to be looser, increasing the likelihood for it to migrate around the cytoplasm (depicted by arrows). G2 cells are fairly small and the loss of tethering of Spg1 could allow its contact with Etd1.

Whilst the data I have already amassed are consistent with my proposed model, there is still much to be done in order to properly test it. In particular a more thorough analysis of the spatiotemporal dynamics of the SIN proteins and the spindle pole body in relation to the nuclear envelope, kinetochore and centromere is needed both in the presence and absence of MHF. Ideally this would be studied by time-lapse microscopy both before and after MHF ablation, with suitable fluorescent protein tags on all of the key components. Unfortunately, the generation of strains with many tags is slow and laborious with the *mhf1/2* null strains proving problematic to work with.

Inactivating mutations in SIN activators like Spg1, Etd1 or Cdc7 produce a characteristic SIN phenotype – multinucleate non-septated cells. If loss of MHF results in precocious activation of the SIN, then the attenuation of SIN components downstream of the point of MHF action should suppress the *mhf1/2* phenotype and instead manifest the SIN phenotype. Initial attempts to investigate this proved unsuccessful due to difficulties working with the various conditional mutants. I tried to create a “conditional” mutant of *etd1* by deleting it from its genomic locus and expressing it from an inducible promoter on a plasmid. Deletion of *etd1* results in cold sensitivity, which in theory should be rescued by the expression of Etd1 from the plasmid. However, when I constructed such a strain (with and without *mhf1/2Δ*), the *etd1Δ* mutant died in the cold room regardless of whether it contained the Etd1 expressing plasmid or not.

Nevertheless, as a first step, my experiments have allowed the detection of early recruitment of Cdc7-GFP to the spindle pole body and have shown that

in the absence of Mhf1, Cdc7-GFP and Spg1-GFP foci are further from the DNA staining material than usual, raising the possibility that the MHF has a previously unknown role in inhibiting the SIN.

Chapter 7: A role for Dcr1 in the maintenance of genome stability

7.1 Introduction

There have been recent reports of a role for Dicer and Drosha in promoting the proposed formation of DSB-induced RNAs (diRNAs), influencing DSB repair in higher eukaryotes (Francia et al. 2012; Wei et al. 2012). Furthermore, in fission yeast, it has been proposed that Dcr1 (the fission yeast orthologue of Dicer) promotes the termination of transcription at sites of replication stress and DNA damage to maintain genome stability (Castel et al. 2014).

Since epistasis analysis (in section 4.2.2) revealed a synergistic interaction between *dcr1*Δ and *mhf2*^{D87A} in repairing MMS-, UV- and cisplatin-induced lesions (Figure 4.4 and Table 4.1), and recognising that the *mhf2*^{D87A} mutant might be disrupted for its DNA repair function (see section 4.2.1), I was intrigued to know whether in yeast, Dcr1 might also influence recombination at stalled replication forks. My investigation was conducted prior to the publication of the Castel *et al.* study.

7.2 Results

7.2.1 Genetic interactions between *dcr1* and key components of the homologous recombination machinery

I first assessed whether the synergistic interaction between *dcr1*Δ and *mhf2*^{D87A} was also observed between *dcr1*Δ and *fml1*Δ. The sensitivity of *dcr1*Δ, *fml1*Δ and *dcr1*Δ *fml1*Δ mutants was tested with a range of genotoxins (Figure 7.1). I found that similar to *dcr1*Δ and *mhf2*^{D87A}, there was a synergistic interaction between *dcr1*Δ and *fml1*Δ for MMS and cisplatin

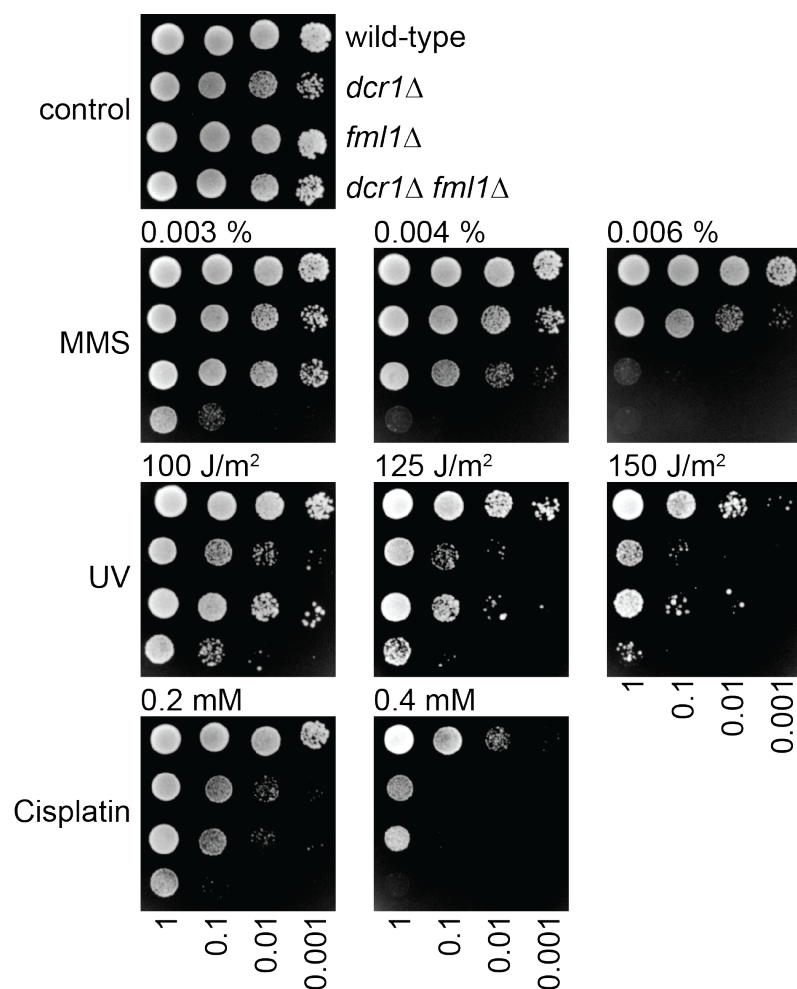


Figure 7.1 Genetic interactions between *dcr1*Δ and *fml1*Δ

Spot assays comparing the growth and genotoxin sensitivities of strains FO656, JN89, MCW4577 and JN87 after 4 days growth at 30 °C.

sensitivity; and an additive interaction with respect to UV sensitivity (Figure 7.1).

Since Fml1 and MHF are thought to act on stalled replication forks to promote recombination and on D-loops to direct repair down the SDSA pathway, I looked to see whether loss of *dcr1* caused a general increased requirement for recombination. To do this I performed epistasis analysis between *dcr1* Δ and a range of key recombination mutants (*rad54* Δ , *rad55* Δ , *sfr1* Δ , *mus81* Δ , *rqh1* Δ and *srs2* Δ) assessing their relative abilities to survive a range of different genotoxic treatments. As mentioned earlier (see section 1.1.2.2), Rad54, Rad55 and Sfr1 are Rad51 mediator proteins, which aid in the process of Rad51 nucleoprotein filament formation and strand invasion during HR (Heyer et al. 2006). Mus81 is a DNA junction-specific endonuclease that can process a variety of recombination intermediate structures (Osman and Whitby 2007), Rqh1 is a member of the RecQ helicase family that are known to catalyse HJ branch migration (Hickson 2003; Chu and Hickson 2009), while Srs2 can abort Rad51-dependent recombination by acting as a Rad51 disruptase (Krejci et al. 2003). Together, Mus81, Rqh1 and Srs2 have roles in the post-synapsis pathway of HR to resolve/disrupt recombination intermediates (see section 1.1.2.3).

In the case of the *dcr1* Δ *rad54* Δ double mutant, a 10-fold reduction in viability is observed compared to the *rad54* Δ single mutant. The interaction between *dcr1* Δ and *rad54* Δ in repairing MMS-induced lesions is epistatic since both *rad54* Δ single and *dcr1* Δ *rad54* Δ double mutants exhibit similar hypersensitivity to MMS (allowing for the difference in growth on the control

plate). However, in the case of UV, HU, CPT and TBZ, even when allowances are made for the difference in growth on the control plate, the *dcr1Δ rad54Δ* double mutant appears to be more sensitive than the *rad54Δ* single mutant. This is especially true for HU and CPT where the interaction between *dcr1Δ* and *rad54Δ* appears to be synergistic (Figure 7.2A and Table 7.1).

The interaction of *dcr1Δ* with *rad55Δ* is similar to its interaction with *rad54Δ*, with the *dcr1Δ rad55Δ* double mutant exhibiting a marked reduction in growth on the control plate relative to the single mutants, as well as heightened genotoxin sensitivity. In the case of MMS, UV, HU and CPT, the increase in sensitivity of the double mutant is clearly synergistic; with cisplatin, it seems additive; whereas, for TBZ, it appears epistatic (Figure 7.2B and Table 7.1).

As for the *dcr1Δ sfr1Δ* double mutant, unlike both *dcr1Δ rad54Δ* and *dcr1Δ rad55Δ*, no reduction in viability is observed compared to either of the *dcr1Δ* or *sfr1Δ* single mutants. However, similar to the other double mutants, it exhibits increased sensitivity to MMS, UV, HU and CPT compared to either of the single mutants, albeit the increase is more modest, appearing to be additive for UV and HU, and synergistic for MMS and CPT. The interaction between *dcr1Δ* and *sfr1Δ* in repairing cisplatin-induced lesions is epistatic since both *dcr1Δ* single and *dcr1Δ sfr1Δ* double mutants exhibit similar levels of hypersensitivity to it (Figure 7.2C and Table 7.1).

Further, the interaction of *dcr1Δ* with *mus81Δ* is similar to its interaction with *sfr1Δ*, with the *dcr1Δ mus81Δ* double mutant exhibiting no reduction in viability compared to either of the *dcr1Δ* or *mus81Δ* single mutants, however, its genotoxin sensitivity is more similar to that of the *dcr1Δ rad55Δ* double

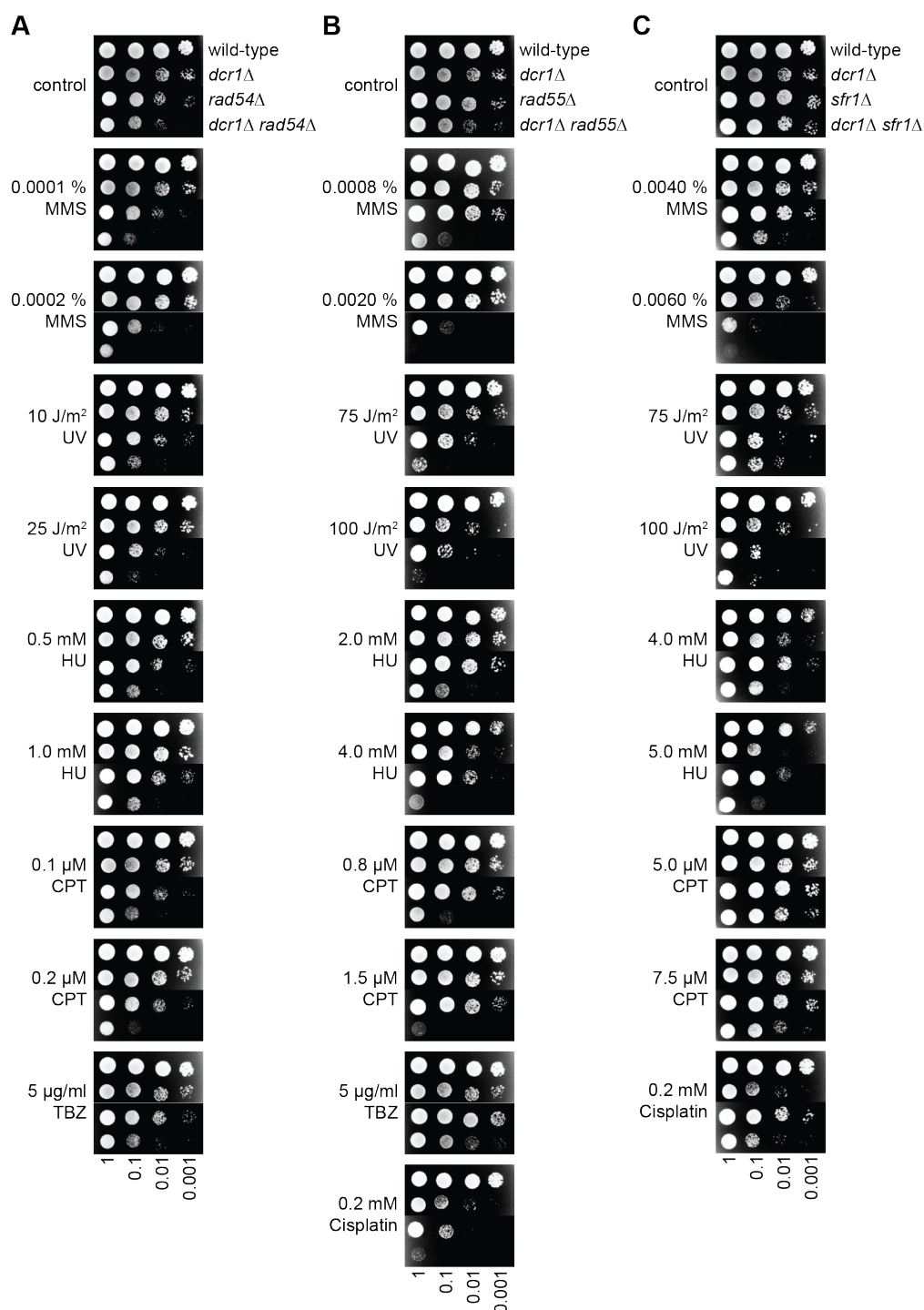


Figure 7.2 Genetic interactions between *dcr1Δ* and *rad54Δ*, *rad55Δ* and *sfr1Δ*

Spot assays comparing the growth and genotoxin sensitivities of the following strains after 4 days growth at 30 °C: (A) FO656, JN89, MCW1230 and JN99; (B) FO656, JN89, MCW6162 and JN105; (C) FO656, JN89, MCW6051 and JN107. The above spot assays were done on the same plate but have been cropped and placed next to each other for ease of presentation.

mutant. In the case of MMS, UV, HU and CPT, the increase in sensitivity of the double mutant is clearly synergistic, whereas with TBZ, it appears to be epistatic (Figure 7.3A and Table 7.1).

Likewise, the *dcr1* Δ *rqh1* Δ double mutant displays no reduction in viability compared to either of the *dcr1* Δ or *rqh1* Δ single mutants. It exhibits a synergistic increase in sensitivity to MMS, but intriguingly appears to be epistatic with *rqh1* Δ with respect to UV, HU and CPT sensitivity (Figure 7.3B and Table 7.1).

