Genetic analysis and meiotic role of the *Saccharomyces cerevisiae RecQ* helicase *SGS1*

PhD thesis

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Acknowledgments

An old saying in French goes a bit like this: "When you are drowning, it is sometimes better not to try to stay afloat but to go straight down, as it will be easier to bounce back up" This sentence could very well be applied to the course of this PhD, the least I could say is that I had a bumpy journey…

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Genetic analysis and meiotic role of the *Saccharomyces cerevisiae RecQ* helicase *SGS1*

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Abstract:

SGS1, a *Saccharomyces cerevisiae* 3′-5′ DNA helicase, is a homologue of the *Escherichia coli RecQ* gene. It is essential for genomic stability both during mitosis and meiosis. The purpose of this thesis is to provide a better understanding of the role of this helicase during meiotic recombination. In meiosis, *SGS1* mutant cells display a decrease in sporulation efficiency and spore viability. In addition, the unusual spore viability pattern observed in *SGS1* mutants cannot be explained solely by meiosis I or meiosis II missegregations. These problems could be partially explained by defects in mitotic chromosome segregation or problems with meiotic S-phase. Cytological experiments demonstrating an increase in synapsis initiation complexes and axial associations in *sgs1*Δ could be explained by an early function of Sgs1p in meiosis, such as the unwinding of inappropriate strand invasion events. Consistent with this, we observed increased gene conversion, increased homeologous recombination and increased interaction between sister chromatids.

Recent observations have suggested that, Sgs1p and Top3p in *S. cerevisiae*, and the human orthologue protein BLM, in conjunction with the Top3α protein, can dissolve double Holliday junctions. Physical analyses of double-strand break repair in meiosis, combined with the genetic analysis of this work, indicate a late function of the Sgs1 protein in the dissolution of double Holliday junctions. We have shown an unusual class of tetrads in which non-sister spores and recombinant spores are dead. We interpret this as a consequence of the failure to untangle intertwined chromatids. This defect in *SGS1* mutant strains could be explained by either the presence of pre-meiotic S-phase catenates, a defect in crossover resolution and/or a defect in the dissolution of closely spaced double Holliday junctions.

Chapter 1: Introduction

Helicases are a specific class of enzymes that play essential roles in the biology of the cell. They facilitate DNA synthesis, DNA repair, recombination and transcription by unwinding double-stranded DNA molecules. Such unwinding is done by breaking the hydrogen bonds between the two DNA strands and requires ATP hydrolysis. This work specifically focuses on one particular class of DNA helicase, the RecQ family and more specifically, its *Saccharomyces cerevisiae* homologue, *SGS1*. Members of this family of helicase are defined by homology to the *RecQ* helicase of *E. coli* (Nakayama *et al.*, 1984). The different domains featured in the RecQ helicase have been conserved through evolution. These include the presence of seven conserved amino acid motifs in the helicase domain, the RecQ Conserved domain (RQC) and the Helicase RNase D C-terminal domain (HRDC). Sequence homology is well conserved between family members in these domains but can vary widely outside (Figure 1.1). All members of the RecQ family display a 3′ to 5′ ATP dependant helicase activity.

This class of helicase has been reported to play a mechanistic role during DNA replication and DNA repair in mitosis. They are also thought to be involved in homologous recombination and checkpoints. However, little is known about the functions of RecQ helicases in meiosis and this work will focus on understanding more accurately the role of *SGS1* during this specialised cell division.

1.1. The RecQ helicase protein family:

RecQ was first identified as a key component of the RecF recombination pathway of *E. coli,* which is activated to maintain conjugal recombination and U.V. resistance in *recBCD* mutants. The RecQ protein has also been reported to play a role in suppressing illegitimate recombination in *E. coli* (Hanada *et al.*, 1997).

By over-expressing the *RecQ* gene on a plasmid and purifying the protein, Umezu *et al.* (1990), identified and characterised the ATP-dependent helicase activity of the RecQ protein. The polarity was characterised by measuring the release of radio-labelled single-stranded DNA fragments from duplex DNA. The RecQ protein unwinds