Lastly, similar to the *dcr1* Δ *mus81* Δ double mutant, *dcr1* Δ *srs2* Δ displays no reduction in viability, and also displays heightened genotoxin sensitivity compared to either of its *dcr1* Δ or *srs2* Δ single mutants. With regards to MMS, UV, HU, CPT and cisplatin, the increase in sensitivity of the double mutant is clearly synergistic; whereas with TBZ, it appears to be epistatic (Figure 7.3C and Table 7.1).

Altogether these data show that in the presence of genotoxins, the absence of Dcr1 causes a problem that can be alleviated by recombination. It is conceivable that in the face of DNA damage, Dcr1 provides a way of dealing with a problem that could otherwise be dealt with by recombination, and that the absence of both Dcr1 and recombination leads to a toxic scenario in the cell.

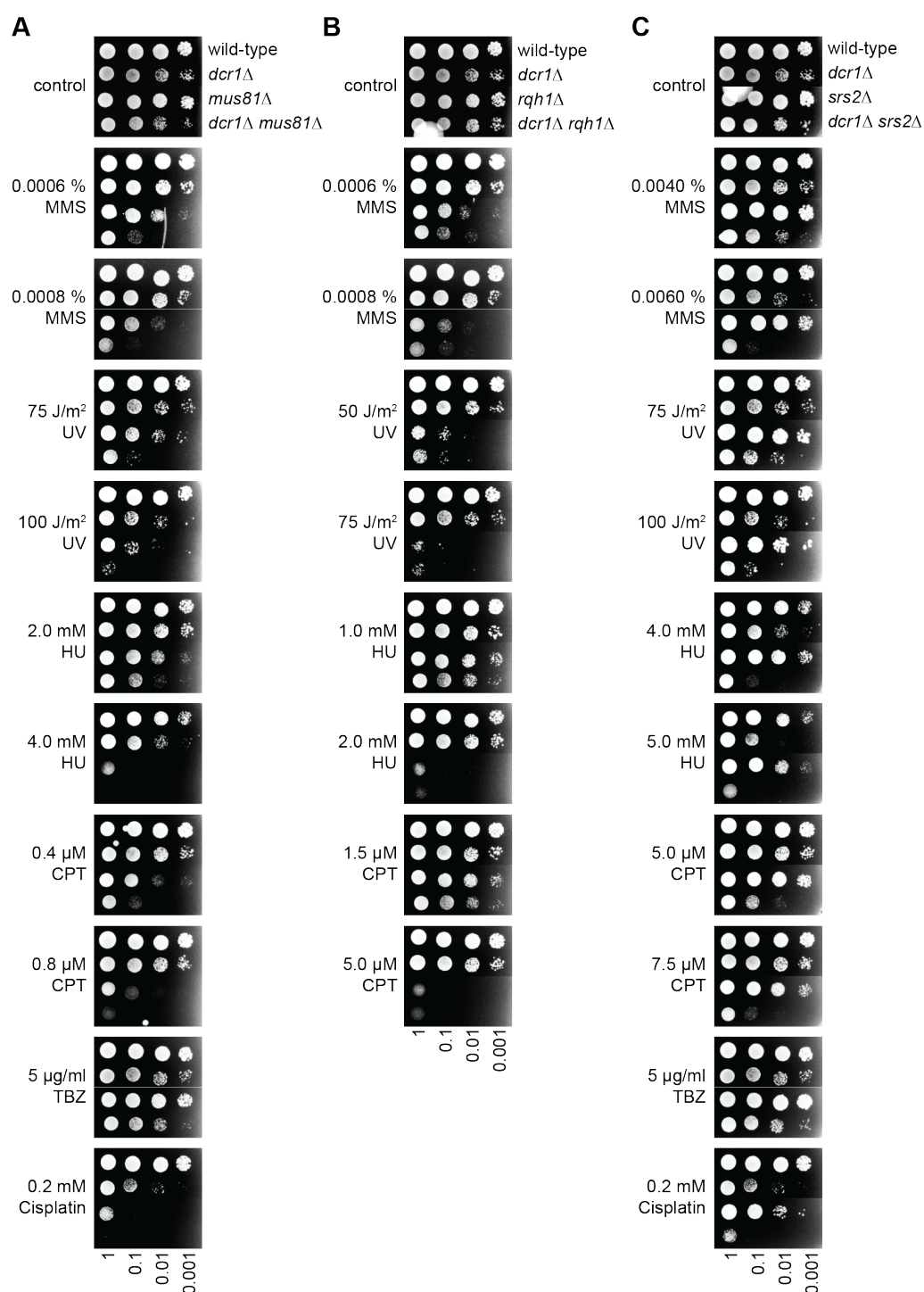


Figure 7.3 Genetic interactions between *dcr1Δ* and *mus81Δ*, *rqh1Δ* and *srs2Δ*

Spot assays comparing the growth and genotoxin sensitivities of the following strains after 4 days growth at 30 °C: (A) FO656, JN89, MCW1779 and JN97; (B) FO656, JN89, MCW1804 and JN101; (C) FO656, JN89, MCW1017 and JN103. The above spot assays were done on the same plate but have been cropped and placed next to each other for ease of presentation.

Table 7.1 Genetic interactions between *dcr1* and key components of the homologous recombination machinery

Genetic interaction data between *dcr1* Δ and *rad54* Δ , *rad55* Δ , *sfr1* Δ , *mus81* Δ , *rqh1* Δ and *srs2* Δ , presented in the same order as Figure 7.2 and Figure 7.3.

Genotoxins	Genotype		
	<i>dcr1</i> Δ <i>rad54</i> Δ	<i>dcr1</i> Δ <i>rad55</i> Δ	<i>dcr1</i> Δ <i>sfr1</i> Δ
MMS	Epistatic ^a effect	Synergistic effect	Synergistic effect
UV	Additive/ Synergistic effect	Synergistic effect	Additive effect
HU	Synergistic effect	Synergistic effect	Additive effect
CPT	Synergistic effect	Synergistic effect	Synergistic effect
TBZ	Additive/ Synergistic effect	Epistatic effect	Undetermined
Cisplatin	Undetermined	Additive effect	Epistatic effect
Genotoxins	Genotype		
	<i>dcr1</i> Δ <i>mus81</i> Δ	<i>dcr1</i> Δ <i>rqh1</i> Δ	<i>dcr1</i> Δ <i>srs2</i> Δ
MMS	Synergistic effect	Synergistic effect	Synergistic effect
UV	Synergistic effect	Epistatic effect	Synergistic effect
HU	Synergistic effect	Epistatic effect	Synergistic effect
CPT	Synergistic effect	Epistatic effect	Synergistic effect
TBZ	Epistatic effect	Undetermined	Epistatic effect
Cisplatin	Additive/ Synergistic effect	Undetermined	Synergistic effect

^a No further reduction in growth, viability or survival observed in the double mutant compared to the single mutant with the strongest phenotype.

7.2.2 Effect of *dcr1*Δ on the frequency of *RTS1*-induced direct repeat recombination

The strong genetic interactions seen between *dcr1*Δ and recombination mutants such as *mhf2*^{D87A} and *fml1*Δ (Figure 4.4, Figure 7.1, Figure 7.2, Figure 7.3 and Table 7.1) suggest that Dcr1 might function in a pathway of DNA repair or damage tolerance that operates in parallel to recombination. Indeed in the absence of Dcr1 there is clearly a greater need for recombination proteins, and as such a *dcr1*Δ mutant might exhibit heightened recombination activity. To test this, I assessed the effect of *fml1*Δ, *dcr1*Δ and *dcr1*Δ *fml1*Δ on direct repeat recombinant frequencies induced by *RTS1* at the endogenous *ade6* locus on chromosome III (Figure 7.4 and Table 7.2). The *fml1*Δ *RTS1*-AO data shown here is the same data as shown in Figure 5.10A and Table 5.1 in section 5.2.6. As mentioned above, similar to what our laboratory has previously shown, when replication forks are blocked in the absence of Fml1, the frequency of conversion-types is significantly reduced by ~6-fold compared to wild-type (Sun et al. 2008) (Figure 7.4 and Table 7.2). In contrast, there is a significant increase in the frequency of deletion-types (~2-fold relative to wild-type) (Figure 7.4 and Table 7.2). In the absence of Dcr1, the frequency of both the conversion-types and deletion-types significantly increased by ~2-fold compared to wild-type (Figure 7.4 and Table 7.2). Finally, the *dcr1*Δ *fml1*Δ double mutant exhibited the same very low-level of conversion-type formation as the *fml1*Δ single mutant. However, surprisingly, the level of deletion-types was ~2-fold more than that in the *fml1*Δ and *dcr1*Δ single mutants ($p < 0.001$ and $p = 0.003$ respectively) (Figure 7.4 and Table 7.2). Taken together, these data indicate that Dcr1 plays a role

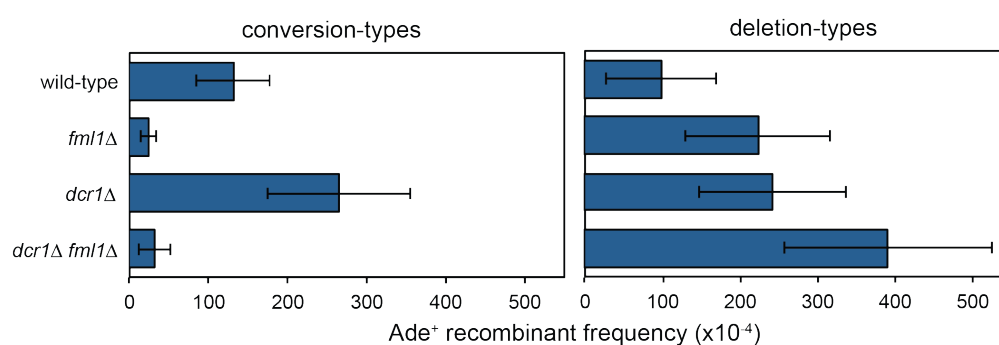


Figure 7.4 Effect of *fml1*Δ, *dcr1*Δ and *dcr1*Δ *fml1*Δ on direct repeat recombination

Bar chart showing the frequency of Ade⁺ recombinants in wild-type, *fml1*Δ, *dcr1*Δ and *dcr1*Δ *fml1*Δ RTS1-AO strains after 5 to 6 days at 30 °C. Error bars represent ± one standard deviation. In order of presentation, the strains are MCW4713, MCW3061, JN91 and JN93. Wild-type data shown in this figure is the same as shown in Figure 3.1B and Table 3.1; and *fml1*Δ RTS1-AO data shown in this figure is the same as shown in Figure 5.10 and Table 5.1.

Table 7.2 Direct repeat recombination frequencies in *fml1*Δ, *dcr1*Δ and *dcr1*Δ *fml1*Δ strains

Direct repeat recombination data presented in the same order as Figure 7.4. Wild-type data is the same as shown in Table 3.1; and *fml1*Δ data is the same as shown in Table 5.1.

Genotype	<i>RTS1</i> orientation	Position of direct repeat	Number of colonies analysed	Total Ade ⁺ recombinant frequency (x10 ⁻⁴)		% Ade ⁺ His ⁺ recombinants		Ade ⁺ His ⁺ recombinant frequency (x10 ⁻⁴)		Ade ⁺ His ⁺ recombinant frequency (x10 ⁻⁴)	
				Mean ^a	<i>P</i> -value ^b	Mean ^a	<i>P</i> -value ^b	Mean ^a	<i>P</i> -value ^b	Mean ^a	<i>P</i> -value ^b
wild-type	AO	Chr III	24	229.0 (±85.6)	-	59.8 (±15.0)	-	130.9 (±46.8)	-	98.1 (±70.8)	-
<i>fml1</i> Δ	AO	Chr III	23	246.4 (±94.0)	0.510	10.6 (±5.2)	< 0.001	23.5 (±10.0)	<0.001	222.9 (±94.0)	<0.001
<i>dcr1</i> Δ	AO	Chr III	10	506.5 (±150.7)	< 0.001	52.7 (±9.1)	0.171	264.7 (±90.7)	<0.001	241.8 (±95.4)	<0.001
<i>dcr1</i> Δ <i>fml1</i> Δ	AO	Chr III	23	422.5 (±148.9)	< 0.001	7.3 (±2.8)	< 0.001	32.0 (±19.4)	<0.001	390.4 (±134.0)	<0.001

^a The values in parentheses are the standard deviations about the mean.

^b *P*-values are derived from independent-sample t-tests comparing the mean values of the respective mutant to the corresponding mean value found in the control strain.

in limiting Fml1-dependent gene conversion and Fml1-independent deletion-types at blocked replication forks.

7.3 Discussion

In this chapter, I have found that in the absence of Dcr1 there is a greater need for recombination to repair/tolerate DNA damage, and this correlates with an increase in *RTS1*-induced recombination. Dcr1 potentially has wide reaching roles in the cell, which could indirectly impact on the need for recombination (e.g. by affecting gene expression or chromatin structure). However, two recent studies suggest that Dcr1 might have a more direct role in promoting recombinational repair as well as removing obstacles to DNA replication that would otherwise elicit a recombinational response.

In fission yeast, it has been proposed that during S phase, collisions occur between RNA Pol II and the replisome at highly transcribed genes, and during antisense transcription of rDNA and transfer DNA (tDNA) genes (which are transcribed by RNA Pol I and Pol III respectively). These collisions lead to Pol II pausing and replication fork stalling. Dcr1 does not prevent collisions from occurring, however, it resolves them by releasing Pol II from the collision site to allow replication resumption (Castel et al. 2014). In the absence of Dcr1, Pol II accumulates at the collision site. Dcr1 dependent small RNAs (sRNAs) have been found at sites of Pol II termination loci, providing evidence for direct Dcr1 activity at sites where collisions are frequent. Interestingly, neither the catalytic activity of Dcr1 nor the recruitment of sRNA products is essential for the release of Pol II. No accumulation of Pol II was observed in a catalytically dead *dcr1* or *ago1* Δ mutants at rDNA or tDNA, indicating that it is

not the RNA interference (RNAi) pathway, but Dcr1 itself that is required for the termination of transcription. In line with this, the TRAMP complex prevents the loading of sRNA from rDNA and tDNA into Ago1 by targeting them for degradation, to prevent these sRNA by-products from entering the RNAi pathway (Buhler et al. 2008). In the absence of Dcr1, Pol II and Rad52 accumulate within rDNA repeats and rDNA copy number decreases, likely resulting from HR. Despite the exclusion of the widely known sRNA biogenesis function of Dcr1 for transcription termination, the appearance of Dcr1-dependent sRNA at termination loci provides evidence for a direct role of Dcr1 at such regions, possibly by serving as a platform for the recruitment of proteins for fork protection and Pol II release (Castel et al. 2014).

On the other hand, in *Arabidopsis* and human cells, it has been shown that sRNAs are produced in the vicinity of DSB sites. These sRNAs have been proposed to function as guide molecules, directing chromatin modifications or recruiting protein complexes to DSB sites for repair. In *Arabidopsis*, the efficiency of DSB repair is greatly diminished with mutations in Dicer-like and *ago2* genes. Also, in human cells, the knock down of *dicer* or *ago2* inhibited DSB repair (Wei et al. 2012). Besides, it has been reported that in zebrafish, mice and human cells, Dicer and Drosha are required to activate the DNA damage response upon oncogene-induced genotoxic stress and exogenous DNA damage (Francia et al. 2012).

Consistent with Castel *et al.*, epistasis analysis reveals that in the absence of Dcr1, there is a much greater requirement for recombination proteins in the face of DNA damage. As much as lesions on the DNA template are able to

stall a replisome, they pose as barriers to RNA polymerases (Tornaletti and Hanawalt 1999; Tornaletti 2005). Conceivably, besides terminating transcription at sites of replication stress, Dcr1 might aid in removing RNA polymerase complexes stalled at lesions, and in the absence of Dcr1, problems with collisions between replisomes, RNA polymerases and lesions are accumulated and exacerbated, requiring a greater need for recombination (Rudolph et al. 2007).

In line with this, in the absence of Dcr1 at an *RTS1* barrier, the frequency of both the conversion-types and deletion-types increased by ~2-fold (Figure 7.4 and Table 7.2). Conceivably the heightened HR seen at the *ade6⁻* direct repeats in Figure 7.4 and Table 7.2 is independent of RNAi since no DSBs are induced at the site. Instead, it is due to the inability of transcription machinery that have collided with replisomes during the transcription of the *his3⁺*, *ade6-L469* or *ade6-M375* genes to be released in the absence of Dcr1 (Figure 7.5B). Alternatively, if a replication fork got blocked at the *RTS1* barrier, and the opposing fork collided with RNA polymerases transcribing *his3⁺*, more time would be provided for recombination to occur at the *RTS1* barrier (Figure 7.5C). A similar effect was observed when origin *III-1253* was deleted to reduce the probability of fork merging at *RTS1-AO* (Figure 7.5A) (unpublished data). Additionally, it is interesting to note that the deletion of *fml1* from a *dcr1Δ* strain resulted in a decrease in conversion-types and an increase in deletion-types. Since the high level of deletion-types in *fml1Δ* is Rad51-independent (Sun et al. 2008), they have to be formed by the Rad52-dependent SSA pathway. It is thus conceivable that Fml1 blocks and/or

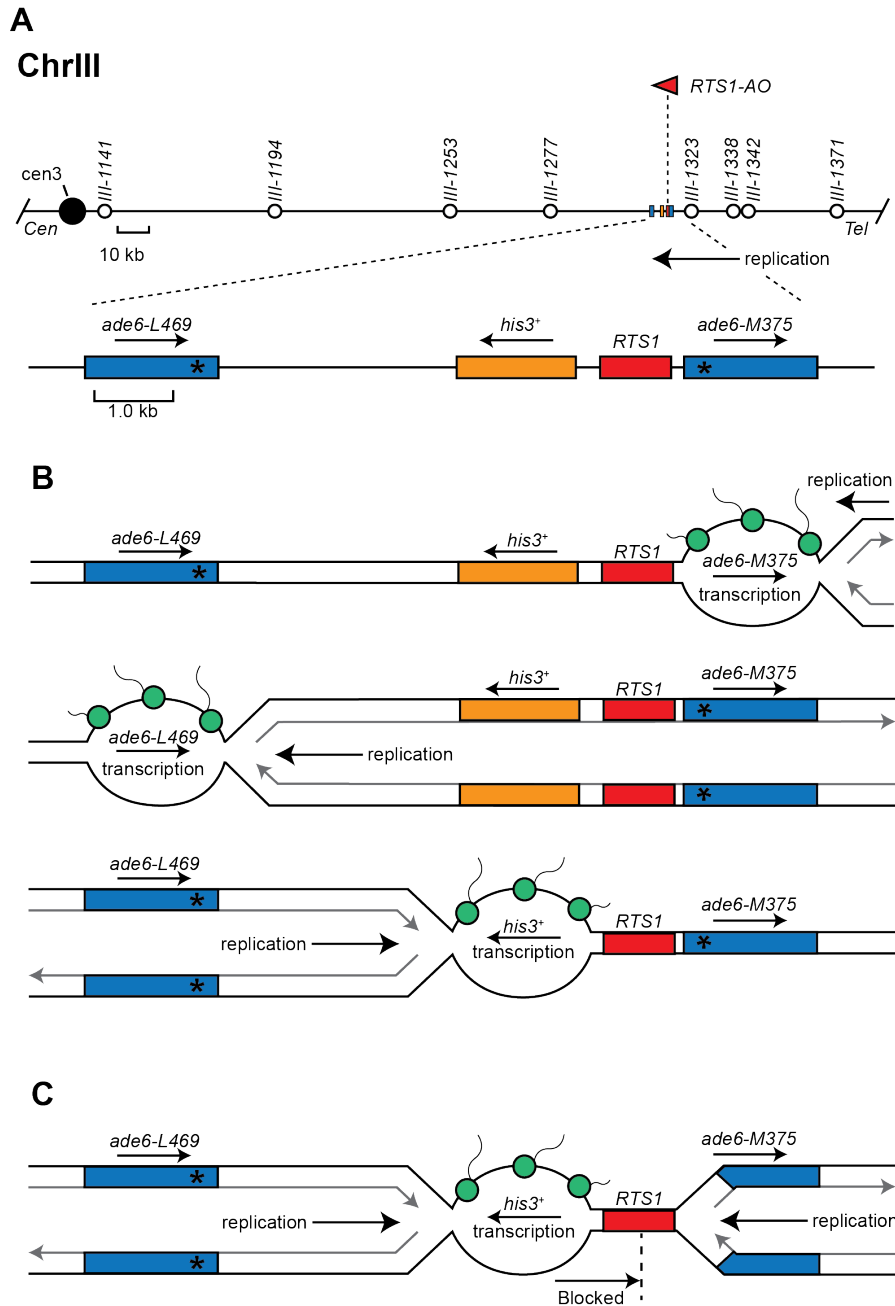


Figure 7.5 Collision of transcription polymerases with DNA replication machinery

A Schematic showing the location of the *ade6⁻* intrachromosomal recombination reporter along with an intervening *his3⁺* gene and *RTS1* polar RFB in relation to nearby replication origins at the endogenous *ade6* locus on chromosome III. Asterisks indicate the position of point mutations in *ade6-L469* and *ade6-M375*. Replication across the direct repeat recombination reporter occurs predominantly in the centromere-proximal direction and will be blocked at *RTS1-AO*. **B** Collision of transcription machinery with replisomes. **C** Replication forks blocked at the *RTS1* barrier and upon colliding with transcription machinery at *his3⁺*.

unwinds Rad52-dependent SSA events that may occur during fork merging to suppress the formation of deletion-types.

To determine the contribution of the *RTS1* barrier to the increased recombination in the absence of Dcr1, the effect of *dcr1* Δ on direct repeat recombination would have to be done on a control strain without the *RTS1* barrier for a comparison to be made. To further verify whether the increased recombination is due to collisions between RNA Pol II and the replisome, the *RTS1* barrier could be replaced with a *tRNA^{GLU}* gene in both orientations, in the presence and absence of Dcr1. When positioned between *ade6⁻* repeats, the *tRNA^{GLU}* gene has been shown to induce recombination when transcription is contra-directional with replication (Steinacher et al. 2012). Lastly, northern blots could be used to monitor the RNA transcript levels of the *his3⁺*, *ade6-L469* and *ade6-M375* genes in the presence and absence of Dcr1. In the absence of Dcr1, it is expected that RNA levels would decrease since collided RNA polymerases are not released to allow more transcription to occur.

Finally, the fact that the interaction between *dcr1* and *rqh1* appears to be epistatic regarding sensitivity to at least some of the genotoxins tested is intriguing. This might reflect a role for the helicase activity of Rqh1 in assisting the clearance of RNA polymerases or the unwinding of transcripts that have annealed to the DNA template in the Dcr1 pathway. Possibly, Dcr1 serves as a platform for the recruitment of Rqh1 and other helicases to release Pol II upon collision with a replisome. A more thorough and quantitative analysis of genotoxin sensitivities, as well as an assessment of recombination

frequencies, is needed to determine whether the interaction between *dcr1* Δ and *rqh1* Δ is truly epistatic.

Chapter 8: Concluding remarks

The first part of this dissertation (Chapter 3) focuses on investigating the importance of DNA replication termination to genome stability. In an effort to study whether replication fork termination is a recombinogenic process, I exploited the well-characterised site-specific *RTS1* barrier (Dalgaard and Klar 2001) in fission yeast to investigate the consequences of fork merging. Through positioning the *RTS1* direct repeat recombination reporter at a site with a cluster of replication origins on chromosome I where time from replication fork initiation to termination is short, I have demonstrated that termination is not normally a recombinogenic process. This is in contrast to the strong induction of recombination seen at the endogenous *ade6* locus on chromosome III where replication is unidirectional (Ahn et al. 2005). On chromosome III, it is likely that the strong induction of recombination is a result of replication restart at the blocked fork instead of fork merging. On the contrary, on chromosome I, it is likely that an opposing fork arrives too rapidly to merge with the fork blocked at *RTS1*, providing insufficient time for recombination to occur. In support of this model, the deletion of origin *III-1253* from chromosome III (Figure 3.1A) to delay fork merging when the *RTS1* recombination reporter was positioned at the *ade6* locus not only led to increases in both the amount and duration of Rad52 foci assembly at impeded *RTS1* forks, it also increased direct repeat recombination (unpublished laboratory data). This is consistent with the notion that when a fork is blocked, recombinases load and act, presumably to assist RDR, and that any resulting recombination is constrained by the arrival of a converging fork. Also, it is unlikely that the increased recruitment of Rad52 foci is due to fork merging since the probability of a converging fork would have decreased with the

deletion of origin *III-1253*. To further support this model, the insertion of a *TER2/3* pause site 10 kb downstream of the *RTS1* RFB on chromosome III to delay the arrival of converging forks at *RTS1*, to provide more time for cells to perform RDR has seen increases in recombination, similar to the deletion of origin *III-1253* (unpublished laboratory data). Altogether, these data seem to support the idea that fork termination generally is not a recombinogenic process. Further experiments, such as ChIP to study Rad52 recruitment to the *RTS1* site, or live cell imaging to look at the timing of colocalisation of Rad52 both in the wild-type and *pfh1-mt* backgrounds would provide a clearer idea of the amount of recombinases that are recruited to the chromosome I site (where forks tend to merge). This presumably could be compared to the amount of Rad52 recruitment to the chromosome III site (where forks are less likely to merge), to provide a measure of whether Rad52 tends to be recruited when forks restart or when forks terminate.

The second part of this dissertation (Chapters 4 to 6) focuses on studying the importance of the MHF complex to genome stability. In Chapter 4, I explored the possibility of a biological significance for the MHF octamer in fission yeast. Our collaborators have found that when three amino acid residues in Mhf2 (serine (S) 59, glutamine (Q) 83 and aspartate (D) 87) are individually mutated to alanine *in vitro*, it disrupts the octamer. I showed that the fission yeast mutants carrying these three point mutations are hypersensitive to TBZ (Figure 4.2), reminiscent of mutants with impaired centromeric function. However, an epistasis analysis between the three octamer point mutants and deletions in other key centromeric factors failed to reveal any consistent synergistic effects. Also, analysis of asynchronously growing cultures of the

three point mutants by epifluorescence microscopy did not reveal any DNA segregation defects in the mutants (data not shown). Some recombination mutants exhibit hypersensitivity to TBZ (unpublished laboratory data), possibly reflecting the importance of recombination in maintaining normal centromeric function. Hence, it is plausible that the shared sensitivity of the *mhf2*^{S59A}, *mhf2*^{Q83A} and *mhf2*^{D87A} point mutants to TBZ reflects a deficiency in centromeric recombination/repair rather than a deficiency in CCAN function. This is consistent with the point mutants being hypersensitive to MMS (albeit to varying degrees), suggesting a DNA repair role for the MHF octamer. Further experiments such as the *RTS1* direct repeat recombination assay, the plasmid gap repair assay and the meiotic crossover assay could be carried out to assess whether the *mhf2*^{S59A} and *mhf2*^{Q83A} mutants are defective in promoting Fml1-dependent gene conversion at stalled replication forks and in crossover avoidance in mitotic and meiotic cells. Any impairment in gene conversion and/or crossover avoidance in these mutants could be compared to that of *mhf2*^{D87A} mutant to determine the degree to which the *mhf2*^{D87A} phenotype is due to a reduced interaction between MHF and Fml1.

To examine the interaction between MHF and Fml1 (Chapter 5), I employed the D87A mutation in Mhf2 and a mutation of tyrosine (Y) 672 together with arginines (R) 674 and 678 to alanine (A) in Fml1 (referred to as the Fml1^{AAA}), which appear to completely disrupt the interaction between MHF and Fml1 when combined *in vitro*. As mentioned earlier, the N-terminal helicase domain of Fml1 (amino acids 1 to 603) does not interact with MHF *in vitro* (unpublished data). I showed that whilst the Fml1^{AAA} had a reduced interaction with MHF *in vitro*, the phenotype of *fml1*^{AAA} is similar to the wild-

type strain *in vivo*. Conceivably the residual interaction that Fml1^{AAA} retains with MHF is sufficient for its role in DNA damage repair/tolerance *in vivo*. Alternatively, it is possible that Fml1^{AAA} interacts with MHF as strongly as the wild-type protein *in vivo*. Consistent with the loss of the MHF interaction domain, the *fml1ΔC*¹⁻⁶⁰³ strain exhibits approximately the same hypersensitivity to MMS, UV and cisplatin as a *fml1Δ* mutant. However, the over-expression of the Fml1 helicase domain, Fml1ΔC¹⁻⁶⁰³, is sufficient to fully complement the genotoxin hypersensitivity of a *fml1Δ* mutant (Sun et al. 2008; Nandi and Whitby 2012). This suggests that when Fml1ΔC¹⁻⁶⁰³ is overexpressed, it can rescue the genotoxin hypersensitivity of a *fml1Δ* mutant independently of MHF. If true, the overexpression of Fml1ΔC¹⁻⁶⁰³ should also rescue the genotoxin hypersensitivity of *mhf2*^{D87A}.