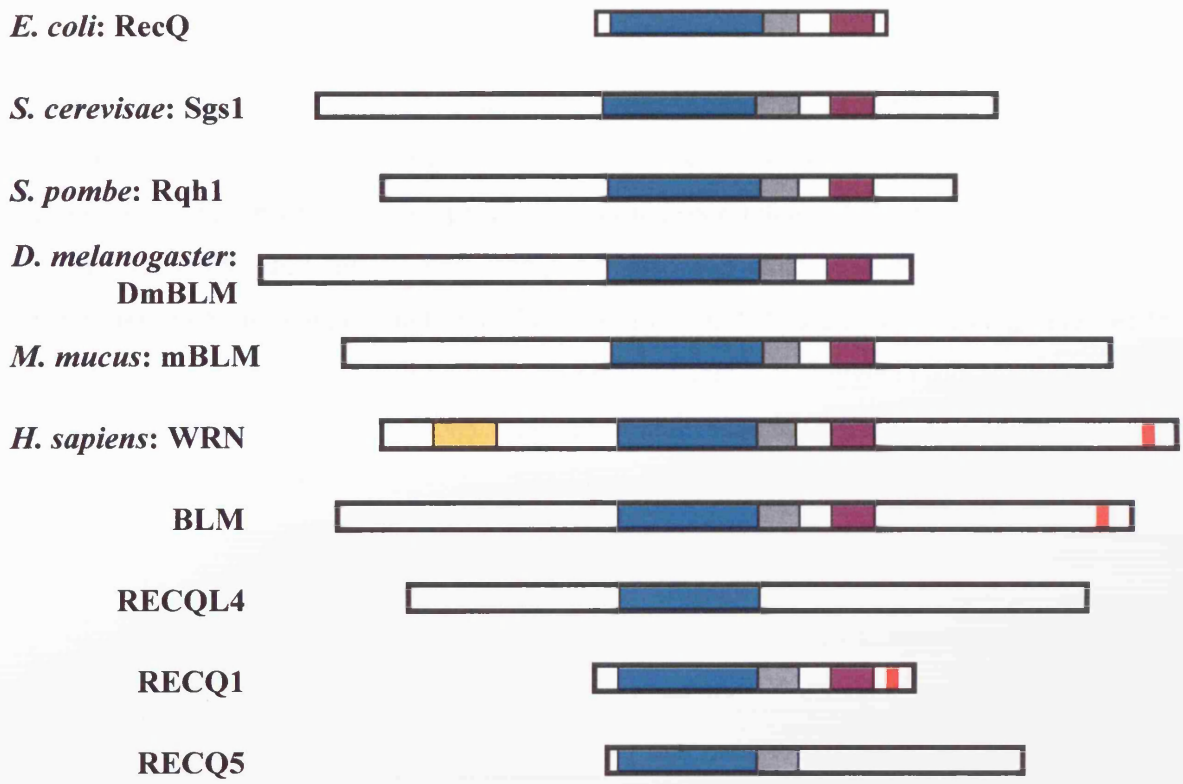


Figure 1.1: Schematic representation of the RecQ protein family.
The different family members are aligned according to their helicase domain.
The conserved domains between the different proteins members share up to 90% homology.
Helicase domain; RecQ Conserved domain; Helicase and RNase D Conserved domain (HRDC); Exonuclease domain; Nuclear localisation domain.

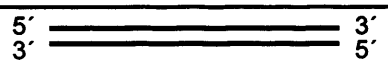
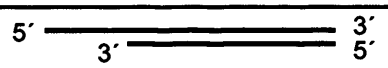
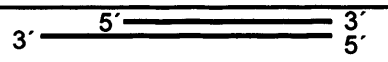
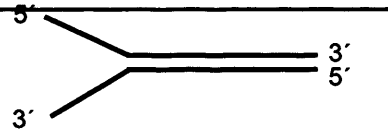
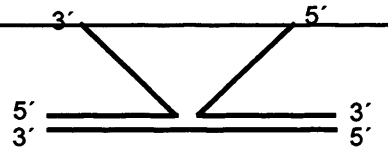
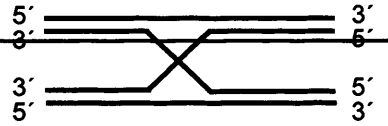
duplex DNA in a 3′ to 5′ direction. This reaction requires ATP and Mg^{2+} cations (Umezu *et al.*, 1990). The ATPase activity is dependent upon the presence of DNA as no ATPase activity could be detected in absence of DNA.

RecQ's role in recombination has been characterised by experiments *in vitro.* Harmon *et al.* (1998) have shown firstly that the RecQ protein in conjunction with the strand exchange protein RecA (*Saccharomyces cerevisiae* homologue, Rad51p) and Single Strand Binding protein (SSB, *S. cerevisiae* homologue, RPA) can initiate homologous recombination by processing double-stranded DNA into single stranded DNA. Secondly, that the *E. coli* RecQ helicase can bind to a wide range of DNA substrates such as 3-way junctions, flayed duplex and 3'-ssDNA overhang. The wide variety of substrates for RecQ helicase proteins (Table 1.1) suggests that they are likely to play multiple roles during DNA replication and recombination process (Harmon and Kowalczykowski, 1998).