I tested the ability of the *mhf2* and *fml1* mutants in promoting recombination in the *RTS1* direct repeat recombination assay. *mhf2*^{D87A} exhibits a similar effect on recombination as *fml1Δ* in decreasing conversion-type formation and increasing deletion-type formation in the presence and absence of *RTS1* fork blockage, albeit not to a similar extent as *fml1Δ* in decreasing the formation of conversion-types. On the other hand, the *fml1ΔC*¹⁻⁶⁰³ and *fml1*^{AAA} *mhf2*^{D87A} mutants resemble *fml1Δ* in terms of conversion-type frequency but retain a deletion-type frequency similar to wild-type. Plausibly, the N-terminal helicase domain of Fml1 (amino acids 1 to 603) is able to unwind D-loops or reset regressed forks during fork merging (as alluded to in section 3.2.6) to suppress the formation of deletion-types only when it's C-terminal domain (amino acids 604 to 834) interacts strongly with MHF, and/or when its

interaction with MHF is completely disrupted, in an “all or nothing” fashion, but not when the interaction is weak.

Based on the effects seen with the *RTS1* and mitotic DSB repair assays, it is likely that in the *mhf2*^{D87A} mutant, interaction between MHF and Fml1 is more greatly impaired than in the *fml1*^{AAA} mutant, which functions like wild-type. Although the *mhf2*^{D87A} mutant has a relatively modest effect on *RTS1*-induced gene conversion compared to *fml1*Δ, it appears to render the MHF complex completely incapable of promoting Fml1-dependent crossover avoidance in vegetative cells. This indicates that the physical interaction between MHF and Fml1 is highly essential for Fml1 to stimulate crossover avoidance. It is currently unclear why MHF is more important for aiding Fml1 in crossover avoidance than in promoting recombination at a blocked fork. As alluded to earlier, Fml1's role at blocked forks is likely to be promoting fork remodelling, whereas in crossover avoidance, it is likely to be catalysing D-loop dissociation. Blocked forks and D-loops are different structures and will be recognised and bound by different proteins *in vivo*. It is plausible that there is a more critical need for MHF in enhancing the ability of Fml1 to bind to a D-loop than a blocked fork *in vivo*. The FAAP24 protein has been shown to target FANCM to structures that mimic intermediates formed during the replication/repair of damaged DNA (Ciccio et al. 2007). A FAAP24 homologue has yet to be identified in fission yeast, however, it is possible that a similar factor may exist to stimulate Fml1's activity at blocked replication forks, reducing the need for MHF. Presumably, this factor (if it exists) is unable to compensate for the loss of MHF at D-loops. Alternatively, it is possible that Fml1's D-loop unwinding activity is preferentially stimulated by the octameric

form of MHF while at blocked forks, its fork reversal activity is preferentially stimulated by the tetrameric form of MHF. If this were true, the phenotypes of the *mhf2*^{S59A} and *mhf2*^{Q83A} mutants would resemble that of *mhf2*^{D87A} in the mitotic and meiotic DSB repair assays, but might be completely wild-type in the *RTS1* direct repeat recombination assay.

In Chapter 6, I introduced a previously unknown role of the MHF complex, which is in inhibiting the SIN. The premature activation of the SIN by the over-expression of Spg1 or the Polo kinase Plo1 leads to septum formation prior to mitosis (Ohkura et al. 1995; Schmidt et al. 1997). This phenotype appears to be the terminal phenotype of the *mhf1*^{TS} mutant at its restrictive temperature. Additionally, a large proportion of cells exhibit abnormally high levels of aberrant staining with calcofluor white, which interacts with the linear (1,3) β-D-glucan that is needed for primary septum formation, indicative of hyperactive SIN. Also, in the absence of Mhf1, there is more recruitment of Cdc7-GFP to the spindle pole body in mononucleate cells and that Cdc7-GFP foci tend to be further away from the DNA in these cells. To explain these observations I propose that MHF has a role in SIN inhibition, and a hypothetical model to explain how it might fulfil this role is given below.

During interphase in fission yeast cells, the SIN components are located in the cytoplasm and the spindle pole body is tethered to the outer nuclear membrane (Ding et al. 1997). The tethering of the spindle pole body to the outer nuclear membrane involves KASH (Klarsicht, ANC-1, Syne Homology) domain proteins, Kms1 and/or Kms2 (Shimanuki et al. 1997; Miki et al. 2004) (Figure 8.1A). Besides the spindle pole body, these KASH domain proteins

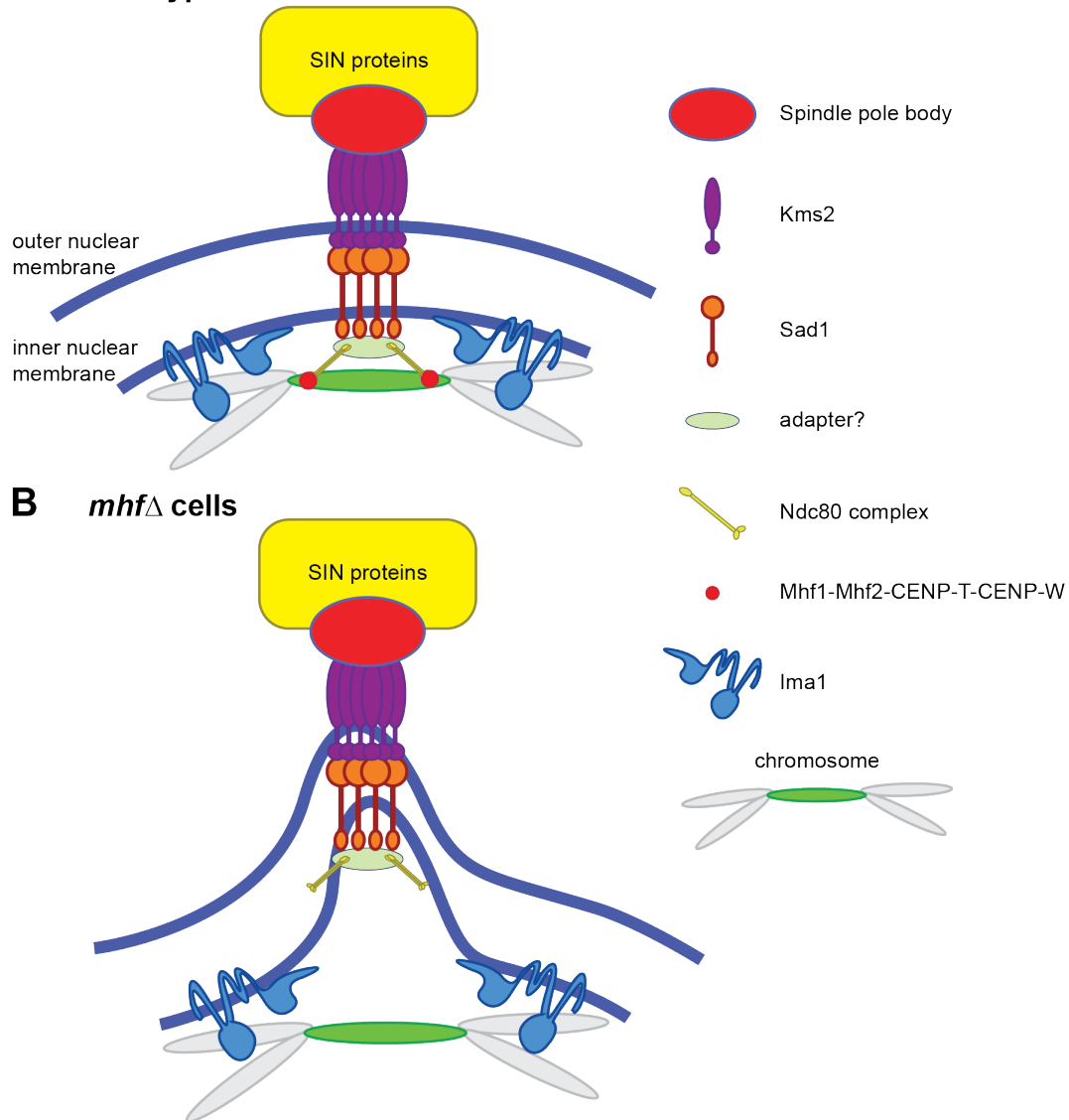
A wild-type cells

Figure 8.1 The physical connection between the centromeres and the SIN proteins in fission yeast

(A) In wild-type cells, the Sad1 (orange)-Kms2 (purple) bridging complex links the spindle pole body (red oval) to the centromeric heterochromatin (green and grey). Ima1 (blue) contributes to the coupling of heterochromatin to the inner nuclear membrane. Ndc80 (yellow) links the centromeres to the Sad1 via an unknown adaptor (light green). The Mhf1-Mhf2-CENP-T-CENP-W complex secures Ndc80 to the chromatin (red circle). (B) In the absence of the Mhf1-Mhf2-CENP-T-CENP-W complex, anchorage of the spindle pole body to the chromatin is looser, increasing the likelihood for it to migrate around the cytoplasm. Adapted from (King et al. 2008).

also connect the major cytoskeletal elements to the nuclear envelope (Starr and Fischer 2005; Wilhelmsen et al. 2006). Kms1 and Kms2 interact with Sad1 (Miki et al. 2004), which is a SUN (Sad1p, UNC-84) domain protein in the inner nuclear membrane, providing a means of linking the spindle pole body and the cytoplasmic cytoskeleton to the nuclear envelope (Figure 8.1A). Additionally, an integral inner nuclear membrane protein, Ima1, links the nuclear envelope site to the heterochromatin to support buffering forces at the nuclear envelope (King et al. 2008) (Figure 8.1A). Hence, there is communication and functional cooperation between the chromatin and the cytoplasmic cytoskeleton throughout the cell cycle. Indeed, in cells harbouring Ndc80 mutations that disrupt its localisation to the centromeres, centromeric heterochromatin is released from the nuclear envelope, leading to structural defects in the nuclear envelope and spindle pole body (King et al. 2008). Ndc80 is responsible for anchoring the spindle pole body to the chromatin and presumably this is mediated in part by its interaction with Mhf1-Mhf2-CENP-T-CENP-W (Figure 8.1A). It is imaginable that the integral SUN and KASH domains act as a “bolt” to secure to the spindle pole body to the nuclear envelope. On the other hand, the heterochromatin act as the “nut” at the end of the bolt to withstand and distribute forces experienced. It is conceivable that the Mhf1-Mhf2-CENP-T-CENP-W complex is acting to secure the centromeres (the “nut”) to the SUN and KASH domain proteins (the “bolt”) to anchor the spindle pole body (and SIN proteins attached to it) firmly to the chromatin. In the absence of MHF, the anchorage of the spindle pole body to the chromatin is looser, increasing the likelihood for it to migrate around the cytoplasm (Figure 8.1B). G2 cells are fairly small and the loss of anchorage of

Spg1 (held constitutively at the spindle pole body) could come into contact with Etd1 (found at the cell cortex) to activate the SIN (Figure 6.16B).

These data open up new paradigms for consideration of how MHF is transformed and swapped between its heterotetrameric (and putative octameric) complex that supports Fml1 and its complex with CENP-T-CENP-W in functioning at the centomere. It is conceivable that one pool of MHF proteins exists in the cell and is mobilised appropriately based on cellular requirements. If this were true, it will be interesting to determine how the mobilisation of proteins to form their different complexes is regulated, possibly via DNA damage reponse or cell cycle regulation.

The final part of this dissertation (Chapter 7) explored a role for Dcr1 in the maintenance of genome stability. An epistasis analysis between *dcr1Δ* and a range of key recombination mutants showed a much greater requirement for recombination proteins in the absence of Dcr1 for the repair/tolerance of DNA damage. Consistent with this, the absence of Dcr1 led to increased recombination at an *RTS1* barrier. Incidentally, Castel *et al.* showed that indeed, Dcr1 has a role in promoting transcription termination at sites of replication stress to maintain genome stability (Castel et al. 2014). Conceivably, the heightened recombination is due to the inability of transcription machinery that have collided with replisomes during the transcription of neighbouring genes to terminate. Future experiments to verify this include the replacement of the *RTS1* barrier with a *tRNA^{GLU}* gene in the presence and absence of Dcr1. This would provide a clearer idea of whether antisense *tRNA^{GLU}* transcription truly triggers recombination in the absence of

Dcr1. Additionally, northern blots to monitor RNA transcript levels of neighbouring genes could be used to assess whether transcription termination has failed in the absence of Dcr1.