In *Saccharomyces cerevisiae* the *SGS1* (Figure 1.1) gene was first identified as a Slow Growth Suppressor of the phenotype due to mutations in the type I Topoisomerase III gene, *TOP3* (Gangloff *et al.*, 1994). In *S. cerevisiae top3* mutants grow slower than wild-type cells and display an increased genomic instability. *top3* suppressors were found to arise spontaneously in yeast cultures where "pseudo-revertants" grew at nearly wild-type rate. These suppressors were shown to be genetic mutations by segregation analysis *i.e.* they were crossed to wild type and the growth phenotype was analysed in meiotic progeny. The segregation observed was consistent with that of two independent genes segregating (¾ of spore colonies with normal growth and ¼ of spore colonies with slow growth). All pseudo-revertants were shown to map to the same gene, which was named *SGS1* (Gangloff *et al.*, 1994). *SGS1* was also identified independently by Watt *et al.* (1995) in a screen for proteins that interacted with Top2p. Nucleotide sequencing indicated that *SGS1* was a member of the RecQ helicase family. Similar to *RecQ*, Sgs1p unwinds duplex DNA or heteroduplex RNA-DNA in an ATP or dATP dependant manner in a 3′ to 5′ direction (Bennett *et al.*, 1998). The ATPase activity is dependent on the presence of single- or double-stranded DNA.

The yeast *Schizosaccharomyces pombe* also possesses a *RecQ* orthologue, *rqh1*$^+$ (Figure 1.1), which has been identified independently by several laboratories. The

Table 1.1: DNA substrates bound and unwound by RecQ helicases.

Substrate[1]		Protein Unwinding activities[2]: RecQ	Sgs1	BLM	WRN	RecQ1
blunt duplex		++	N.D	∅	∅	∅
5′ ssDNA overhang		++	∅	∅	∅	∅
3′ ssDNA overhang		++	++	+	+	++
Flayed duplex		++	++	++	++	N.D
3-way junction		++	++	N.D	N.D	N.D
4-way junction		++	++	+++	++	N.D

Note: [1]: DNA substrates names and schematic representations are presented next to one another. These substrates were made from synthetic oligonucleotides of various length. [2]:Binding and unwinding activities of the different RecQ helicases have been tested *in vitro*. N.D: Not Determined; ∅: No unwinding detected; +: detectable but very limited unwinding; ++: robust unwinding; +++: robust unwinding and preference for that particular substrate (adapted from Bachrati and Hickson (2003)).

rqh1$^+$ gene, previously known as *hus2*$^+$, was first identified in a genetic screen for mutants sensitive to hydroxyurea during mitotic growth (Enoch *et al.*, 1992). Sequence analysis of *hus2*$^+$, thereafter renamed *rqh1*$^+$, have shown that it shares homology with the RecQ DNA helicase family (Stewart *et al.*, 1997). *rqh1*$^+$ encodes a 1328 amino acid protein. *S. pombe* cells deleted for *rqh1*$^+$ undergo aberrant mitosis when cultured with HU or exposed to UV irradiation. The phenotype of *rqh1*$^-$ mutant resembles the morphology of "cut" mutants where the septum is formed before completion of mitosis leaving an anucleate daughter cell.

In humans, five RecQ homologues have been identified to date (Figure 1.1). These are *BLM*, *WRN*, *RECQL4*, *RECQ1* and *RECQ5* genes (Cui *et al.*, 2003; Ellis *et al.*, 1995; Kitao *et al.*, 1998; Kitao *et al.*, 1999a; Puranam and Blackshear, 1994; Yu *et al.*, 1996). When mutated, three of these genes give rise to cancer predisposition syndromes such as Bloom's syndrome (mutation in *BLM*), Werner's syndrome (mutation in *WRN*) and Rothmund-Thompson's syndrome (mutation in *RECQL4*). Mutations in these RecQ orthologues are all recessive. To date no human genetic disorder has been associated with either *RECQ1* or *RECQ5*. The *BLM* gene was identified and mapped by analysis of consanguineous families affected by the Bloom's syndrome (German *et al.*, 1994; Karow *et al.*, 1997). Patients deficient for BLM protein have a well-characterised phenotype with short stature, sun-sensitive skin, type II diabetes from an early age and immunodeficiency. They are also prone to all the cancers known to man. The life expectancy of a Bloom's patient is short, not exceeding 30 years old. Bloom's male patients are infertile while women are sub-fertile. Mutations in the *BLM* gene are in most cases missense, frameshift, nonsense and splicing mutations which all result in the lost of function of the BLM protein (Hickson, 2003).

Although the prevalence of the Werner's syndrome is only 1 in 1,000,000 individuals, this syndrome is well known for its premature aging phenotype, which begins at puberty. All patients display phenotypes usually associated with the elderly such as greying and thinning of hair, cataracts, atherosclerosis, osteoporosis and sarcomas. Other phenotypes include type II diabetes and hypogonadism. On average, the life expectancy of Werner's patients does not exceed forty. The causes of death are often due to cardiovascular failure or cancer (Epstein, 1966). As with Bloom's, the majority of mutations in the *WRN* gene lead to complete loss of function.

Rothmund-Thompson's syndrome was identified in 1868 by Auguste Rothmund and in 1923 by Sidney Thompson. They both reported a peculiar rash but while Rothmund described a high incidence of juvenile cataracts, Thompson reported skeletal abnormalities in addition to the rash. In 1957 William Taylor proposed that both disorders were attributed to a single genetic predisposition, which he named the Rothmund-Thompson's syndrome. The *RECQL4* protein (Figure 1.1), when mutated, is responsible for this condition (Kitao *et al.*, 1998; Kitao *et al.*, 1999b). Patients affected by the Rothmund-Thompson's syndrome are characterised by a sun-sensitive rash from an early age, juvenile cataracts, radial ray defects, sparse hair eyebrows and eyelashes. They also display bone abnormalities and a predisposition to malignancies, particularly bone cancer (osteosarcoma) and non-melanoma skin cancer (Wang *et al.*, 1999). Most of the mutations in affected patients occur in the helicase domain and are predicted to produce truncated proteins lacking this domain (Kitao *et al.*, 1999a).

Neither *RecQ1* nor *RecQ5* are associated with diseases in humans. *RECQ1* was first cloned and characterised by Puranam *et al.* (1994) and *RECQ5* by Kitao *et al.* (1998). Both open reading frames are rather short when compared to *BLM*, *WRN* and *RECQL4.* The *RECQ1* cDNA is only 1977 base pairs long encoding a protein of 659 amino acids in length (Figure 1.1) (Puranam and Blackshear, 1994), while the open reading frame of the *RECQ5* gene is of 1233 base pairs encoding a 410 amino acids protein (Figure 1.1) (Kitao *et al.*, 1998). RECQ1 displaces short-length double-stranded DNA and its processivity is greatly increased by human replication protein A (hRPA). RECQ1p also requires an overhanging 3′ tail longer than 10 base pairs to load onto the DNA. Interestingly, D-loop DNA can also be a substrate for RECQ1 (Cui *et al.*, 2003).

In mice several RecQ proteins have been identified based on their homology to the human *RecQ* genes. The mouse *BLM* gene was identified by screening a spermatocyte cDNA mice library with a DNA probe made of the 5′end of human *BLM* (Seki *et al.*, 1998). Seki *et al.* also examined the expression of the *mBLM* gene in different tissues. *mBLM* is preferentially expressed in the testis from 12 to 14 days after birth when the cells are in pachytene. Such expression suggests that *mBLM* is involved in meiosis. *mBLM* was also independently identified using the same techniques by Chester *et al.* (1998). To further characterise its function, gene replacement techniques on mice

embryonic stem cells were used to create mutant alleles of the mouse *BLM* gene (Chester *et al.*, 1998; Chester *et al.*, 2006; Luo *et al.*, 2000). According to Chester *et al.* (1998) homozygosity for *blm* is lethal as $blm^{-/-}$ embryos die *in utero*. As in human Bloom's cells, mice $blm^{-/-}$ embryonic cells have an increased level of sister chromatid exchanges (Chester *et al.*, 1998). Interestingly, Luo *et al.* (2000) reported three different *blm* alleles, created using targeted-vector replacement techniques (Blm^{m1Brd}, Blm^{m2Brd} and Blm^{m3Brd}). While Blm^{m1Brd} is a complete deletion of the exon 2 of the *BLM* gene, Blm^{m2Brd} is the result of a complex insertion event of the targeting plasmid. This allele carries three copies of the targeting vector and lead to an aberrant transcript with four copies of exon 3. The third allele Blm^{m3Brd} is a derivative of Blm^{m2Brd} after excision of the insertion cassette by Cre-LoxP-mediated deletion (Luo *et al.*, 2000). The homozygote $Blm^{m2Brd/m2Brd}$ mouse did not survive to term as previously reported by Chester *et al.* (1998), while the $Blm^{m3Brd/m3Brd}$ mice are viable and represent a model for the human Bloom's syndrome. In these mice, both sister chromatid exchange (SCE) and loss of heterozygozity (LOH) are elevated (10-fold and 18-fold, respectively) compared to wild type mice, leading to increased tumorigenicity *in vivo* (Luo *et al.*, 2000). Recently, Chester et al. (2006) created a conditional *BLM* knockout mice using a Cre protein-mediated deletion of the BLM exon 8 flanked by *loxP* sites. This deletion can be used to delete *mBLM* in specific tissues in embryo or adult mice. An increase of SCE (6.5-fold) was also reported in this Blm^{m4Ches} mouse (Chester *et al.*, 2006). Interestingly, as in *S. cerevisiae*, LOH events were preferentially associated with chromosome loss in the Blm^{m4Ches} mouse (Chester *et al.*, 2006).