Appendix

Table S2.1 List of *S. pombe* strains used in this study

Name	Mating Type	Genotype	Source
JN6	<i>h</i> -	<i>ChrII(86,494-86,502)::ade6-L469/pUc8/his3⁺/RTS1-AO/ade6-M375-KanMX6 ade6-D20 ura4-D18 leu1-32 his3-D1</i> (diploid)	This study
JN8	<i>h</i> +	<i>rtf1Δ::ura4⁺ ChrI(80,355-80,423)::ade6-L469/pUC8/his3⁺/RTS1-AO/ade6-M375-kanMX6 ade6-D20 ura4-D18 leu1-32 his3-D1</i>	This study
JN11	<i>h</i> +	<i>rtf1Δ::ura4⁺ ChrII(86,494-86,502)::ade6-L469/pUC8/his3⁺/RTS1-IO/ade6-M375-kanMX6 ade6-D20 ura4-D18 leu1-32 his3-D1</i>	This study
JN13	<i>h</i> +	<i>mhf1^{D75V}::kanMX6 ura4-D18 leu1-32 his3-D1 arg3-D4</i>	This study
JN16	<i>h</i> +	<i>rtf1Δ::ura4⁺ ura4-D18 leu1-32 his3-D1 arg3-D4</i>	This study
JN18	<i>h</i> +	<i>rtf1Δ::ura4⁺ ChrII(86,494-86,502)::ade6-L469/pUC8/his3⁺/RTS1-AO/ade6-M375-kanMX6 ade6-D20 ura4-D18 leu1-32 his3-D1</i>	This study
JN21	<i>h</i> +	<i>mhf2^{D87A}::KanMX6 ura4-D18 leu1-32 his3-D1 arg3-D4 ade-6</i>	This study
JN27	<i>h</i> -	<i>ChrI(80,355-80,423)::ade6-L469/pUC8/his3⁺/ade6-M375-kanMX6 ade6-D20 ura4-D18 leu1-32 his3-D1</i>	This study
JN29	<i>h</i> -	<i>ChrI(80,355-80,423)::ade6-L469/pUC8/his3⁺/RTS1-AO/ade6-M375-kanMX6 ade6-D20 ura4-D18 leu1-32 his3-D1 arg3-D4</i>	This study
JN30	<i>h</i> +	<i>rtf1Δ::ura4⁺ ade6-D20 ura4-D18 leu1-32 his3-D1 ChrI(80,355-80,423)::ade6-L469/pUC8/his3⁺/RTS1-IO/ade6-M375-kanMX6</i>	This study

JN31	<i>h</i> -	<i>ade6-D20 ura4-D18 leu1-32 his3-D1</i> <i>ChrII(86,494-86,502)::ade6-</i> <i>L469/pUc8/his3⁺/no RTS1/ade6-M375-</i> <i>kanMX6</i>	This study
JN33	<i>h</i> -	<i>ChrII(86,494-86,502)::ade6-</i> <i>L469/pUC8/his3⁺/RTS1-IO/ade6-M375-</i> <i>kanMX6 ade6-D20 ura4-D18 leu1-32 his3-</i> <i>D1 arg3-D4</i>	This study
JN34	<i>h</i> +	<i>ChrI(80,355-80,423)::ade6-</i> <i>L469/pUC8/his3⁺/RTS1-IO/ade6-M375-</i> <i>kanMX6 ade6-D20 ura4-D18 leu1-32 his3-</i> <i>D1</i>	This study
JN39	<i>h</i> -	<i>mhf2^{D87A}::kanMX6 fml1Δ::hphMX4 ura4-</i> <i>D18 leu1-32 his3-D1 arg3-D4</i>	This study
JN42	<i>h</i> +	<i>ChrII(86,494-86,502)::ade6-</i> <i>L469/pUC8/his3⁺/no RTS1/ade6-M375-</i> <i>kanMX6 ade6-D20 ura4-D18 leu1-32 his3-</i> <i>D1</i>	This study
JN46	<i>h</i> +	<i>ChrII(86,494-86,502)::ade6-</i> <i>L469/pUC8/his3⁺/RTS1-AO/ade6-M375-</i> <i>kanMX6 ade6-D20 ura4-D18 leu1-32 his3-</i> <i>D1</i>	This study
JN53	<i>h</i> +	<i>fml1ΔC¹⁻⁶⁰³::natMX4 mhf2^{D87A}::kanMX6</i> <i>ura4-D18 leu1-32 his-D1 arg3-D4 ade+? or</i> <i>ade6-M210? or ade6-M216?</i>	This study
JN56	<i>h</i> -	<i>mhf2^{D87A}::hphMX4 ura4-D18 leu1-32 his3-</i> <i>D1 arg3-D4</i>	This study
JN57	<i>h</i> +	<i>dcr1Δ::nat^R mhf2^{S59A}::kanMX6 ade6-210</i> <i>ura4-D18 leu1-32 his3-D1 arg3-D4</i>	This study
JN59	<i>h</i> +	<i>dcr1Δ::nat^R mhf2^{Q83A}::kanMX6 ade6-210</i> <i>ura4-D18 leu1-32 his3-D1 arg3-D4</i>	This study
JN61	<i>h</i> +	<i>sir2Δ::nat^R mhf2^{S59A}::kanMX6 ade6-210</i> <i>ura4-D18 leu1-32 his3-D1 arg3-D4</i>	This study
JN63	<i>h</i> +	<i>sir2Δ::nat^R mhf2^{Q83A}::kanMX6 ade6-210</i> <i>ura4-D18 leu1-32 his3-D1 arg3-D4</i>	This study
JN65	<i>h</i> +	<i>swi6Δ::nat^R mhf2^{S59A}::kanMX6 ade6-210</i> <i>ura4-D18 leu1-32 his3-D1</i>	This study

JN67	<i>h+</i>	<i>swi6Δ::nat^R mhf2^{Q83A}::kanMX6 ade6-210 ura4-D18 leu1-32 his3-D1</i>	This study
JN70	<i>h-</i>	<i>dcr1Δ::nat^R mhf2^{D87A}::kanMX6 ade6-210 ura4-D18 leu1-32 his3-D1 arg3-D4</i>	This study
JN71	<i>h+</i>	<i>sir2Δ::nat^R mhf2^{D87A}::kanMX6 ade6-210 ura4-D18 leu1-32 his3-D1 arg3-D4</i>	This study
JN73	<i>h+</i>	<i>swi6Δ::nat^R mhf2^{D87A}::kanMX6 ade6-210 ura4-D18 leu1-32 his3-D1 arg3-D4</i>	This study
JN79	<i>h+</i>	<i>mhf1^{D75V}::kanMX6 wee1::ura4⁺ ura4-D18 leu1-32 his3-D1 arg3-D4</i>	This study
JN81	<i>h+</i>	<i>mhf2Δ::natMX4 mhf1⁺::GFP-kanMX6 mis6⁺::mCHERRY-ura4⁺ ura4-D18 leu1-32 his3-D1 arg3-D4</i>	This study
JN84	<i>h-</i>	<i>mhf2^{D87A}::hghMX mhf1⁺::GFP-kanMX6 mis6⁺::mCHERRY-ura4⁺ ura4-D18 leu1-32 his3-D1 arg3-D4</i>	This study
JN86	<i>h+</i>	<i>fml1ΔC¹⁻⁶⁰³::natMX4 ura4-D18 leu1-32 his3-D1 arg3-D4</i>	This study
JN87	<i>h-</i>	<i>dcr1Δ::nat^R fml1Δ::hphMX4 ura4-D18 leu1-32 his3-D1 arg3-D4</i>	This study
JN89	<i>h+</i>	<i>dcr1Δ::nat^R ura4-D18 leu1-32 his3-D1 arg3-D4</i>	This study
JN91	<i>h+</i>	<i>dcr1Δ::nat^R ura4-D18 leu1-32 his3-D1 arg3-D4 ade6-L469/int::pUC8/his3⁺/RTS1-AO/ade6-M375</i>	This study
JN93	<i>h+</i>	<i>dcr1Δ::nat^R fml1Δ::hphMX4 ade6-L469/int::pUC8/his3⁺/RTS1-AO/ade6-M375 ura4-D18 leu1-32 his3-D1 arg3-D4</i>	This study
JN97	<i>h+</i>	<i>dcr1Δ::nat^R mus81Δ::arg3⁺ ura4-D18 leu1-32 his3-D1 arg3-D4</i>	This study
JN99	<i>h+</i>	<i>dcr1Δ::nat^R rad54Δ::ura4⁺ ura4-D18 leu1-32 his3-D1 arg3-D4</i>	This study
JN101	<i>h+</i>	<i>dcr1Δ::nat^R rqh1Δ::ura4⁺ ura4-D18 leu1-32 his3-D1 arg3-D4</i>	This study
JN103	<i>h+</i>	<i>dcr1Δ::nat^R srs2Δ::ura4⁺ ura4-D18 leu1-32 his3-D1 arg3-D4</i>	This study

JN105	<i>h+</i>	<i>dcr1Δ::nat^R rhp55Δ::hphMX4 ura4-D18 leu1-32 his3-D1 arg3-D4</i>	This study
JN107	<i>h+</i>	<i>dcr1Δ::nat^R sfr1D-11::hphMX4 ura4-D18 leu1-32 his3-D1 arg3-D4</i>	This study
JN109	<i>h+</i>	<i>mhf2^{S59A,Q83A,D87A}::KanMX6 ura4-D18 leu1-32 his3-D1 arg3-D4 ade6-M210 or 216</i>	This study
JN114	<i>h-</i>	<i>leu1-32::pJK148-leu1+-pfh1-m21 pfh1D::hphMX4 ChrI(80,355-80,423)::ade6-L469/pUC8/his3⁺/ade6-M375-kanMX6 ade6-D20 ura4-D8 leu1-32 his3-D1 arg3-D4</i>	This study
JN116	<i>h-</i>	<i>leu1-32::pJK148-leu1+-pfh1-m21 pfh1D::hphMX4 ChrI(80,355-80,423)::ade6-L469/pUC8/his3⁺/ RTS1-AO/ade6-M375-KanMX6 ade6-D20 ura4-D8 leu1-32 his3-D1 arg3-D4</i>	This study
JN118	<i>h-</i>	<i>fml1Δ::natMX4 ChrI(80,355-80,423)::ade6-L469/pUC8/his3⁺/ade6-M375-kanMX6 ade6-D20 ura4-D8 leu1-32 his3-D1 arg3-D4</i>	This study
JN120	<i>h-</i>	<i>fml1Δ::natMX4 ChrI(80,355-80,423)::ade6-L469/pUC8/his3⁺/ RTS1-AO/ade6-M375-kanMX6 ade6-D20 ura4-D8 leu1-32 his3-D1 arg3-D4</i>	This study
FO656	<i>h+</i>	<i>ura4-D18 leu1-32 his3-D1 arg3-D4</i>	Lab strain
FO672	<i>h-</i>	<i>rad54Δ::ura4⁺ ura4-D18 leu1-32 his3-D1 arg3-D4</i>	Lab strain
FO808	<i>h-</i>	<i>ura4-D18 leu1-32 his3-D1 arg3-D4</i>	Lab strain
FO1779	<i>h+</i>	<i>rtf1Δ::ura4⁺ ura4-D18 leu1-32 arg3-D4</i>	Lab strain
FO1780	<i>h-</i>	<i>rtf1Δ::ura4⁺ ura4-D18 leu1-32 his3-D1</i>	Lab strain
MCW1016	<i>h-</i>	<i>srs2Δ::ura4⁺ ura4D-18 leu1-32 his3-D1 arg3-D4</i>	Lab strain

MCW1017	<i>h+</i>	<i>srs2Δ::ura4⁺ ura4D-18 leu1-32 his3-D1 arg3-D4</i>	Lab strain
MCW1193	<i>h+</i>	<i>ade6-M26 ura4D18 leu1-32 his3-D1 arg3-D4</i>	Lab strain
MCW1230	<i>h+</i>	<i>rad54Δ::ura4⁺ ura4-D18 leu1-32 his3-D1 arg3-D4</i>	Lab strain
MCW1239	<i>h-</i>	<i>rqh1Δ::ura4⁺ ura4-D18 leu1-32 his3-D1 arg3-D4</i>	Lab strain
MCW1433	<i>h-</i>	<i>ade6-L469/pUC8/his3⁺/RTS1-AO/ade6-M375 ura4-D8 leu1-32 his3-D1 arg3-D4</i>	Lab strain
MCW1779	<i>h+</i>	<i>mus81Δ::arg3⁺ ura4-D18 leu1-32 his3-D1 arg3-D4</i>	Lab strain
MCW1804	<i>h+</i>	<i>rqh1Δ::ura4⁺ ura4-D18 leu1-32 his3-D1 arg3-D4</i>	Lab strain
MCW2080	<i>h+</i>	<i>fml1Δ::natMX4 ura4-D18 leu1-32 his3-D1 arg3-D4</i>	Lab strain
MCW2096	<i>h+</i>	<i>fml1Δ::natMX4 arg3-D4 his3-D1 leu1-32 ura4-D18 ade6-M26</i>	Lab strain
MCW2537	<i>h-</i>	<i>mus81Δ::arg⁺ ura4-D18 leu1-32 his3-D1 arg3-D4</i>	Lab strain
MCW3059	<i>h+</i>	<i>fml1Δ::natMX4 ura4-D18 leu1-32 his3-D1 arg3-D4 ade6-L469/pUC/his3⁺/RTS1-IO/ade6-M375</i>	Lab strain
MCW3061	<i>h+</i>	<i>fml1Δ::natMX4 ura4-D18 leu1-32 his3-D1 arg3-D4 ade6-L469/pUC/his3⁺/RTS1-AO/ade6-M375</i>	Lab strain
MCW3200	<i>h-smt0</i>	<i>his3⁺-aim ade6-469 ura4-D18 his3-D1 arg3-D4</i>	Lab strain
MCW3202	<i>h+S</i>	<i>ura4⁺-aim2 ade6-3083 ura4-D18 leu1-32 his3-D1</i>	Lab strain
MCW4173	<i>h-smt0</i>	<i>fml1Δ::natMX4 his3⁺-aim ade6-469 ura4-D18 his3-D1 arg3-D4</i>	Lab strain
MCW4174	<i>h+S</i>	<i>fml1Δ::natMX4 ura4⁺-aim2 ade6-3083 ura4-D18 leu1-32 his3-D1</i>	Lab strain

MCW4405	<i>h+</i>	<i>fml1ΔC¹⁻⁶⁰³::natMX4 ura4-D18 leu1-32 his3-D1 arg3-D4</i> (diploid)	Lab strain
MCW4577	<i>h+</i>	<i>fml1Δ::hphMX4 ura4-D18 leu1-32 his3-D1 arg3-D4</i>	Lab strain
MCW4593	<i>h+/h-</i>	<i>ade6-M210/ade6-M216 ura4-D18/ura4-D18 leu1-32/leu1-32 his3-D1/his3-D1 arg3-D4/arg3-D4</i>	Lab strain
MCW4639	<i>h+</i>	<i>mhf1Δ::kanMX6 ura4-D18 leu1-32 his3-D1 arg3-D4</i>	Lab strain
MCW4708	<i>h-</i>	<i>fml1Δ::hphMX4 ura4-D18 leu1-32 his3-D1 arg3-D4</i>	Lab strain
MCW4712	<i>h+</i>	<i>ade6-L469/pUC8/ his3⁺/RTS1-IO/ade6-M375 ura4-D8 leu1-32 his3-D1 arg3-D4</i>	Lab strain
MCW4713	<i>h+</i>	<i>ade6-L469/pUC8/ his3⁺/RTS1-AO/ade6-M375 ura4-D8 leu1-32 his3-D1 arg3-D4</i>	Lab strain
MCW4777	<i>h-</i>	<i>mhf2Δ::natMX4 ura4-D18 leu1-32 his3-D1 arg3-D4</i>	Lab strain
MCW5112	<i>h+</i>	<i>mhf2Δ::natMX4 ura4-D18 leu1-32 his3-D1 arg3-D4</i>	Lab strain
MCW5390	<i>h-</i>	<i>wee1::ura4⁺ leu1-32 ura4-D18</i>	Lab strain
MCW5448	<i>h+</i>	<i>leu1-32::pJK148-leu1+-pfh1-m21 pfh1Δ::hphMX4 ura4-D8 leu1-32 his3-D1 arg3-D4</i>	Lab strain
MCW5671	<i>h-</i>	<i>ade6-D20 ura4-D18 leu1-32 his3-D1 ade6-M375 integrated on ChrI between 80-81kb</i>	This study
MCW5673	<i>h-</i>	<i>ade6-D20 ura4-D18 leu1-32 his3-D1 ade6-M375::kanMX6 integrated on ChrII between 86-87kb</i>	This study
MCW5674	<i>h+</i>	<i>rtf1Δ::ura4+ ade6-D20 leu1-32 his3-D1 ade6-M375 integrated on Chr1 between 80-81kb</i>	This study
MCW5676	<i>h+</i>	<i>rtf1Δ::ura4+ ade6-D20 leu1-32 his3-D1 ade6-M375 integrated on Chr2 between 86-87kb</i>	This study

MCW5677	<i>h+</i>	<i>mhf2⁺::kanMX ura4-D18 leu1-32 his3-D1 arg3-D4</i>	Lab strain
MCW5846	<i>h+</i>	<i>mhf1⁺::GFP-kanMX6 mis6⁺mCHERRY::ura4⁺ ura4-D18 leu1-32 his3-D1 arg3-D4</i>	Lab strain
MCW5932	<i>h+</i>	<i>fml1ΔC¹⁻⁶⁰³::natMX4 ura4-D18 leu1-32 arg3-D4 ade6-L469/pUC8/his3⁺/RTS1-AO/ade6-M375</i>	Lab strain
MCW5991	<i>h+</i>	<i>mhf1⁺::kanMX6 his3-D1 arg3-D4 leu1-32 ura4-D18</i>	Lab strain
MCW6001	<i>h+</i>	<i>fml1ΔC¹⁻⁶⁰³::natMX4 ura4-D18 his3-D1 leu1-32 arg3-D4 ade6-M26</i>	Lab strain
MCW6014	<i>h-smt0</i>	<i>sfr1D-11::hphMX4 ura4-D18 leu1-32 his3-D1 arg3-D4</i>	Lab strain
MCW6051	<i>h+S</i>	<i>sfr1D-11::hphMX4 ura4-D18 leu1-32 his3-D1 arg3-D4</i>	Lab strain
MCW6141	<i>h-smt0</i>	<i>fml1ΔC¹⁻⁶⁰³::natMX4 his3⁺-aim ade6-469 ura4-D18 his3-D1 arg3-D4</i>	Lab strain
MCW6142	<i>h+S</i>	<i>fml1ΔC¹⁻⁶⁰³::natMX4 ura4⁺-aim2 ade6-3083 ura4-D18 leu1-32 his3-D1</i>	Lab strain
MCW6162	<i>h+</i>	<i>rad55Δ::hphMX4 ura4-D18 leu1-32 his3-D1 arg3-D4</i>	Lab strain
MCW6163	<i>h-smt0</i>	<i>rad55Δ::hphMX4 ura4-D18 leu1-32 his3-D1 arg3-D4</i>	Lab strain
MCW6321	<i>h+</i>	<i>mhf2^{D87A}::kanMX6 ura4-D18 leu1-32 his3-D1 arg3-D4</i>	This study
MCW6366	<i>h-</i>	<i>mhf2^{D87A}::kanMX6 ura4-D18 leu1-32 his3-D1 arg3-D4</i>	This study
MCW6367	<i>h-</i>	<i>mhf2^{D87A}::kanMX6 ura4-D18 leu1-32 his3-D1 arg3-D4</i>	This study
MCW6376	<i>h+</i>	<i>mhf2^{S59A}::kanMX6 ura4-D18 leu1-32 his3-D1 arg3-D4</i>	This study
MCW6379	<i>h+</i>	<i>mhf2^{Q83A}::kanMX6 ura4-D18 leu1-32 his3-D1 arg3-D4</i>	This study

MCW6450	<i>h+</i>	<i>cenp-TΔ::kanMX6 ura4-D18 leu1-32 his3-D1 arg3-D4</i>	This study
MCW6455	<i>h-</i>	<i>cenp-WΔ::natMX4 ura4-D18 leu1-32 his3-D1 arg3-D4</i>	This study
MCW6675	<i>h-</i>	<i>spg1⁺::GFP-kanMX6 ura4-D18 leu1-32 his3-D1 arg3-D4</i>	This study
MCW6717	<i>h-</i>	<i>mhf2Δ::natMX4 spg1⁺::GFP-kanMX6 ura4-D18 leu1-32 his3-D1 arg3-D4</i>	This study
MCW6724	<i>h-</i>	<i>dcr1Δ::nat^R ade6-210 leu1-32 his3D</i>	From Robin Allshire
MCW6725	<i>h-</i>	<i>sir2::nat^R ade6-210 leu1-32 his3D</i>	From Robin Allshire
MCW6728	<i>h-</i>	<i>swi6::nat^R ade6-210 leu1-32 his3D</i>	From Robin Allshire
MCW6809	<i>h+</i>	<i>mhf1Δ::kanMX6 cdc7⁺::GFP-ura4⁺ ura4-D18 leu1-32 his3-D1</i>	This study
MCW6864	<i>h+</i>	<i>cdc7⁺::GFP-ura4⁺ ura4-D18 leu1-32 his3-D1 arg3-D4</i>	This study
MCW6899	<i>h+</i>	<i>mhf2Δ::natMX4 cenp-TΔ::kanMX6 ura4-D18 leu1-32 his3-D1 arg3-D4</i>	This study
MCW6908	<i>h+</i>	<i>cenp-TΔ::kanMX6 cenp-WΔ::natMX4 ura4-D18 leu1-32 his3-D1 arg3-D4</i>	This study
MCW6976	<i>h+</i>	<i>fml1^{AAA}::natMX4 ura4-D8 leu1-32 his3-D1 arg3-D4</i>	This study
MCW7026	<i>h+</i>	<i>mhf1Δ::kanMX6 cenp-WΔ::natMX4 ura4-D18 leu1-32 his3-D1 arg3-D4</i>	This study
MCW7052	<i>h- smt0</i>	<i>fml1^{AAA}::natMX4 his3⁺-aim ade6-L469 ura4-D8 his3-D1 arg3-D4</i>	This study
MCW7055	<i>h+</i>	<i>fml1^{AAA}::natMX4 ade6-M26 ura4-D8 leu1-32 his3-D1 arg3-D4</i>	This study
MCW7057	<i>h+</i>	<i>fml1^{AAA}::natMX4 mhf2^{D87A}::KanMX6 ura4-D8 leu1-32 his3-D1 arg3-D4</i>	This study

MCW7059	<i>h+</i>	<i>fml1^{AAA}::natMX4 ura4-D8 leu1-32 his3-D1 arg3-D4 ade6-L469/pUC/his3⁺/RTS1-IO/ade6-M375</i>	This study
MCW7062	<i>h+</i>	<i>fml1^{AAA}::natMX4 ura4-D8 leu1-32 his3-D1 arg3-D4 ade6-L469/pUC/his3⁺/RTS1-AO/ade6-M375</i>	This study
MCW7106	<i>h+</i>	<i>mhf2^{D87A}::kanMX6 ura4-D8 leu1-32 his3-D1 arg3-D4 ade6-L469/pUC/his3⁺/RTS1-IO/ade6-M375</i>	This study
MCW7108	<i>h+</i>	<i>mhf2-D87A::KanMX6 fml1-AAA-natMX4 ura4-D8 leu1-32 his3-D1 arg3-D4 ade6-L469/pUC/his3⁺/RTS1-IO/ade6-M375</i>	This study
MCW7136	<i>h+</i>	<i>mhf2^{D87A}::kanMX6 ura4-D8 leu1-32 his3-D1 arg3-D4 ade6-L469/pUC/his3⁺/RTS1-AO/ade6-M375</i>	This study
MCW7138	<i>h+</i>	<i>mhf2^{D87A}::kanMX6 fml1^{AAA}::natMX4 ura4-D8 leu1-32 his3-D1 arg3-D4 ade6-L469/pUC/his3⁺/RTS1-AO/ade6-M375</i>	This study
MCW7151	<i>h-</i>	<i>mhf2^{D87A}::kanMX6 his3⁺-aim ade6-L469 ura4-D8 his3-D1 arg3-D4</i>	This study
MCW7153	<i>h-</i>	<i>mhf2^{D87A}::kanMX6 fml1^{AAA}::natMX4 his3⁺-aim ade6-L469 ura4-D8 his3-D1 arg3-D4</i>	This study
MCW7174	<i>h+</i>	<i>mhf2^{D87A}::kanMX6 ade6-M26 ura4-D18 leu1-32 his3-D1 arg3-D1</i>	This study
MCW7176	<i>h+</i>	<i>fml1^{AAA}::natMX4 mhf2^{D87A}::kanMX6 ade6-M26 ura4-D18 leu1-32 his3-D1 arg3-D1</i>	This study
MCW7178	<i>h+</i>	<i>fml1^{AAA}::natMX4 ura4⁺-aim2 ade6-3083 ura4-D18 leu1-32 his3-D1</i>	This study
MCW7180	<i>h+</i>	<i>mhf2^{D87A}::kanMX6 ura4⁺-aim2 ade6-3083 ura4-D18 leu1-32 his3-D1</i>	This study
MCW7182	<i>h+</i>	<i>fml1^{AAA}::natMX4 mhf2^{D87A}::kanMX6 ura4⁺-aim2 ade6-3083 ura4-D18 leu1-32 his3-D1</i>	This study
MCW7189	<i>h+</i>	<i>fml1ΔC¹⁻⁶⁰³::natMX4 ura4-D18 leu1-32 his3-D1 arg3-D4 ade6-L469/pUC/his3⁺/RTS1-IO/ade6-M375</i>	This study

Table S2.2 *S. pombe* strain construction

Name	Mode of Construction		Method of Strain Validation			Phenotypic characterisation		
	Transformation of linearised DNA	Cross	Colony PCR	Southern Blot	DNA Sequencing	Microscopic Appearance	FACs	Genotoxin spot assay
JN6	Transformation of pMW701 into MCW5673		oMW717 and oMW1348 showed correct integration	No triplication, construct was integrated into the correct site.		Diploid	Diploid	
JN8	Transformation of pMW701 into MCW5674		oMW717 and oMW1348 showed correct integration	No triplication, construct was integrated into the correct site.		Haploid	Haploid	
JN11	Transformation of pMW700 into MCW5676			No triplication, construct was integrated into the correct site.		Haploid	Haploid	
JN13	Transformation of pJN1 into FO656		oMW1427 and oMW285 showed correct integration		Sequenced with oMW1427 confirmed point mutation.	Haploid		Yes
JN16		Crossing FO1779 and FO1780				Haploid		
JN18		Crossing JN6 and JN16		Parental strain JN6 did not have triplication and construct was integrated into the correct site.		Haploid	Haploid	
JN21	Transformation of pJN4 into MCW4593 and sporulated		oMW1431 and oMW285 showed correct integration		Sequenced with oMW1248 confirmed point mutation	Haploid	Haploid	Yes
JN27	Transformation of pFOX2 into MCW5671			No triplication, construct was integrated into the correct site.		Haploid	Haploid	

JN29		Crossing JN8 and FO808		Parental strain JN8 did not have triplication and construct was integrated into the correct site.		Haploid	Haploid	
JN30	Transformation of pFOX2 into MCW5674			No triplication, construct was integrated into the correct site.		Haploid	Haploid	
JN31	Transformation of pFOX2 into MCW5673			No triplication, construct was integrated into the correct site.		Diploid	Diploid	
JN33		Crossing JN11 and FO808		No triplication, construct was integrated into the correct site.		Haploid	Haploid	
JN34		Crossing JN30 and FO808		Parental strain JN30 did not have triplication and construct was integrated into the correct site.		Haploid	Haploid	
JN39		Crossing JN21 and MCW4577				Haploid		Yes
JN42		Crossing JN29 and FO656		Parental strain JN31 did not have triplication and construct was integrated into the correct site.		Haploid	Haploid	
JN46		Crossing JN18 and FO808		Parental strain JN18 did not have triplication and construct was integrated into the correct site.		Haploid	Haploid	
JN53		Crossing MCW4405 and JN21				Haploid		Yes
JN56	Marker swap to replace kanMX6 with hphMX4					Haploid		Yes
JN57		Crossing MCW6376 with MCW6724.				Haploid		Yes

JN59		Crossing MCW6379 and MCW6724				Haploid		Yes
JN61		Crossing MCW6376 and MCW6725				Haploid		Yes
JN63		Crossing MCW6379 and MCW6725				Haploid		Yes
JN65		Crossing MCW6376 with MCW6728				Haploid		Yes
JN67		Crossing MCW6379 with MCW6728				Haploid		Yes
JN70		Crossing MCW6724 and MCW6321				Haploid		Yes
JN71		Crossing MCW6321 and MCW6725				Haploid		Yes
JN73		Crossing MCW6321 and MCW6728				Haploid		Yes
JN79		Crossing JN13 and MCW5390				Haploid		Yes
JN81		Crossing MCW4777 and MCW5846				Haploid. GFP and mCherry foci clearly seen.		Yes
JN84		Crossing JN56 and MCW5846				Haploid. GFP and mCherry foci clearly seen.		Yes

JN86		Crossing MCW4405 and FO808	oMW848 and oMW1004 confirmed parental strain MCW4405 had truncation			Haploid		Yes
JN87		Crossing MCW6724 and MCW4577	Parental strain MCW6724 had deletion			Haploid		Yes
JN89		Crossing MCW6724 and MCW4577	oMW1636 and oMW1637; oMW1638 and oMW1639 confirmed deletion			Haploid		Yes
JN91		Crossing JN89 and MCW1433	Parental strain MCW6724 had deletion			Haploid		Yes
JN93		Crossing JN87 and MCW1433	Parental strain JN87 was made from MCW6724, which had deletion			Haploid		Yes
JN97		Crossing JN89 and MCW2537				Haploid		Yes
JN99		Crossing JN89 and FO672				Haploid		Yes
JN101		Crossing JN89 and MCW1239				Haploid		Yes
JN103		Crossing JN89 and MCW1016				Haploid		Yes
JN105		Crossing JN89 and MCW6163				Haploid		Yes
JN107		Crossing JN89 and MCW6014				Haploid		Yes

JN109	Transformation of pJN5 into MCW4593 followed by sporulation					Haploid		Yes
JN114		Crossing MCW5448 and JN27		Parental strain JN27 did not have triplication and construct was integrated into the correct site.		Haploid	Haploid	Yes
JN116		Crossing MCW5448 and JN29		Parental strain JN29 was made from JN8, which did not have triplication and construct was integrated into the correct site.		Haploid	Haploid	Yes
JN118		Crossing MCW2080 and JN27		Parental strain JN27 did not have triplication and construct was integrated into the correct site.		Haploid	Haploid	Yes
JN120		Crossing MCW2080 and JN29		Parental strain JN29 was made from JN8, which did not have triplication and construct was integrated into the correct site.		Haploid	Haploid	Yes

Table S3.1 Origin firing around *RTS1* at Chromosome I 80355-80423 bp

Origin Name	Start ^a	End ^a	Distance from <i>RTS1</i> (kb)	Time from <i>RTS1</i> (min) ^b	Status (ARS) ^c	Origin Location Assignments	References for this Origin	Origin Efficiency ^d	Replication Time (min) ^e
Origins upstream of <i>RTS1</i> site:									
<i>I-20</i>	20109	20609	66.8	22.3	Dubious	A+T-rich islands, Pu-seq	(Segurado et al. 2003; Daigaku et al. 2015)	28.43 %	ND
<i>I-32</i>	31950	-	55.2	18.4	-	Pu-seq	(Daigaku et al. 2015)	19.78 %	ND
<i>I-44</i>	43650	44150	43.3	14.4	Dubious	A+T-rich islands, Pu-seq	(Segurado et al. 2003; Daigaku et al. 2015)	48.01 %	ND
<i>I-51</i>	50226	51226	36.5	12.2	Dubious	HU and microarrays	(Hayashi et al. 2007)	ND	ND
<i>I-54</i>	53600	54600	31.8	10.6	Dubious	HU and microarrays, Pu-seq	(Hayashi et al. 2007; Daigaku et al. 2015)	0.84 %	ND
<i>I-62</i>	61633	62133	25.3	8.4	Likely	ChIP, A+T-rich islands, Pu-seq	(Segurado et al. 2003; Hayashi et al. 2007; Daigaku et al. 2015)	61.10 %	ND
<i>I-70</i>	69625	70125	17.3	5.8	Likely	ChIP, A+T-rich islands, Pu-seq	(Segurado et al. 2003; Hayashi et al. 2007; Daigaku et al. 2015)	17.74 %	ND
<i>I-74</i>	73976	74976	12.7	4.2	Dubious	HU and microarrays, Pu-seq	(Hayashi et al. 2007; Daigaku et al. 2015)	3.06 %	ND

^a Genomic location according to OriDB, a DNA replication origin database.

^b Assuming rate of 3 kb/min at 30°C in minimal media. Previously, replication rate was measured to be 2.8 kb/min at 25 °C in minimal media Heichinger C, Penkett CJ, Bahler J, Nurse P. 2006. Genome-wide characterization of fission yeast DNA replication origins. *EMBO J* **25**: 5171-5179.

^c Confirmed – origin has been cloned and tested by Autonomously Replicating Sequence (ARS) assay, or has been detected by 2D gel analysis. Likely – origin identified by two (or more) microarray studies but not yet confirmed. Dubious – origin identified by only one microarray study Nieduszynski CA, Hiraga S, Ak P, Benham CJ, Donaldson AD. 2007. OriDB: a DNA replication origin database. *Nucleic Acids Res* **35**: D40-46, Siow CC, Nieduszynski SR, Muller CA, Nieduszynski CA. 2012. OriDB, the DNA replication origin database updated and extended. *Ibid*. **40**: D682-686.

^d Origin efficiency in the mitotic S-phase Daigaku Y, Keszthelyi A, Muller CA, Miyabe I, Brooks T, Retkute R, Hubank M, Nieduszynski CA, Carr AM. 2015. A global profile of replicative polymerase usage. *Nat Struct Mol Biol*.

^e Time of 50% replication along the nucleotide sequence of the origin during mitosis in minimal media Heichinger C, Penkett CJ, Bahler J, Nurse P. 2006. Genome-wide characterization of fission yeast DNA replication origins. *EMBO J* **25**: 5171-5179. 70 - 80 min is early in S-phase, while >80 min is late in S-phase. The overall length of S-phase in fission yeast is 20 min in minimal media Mitchison JM, Creanor J. 1971. Further measurements of DNA synthesis and enzyme potential during cell cycle of fission yeast *Schizosaccharomyces pombe*. *Exp Cell Res* **69**: 244-247.

<i>I-79</i>	78850	79350	8.3	2.8	Likely	ChIP, A+T-rich islands, Pu-seq	(Segurado et al. 2003; Hayashi et al. 2007; Daigaku et al. 2015)	11.83 %	ND
Origins downstream of <i>RTS1</i> site:									
<i>I-85</i>	84850	85350	8.3	2.8	Likely	ChIP, HU and microarrays, A+T-rich islands, Pu-seq	(Segurado et al. 2003; Feng et al. 2006; Hayashi et al. 2007; Daigaku et al. 2015)	10.99 %	ND
<i>I-88</i>	87476	88476	11.1	3.7	Likely	ChIP, HU and microarrays, Pu-seq	(Feng et al. 2006; Hayashi et al. 2007; Daigaku et al. 2015)	17.00 %	ND
<i>I-95</i>	94350	95350	18.0	6	Likely	ChIP, HU and microarrays, Pu-seq	(Heichinger et al. 2006; Hayashi et al. 2007; Daigaku et al. 2015)	26.19 %	80
<i>I-107a</i>	107250	-	30.6	10.2	-	Pu-seq	(Daigaku et al. 2015)	9.51 %	ND
<i>I-107b</i>	107250	-	30.6	10.2	-	Pu-seq	(Daigaku et al. 2015)	7.90 %	ND
<i>I-107c</i>	107850	-	31.2	10.4	-	Pu-seq	(Daigaku et al. 2015)	7.85 %	ND
<i>I-113</i>	112209	114006	36.3	12.1	Confirmed	ChIP, HU and microarrays, A+T-rich islands and 2D gels, Pu-seq	(Segurado et al. 2003; Feng et al. 2006; Heichinger et al. 2006; Hayashi et al. 2007; Daigaku et al. 2015)	52.83 %	79
<i>I-124</i>	124138	124638	47.5	15.8	Likely	HU and microarrays, A+T-rich islands, Pu-seq	(Segurado et al. 2003; Feng et al. 2006; Heichinger et al. 2006; Daigaku et al. 2015)	52.83 %	78
<i>I-130</i>	129780	130280	53.2	17.7	Likely	A+T-rich islands, Pu-seq	(Segurado et al. 2003; Daigaku et al. 2015)	2.94 %	ND

Origin firing around *RTS1* at Chromosome II 86494-86402 bp

Origin Name	Start ^a	End ^a	Distance from <i>RTS1</i> (kb)	Time from <i>RTS1</i> (min) ^b	Status (ARS) ^a	Origin Location Assignments	References for this Origin	Origin Efficiency ^c	Replication Time (min) ^d
Origins upstream of <i>RTS1</i> site:									
<i>II-61</i>	61350	-	31.9	10.6	Dubious	Pu-seq	(Daigaku et al. 2015)	11.49 %	ND
<i>II-63</i>	62126	63126	30.7	10.2	Dubious	ChIP	(Hayashi et al. 2007)	ND	ND
<i>II-66</i>	66198	66698	26.8	8.9	Likely	ChIP, A+T-rich islands, Pu-seq	(Segurado et al. 2003; Hayashi et al. 2007; Daigaku et al. 2015)	11.81 %	ND
<i>II-68</i>	67791	68291	25.3	8.4	Dubious	A+T-rich islands	(Segurado et al. 2003)	ND	ND
<i>II-70</i>	69376	70376	23.4	7.8	Dubious	ChIP	(Hayashi et al. 2007)	ND	ND
<i>II-75</i>	74379	74879	18.6	6.2	Likely	ChIP, A+T-rich islands, Pu-seq	(Segurado et al. 2003; Hayashi et al. 2007; Daigaku et al. 2015)	31.73 %	ND
<i>II-78</i>	77626	78626	15.2	5.1	Dubious	HU and microarrays, Pu-seq	(Hayashi et al. 2007; Daigaku et al. 2015)	15.18 %	ND
<i>II-81</i>	80226	81226	12.6	4.2	Likely	ChIP	(Hayashi et al. 2007)	ND	ND
<i>II-86a</i>	85845	86345	7.2	2.4	Likely	ChIP, HU and microarrays, A+T-rich islands, Pu-seq	(Segurado et al. 2003; Feng et al. 2006; Hayashi et al. 2007; Daigaku et al. 2015)	6.30 %	ND
<i>II-86b</i>	86250	-	7.0	2.3	-	Pu-seq	(Daigaku et al. 2015)	7.03 %	ND
Origins downstream of <i>RTS1</i> site:									
<i>II-90</i>	89868	90368	7.2	2.4	Likely	ChIP, HU and microarrays, A+T-rich islands, Pu-seq	(Segurado et al. 2003; Feng et al. 2006; Hayashi et al. 2007; Daigaku et al. 2015)	23.46 %	ND

<i>II-94</i>	93972	94472	11.3	3.8	Likely	HU and microarrays, A+T-rich islands	(Segurado et al. 2003; Heichinger et al. 2006)	1.70 %	ND
<i>II-97</i>	96780	97280	14.1	4.7	Likely	HU and microarrays, A+T-rich islands	(Segurado et al. 2003; Heichinger et al. 2006)	15 %	76
<i>II-100</i>	99534	100034	16.9	5.6	Likely	A+T-rich islands, Pu-seq	(Segurado et al. 2003; Daigaku et al. 2015)	ND	ND
<i>II-103</i>	102504	103004	19.8	6.6	Likely	A+T-rich islands	(Segurado et al. 2003)	16.79 %	ND
<i>II-104</i>	103226	104226	20.8	6.9	Likely	ChIP, HU and microarrays, A+T-rich islands	(Segurado et al. 2003; Feng et al. 2006; Hayashi et al. 2007)	ND	ND
<i>II-109</i>	108849	109349	26.2	8.7	Likely	HU and microarrays, A+T-rich islands	(Segurado et al. 2003; Feng et al. 2006)	ND	ND
<i>II-111</i>	110600	111600	28.2	9.4	Likely	ChIP, HU and microarrays, Pu-seq	(Feng et al. 2006; Hayashi et al. 2007; Daigaku et al. 2015)	42.43 %	ND

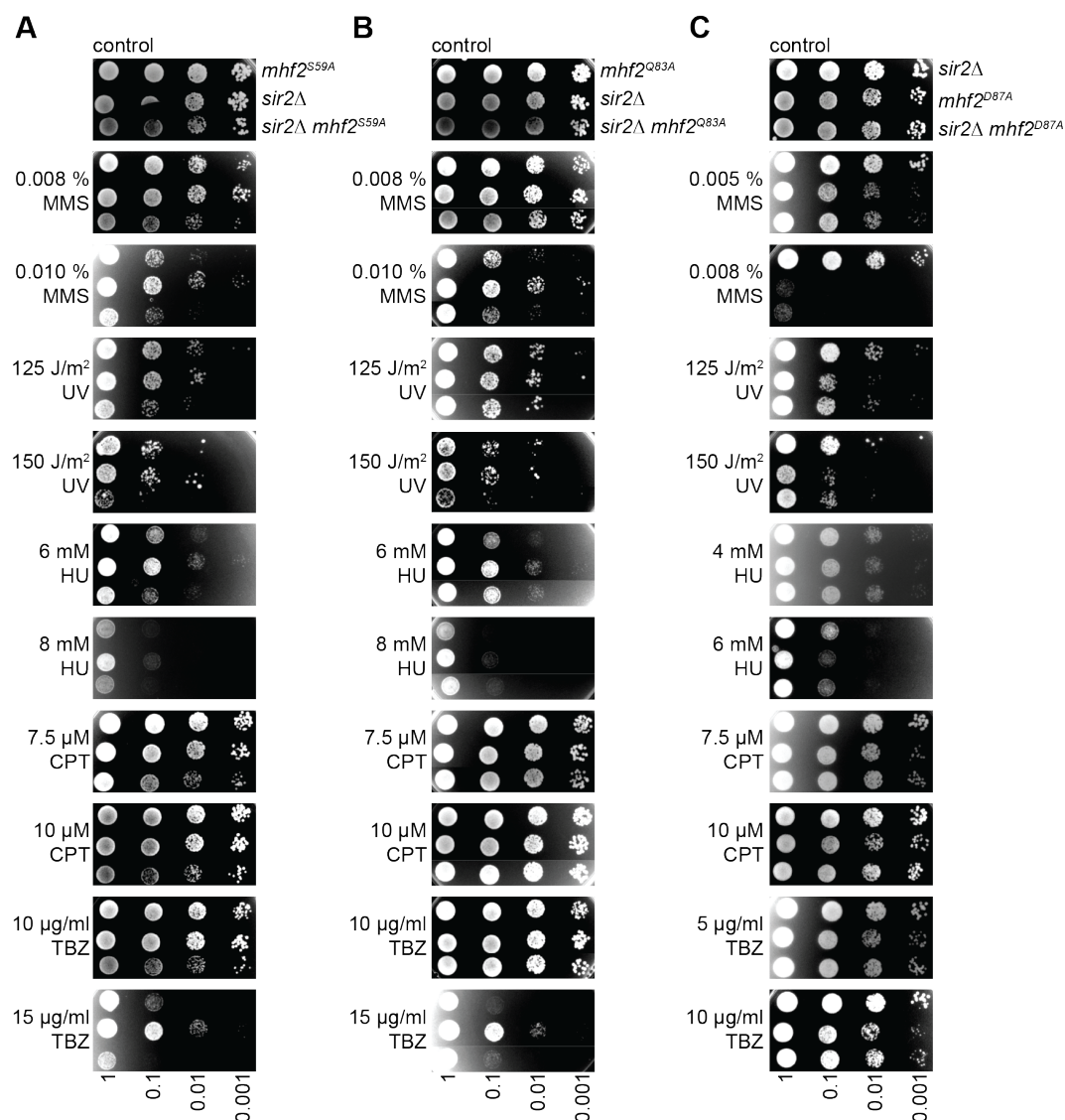


Figure S4.1 Genetic interactions between *mhf2*^{S59A}, *mhf2*^{Q83A} and *mhf2*^{D87A} point mutants and *sir2*Δ

Crude spot assays comparing the growth and genotoxin sensitivities of the following strains after 4 days growth at 30 °C: (A) MCW6376, MCW6725 and JN61; (B) MCW6379, MCW6725 and JN63; (C) MCW6725, MCW6321 and JN71. The crude spot assays from (B) were done on the same plate but have been cropped and placed next to each other for ease of presentation.

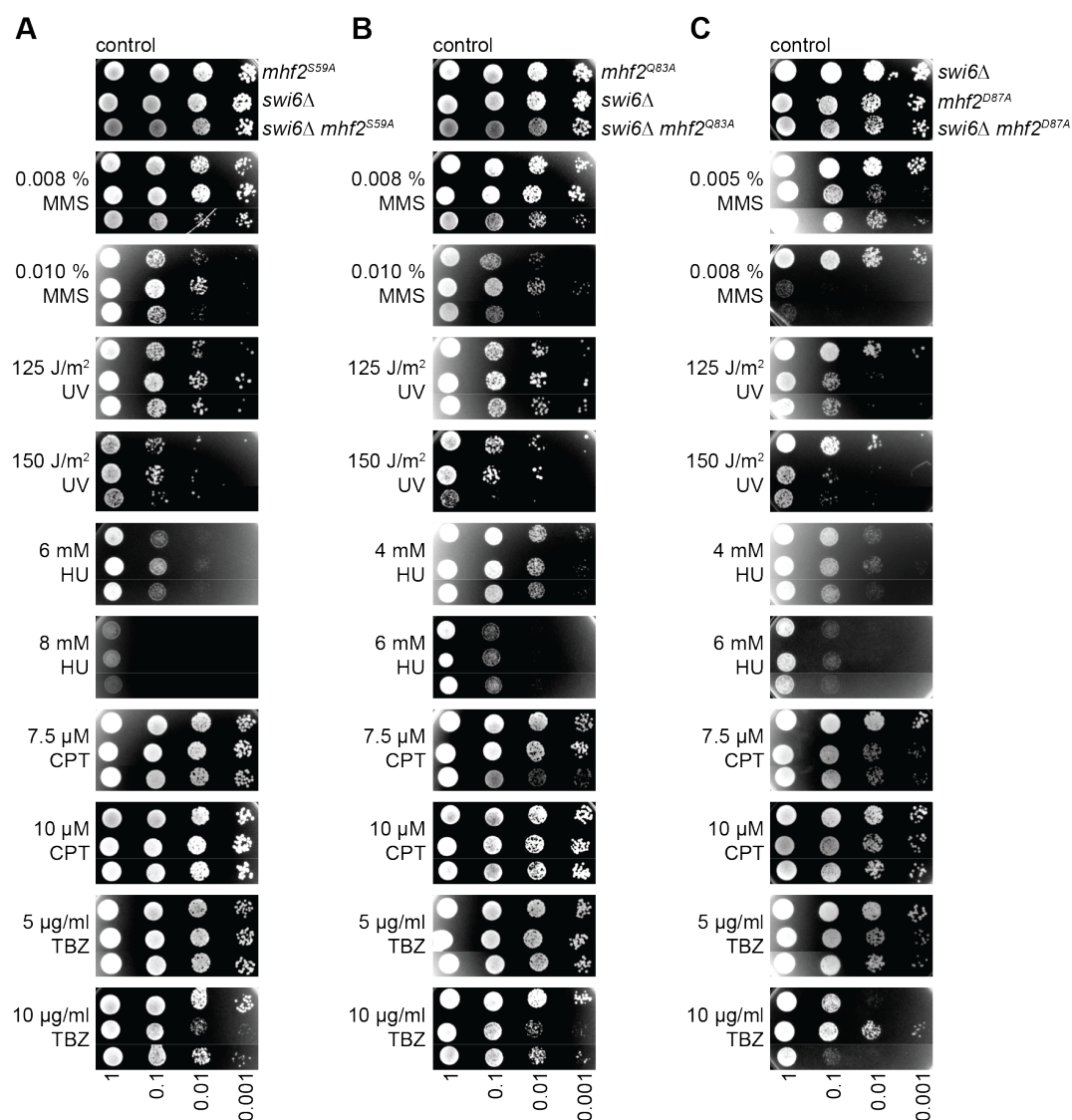


Figure S4.2 Genetic interactions between *mhf2*^{S59A}, *mhf2*^{Q83A} and *mhf2*^{D87A} point mutants and *swi6*Δ

Crude spot assays comparing the growth and genotoxin sensitivities of the following strains after 4 days growth at 30 °C: (A) MCW6376, MCW6728 and JN65; (B) MCW6379, MCW6728 and JN67; (C) MCW6728, MCW6321 and JN73. The crude spot assays from (B) and (C) were done on the same plate but have been cropped and placed next to each other for ease of presentation.

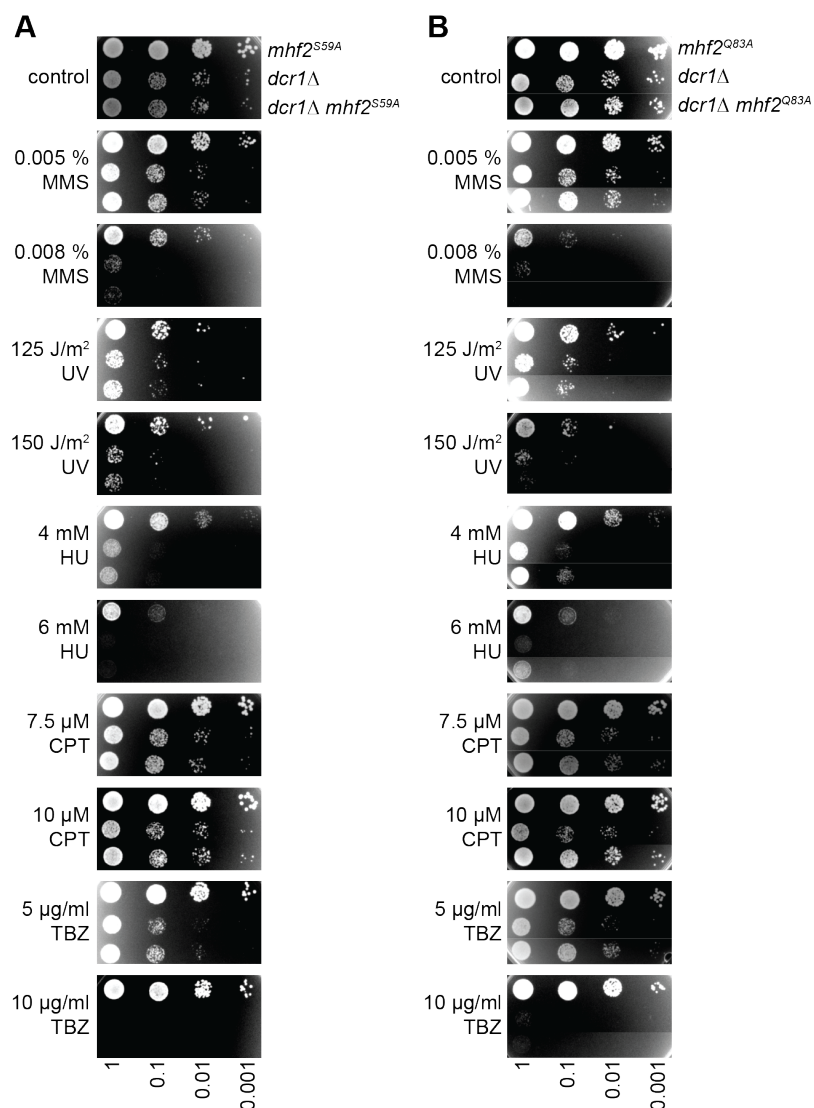


Figure S4.3 Genetic interactions between *mhf2^{S59A}* and *mhf2^{Q83A}* point mutants and *dcr1Δ*

Crude spot assays comparing the growth and genotoxin sensitivities of the following strains after 4 days growth at 30 °C: (A) MCW6376, MCW6724 and JN57; (B) MCW6379, MCW6724 and JN59. The crude spot assays from (B) were done on the same plate but have been cropped and placed next to each other for ease of presentation.

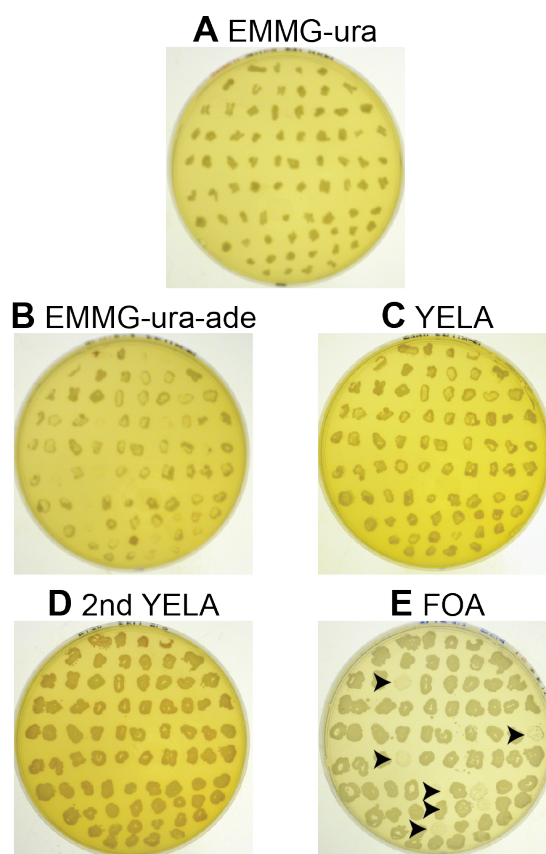


Figure S5.1 Plasmid gap repair assay

An example of the analysis of plasmid gap repair assay transformants. (A) Ura^+ transformants are first patched onto minimal media lacking uracil (EMMG-ura). (B) The number of Ura^+ transformants that are Ade^+ is assessed by replica plating the EMMG-ura plate onto minimal media lacking uracil and adenine (EMMG-ura-ade). (C and D) Two rounds of replica plating onto non-selective media (YELA) are performed to allow loss of non-integrated plasmid DNA. (E) The second YELA plate is replica plated onto FOA to identify stable and unstable transformants. Stable transformants remain Ura^+ and therefore are unable to grow on FOA (examples indicated by black arrowheads), whereas unstable transformants lose the ura4^+ gene and are therefore able to grow on FOA.

Table S6.1 *mhf1* isolates that are temperature sensitive for growth at 37 °C

TS denotes temperature sensitive.

#	TS at 37 °C for growth	MMS sensitive at 28 °C	Colony PCR	Sequenced
49	✓	X		
55	✓	X		
62	✓	✓		
68	✓	X		
138	✓	✓	✓	✓
153	✓	✓	✓	✓
203	✓	X		
244	✓	X		
255	✓	X	✓	✓
259	✓	✓		
260	✓	✓	✓	✓
268	✓	X		
280	✓	✓		
303	✓	X		
320	✓	✓		
343	✓	✓	✓	✓
348	✓	X		
374	✓	X		
375	✓	✓		
443	✓	X		
458	✓	✓		
468	✓	X		
480	✓	✓		
506	✓	X	✓	✓
524	✓	X		

Table S6.2 *mhf1* isolates that are not temperature sensitive for growth at 37 °C, but genotoxin sensitive

TS denotes temperature sensitive.

#	MMS sensitive at 28 °C	MMS sensitive at 37 °C
12	✓	✓
14	X	✓
21	X	✓
41	✓	✓
51	✓	✓
58	✓	✓
72	✓	✓
79	X	✓
93	✓	✓
110	✓	✓
118	✓	✓
126	X	✓
140	X	✓
180	X	✓
185	✓	✓
187	✓	✓
206	✓	✓
225	X	✓
232	✓	✓
249	X	✓
264	✓	✓
272	X	✓
282	✓	✓
291	X	✓
296	X	✓
297	X	✓
307	X	✓
363	✓	✓
376	✓	✓
377	✓	✓
398	✓	✓
404	X	✓
412	X	✓
428	X	✓
448	X	✓
452	✓	✓
479	X	✓
485	✓	✓
491	✓	✓
516	X	✓
520	X	✓
523	✓	✓
525	X	✓

Table S6.3 *mhf2* isolates that are temperature sensitive for growth at 37 °C

TS denotes temperature sensitive.

#	TS at 37 °C for growth	MMS sensitive at 28 °C	Colony PCR	Sequenced
9	✓	✓	✓	✓
11	✓	✓	✓	
12	✓	✓		
24	✓	X		
44	✓	✓		
48	✓	✓	✓	
57	✓	✓	✓	
67	✓	X	✓	✓
126	✓	X		
133	✓	✓		
140	✓	X		
217	✓	✓		
225	✓	X		
237	✓	✓		
245	✓	X		
255	✓	X		
258	✓	✓		
276	✓	✓		
279	✓	✓		
280	✓	✓		
282	✓	✓		
288	✓	✓		
332	✓	✓		
339	✓	X		
353	✓	X		

Table S6.4 *mhf2* isolates that are not temperature sensitive for growth at 37 °C, but genotoxin sensitive

TS denotes temperature sensitive.

#	MMS sensitive at 28 °C	MMS sensitive at 37 °C	#	MMS sensitive at 28 °C	MMS sensitive at 37 °C
2	✓	✓	178	✓	✓
3	✓	✓	182	X	✓
4	✓	✓	183	X	✓
13	✓	✓	187	✓	✓
18	✓	✓	192	X	✓
27	✓	✓	199	✓	✓
31	✓	✓	203	✓	✓
47	✓	✓	209	X	✓
51	✓	✓	214	X	✓
54	✓	✓	221	X	✓
58	✓	✓	228	X	✓
59	✓	✓	235	✓	✓
76	✓	✓	239	X	✓
86	✓	✓	252	✓	✓
88	X	✓	261	✓	✓
92	X	✓	275	✓	✓
93	X	✓	290	✓	✓
94	X	✓	292	✓	✓
104	✓	✓	312	X	✓
122	X	✓	314	X	✓
130	X	✓	317	X	✓
138	X	✓	318	X	✓
155	X	✓	329	X	✓
156	✓	✓	336	X	✓
162	X	✓	351	X	✓
167	X	✓	360	✓	✓
168	✓	✓	364	X	✓
169	✓	✓			

Table S6.5 Mutations in *mhf1* and *mhf2* temperature sensitive isolates

TS denotes temperature sensitive.

#	Mutations in <i>mhf1</i>		#	Mutations in <i>mhf2</i>	
	DNA changes	Amino acid changes		DNA changes	Amino acid changes
138	A to T 58 A to T 224	N20Y D75V	9	G to T 214	E72→STOP
153	G to A 25 T to C 55	A9T C19R	67	T to C 212	L71S
255	A to T 87 A to T 224 A to G 302	S29S (silent) D75V K101R			
260	T to G 35 G to T 109 T to A 184	F12C D37Y F62I			
343	A to C 194	H65P			
506	T to C 41 T to C 85 T to C 107 T to C 212	V14A S29P V36A V71A			

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