Identification of the mouse Werner homologue was also carried out by screening a mouse cDNA library. The amino acid sequence of the protein shares high homology with the human WRN protein. As an example, the helicase domain of the mouse and human Werner helicase are 95% identical while the overall identity of both proteins is greater than 70% (Imamura *et al.*, 1997). Homozygotes $wrn^{-/-}$ mutant mice are viable but their life expectancy is greatly reduced compared to wild-type mice. At the cellular level, homozygous mutant ES cells display an increased sensitivity to topoisomerase inhibitor and a reduced growth rate (Lebel and Leder, 1998). Mann *et al.* (2005) have created a viable mice model of type II Rothmund-Thompson syndrome. In such mice and the cell lines derived from it, chromosome instability is increased via an

increase in aneuploidy whereas intra-chromosomal aberrations are not detected (Mann *et al.*, 2005).

Members of the RecQ family have also been identified in *Drosophila melanogaster*. *DmBLM* (Figure 1.1) encodes a putative 1487 amino acid protein that exhibits the same properties as RecQ homologues (Kusano *et al.*, 1999). Interestingly, *DmBLM* corresponds to the previously characterised gene *mus309* (Kusano *et al.*, 1999) first identified in a screen for mutagen sensitivity (Boyd *et al.*, 1981). Mutations in *DmBLM* cause partial male sterility and complete female sterility (Kusano *et al.*, 2001). This difference of sterility between male and female drosophila is opposite with that of *blm* in humans. Such difference might be explained by the absence of recombination in *D. melanogaster* males or the pausing of meiotic division at the meiosis I/meiosis II transition in women.

In *Arabidopsis thaliana*, at least seven genes have been identified that belong to the RecQ helicase family. They are located on four different chromosomes and are named *AtRecQl1*, *2*, *3*, *4A*, *4B* and *AtRecQ5*. The seventh homologue is called *AtRecQsim* (for similar) due to an insertion in the helicase domain (Hartung *et al.*, 2000). Their biological functions remain unclear as of this writing.

1.2. Repair of broken DNA via homologous recombination:

The repair of broken DNA is essential for the accurate transmission of replicated molecules to daughter cells. This repair is often done using homologous recombination, which refers to any exchange of genetic material between homologous DNA sequences. Homologous recombination involves reciprocal transfer of genetic information between the two DNA molecules (crossover) but also non-reciprocal exchange of genetic material from one molecule to another (gene conversion and break-induced replication) or intra-molecular deletion events (single-strand annealing). Different templates can also be used as donors of genetic information during homologous repair. These can either be sister chromatids, homologues or repeat DNA sequences at allelic or non-allelic loci. Homologous recombination is one of the major repair mechanisms for double-strand breaks (DSBs), the other mechanism being non-homologous end joining. During mitosis, DSBs can be induced endogenously via HO endonuclease during mating-type switching (Haber, 1992), or exogenously as a result of γ-irradiation. DSBs are also generated when replication forks collapse when they encounter single-strand gaps in the template (Kuzminov, 2001). In addition, it has also been shown that during replication, DSBs can arise from nicks in the DNA (Cortes-Ledesma and Aguilera, 2006).

1.2.1. Repair of collapsed forks via break-induced replication:

Break-induced replication (BIR) is a recombination-dependent replication process that leads to the non-reciprocal exchange of genetic material between two DNA molecules. Genetic evidence for such a mechanism came from the detection of gene conversion events over hundred of kilobases from the DSB site (Golin and Esposito, 1984; Voelkel-Meiman and Roeder, 1990). The repair of collapsed replication forks and irradiation-induced DSB has been attributed to this specific recombination pathway (Kraus *et al.*, 2001). BIR can lead to loss of heterozygosity due to the intrinsic non-reciprocity of its mechanism. Break-induced replication starts as a one-ended recombination event between a donor and recipient DNA molecules as one would see in a collapsed replication fork (Figure 1.2 A and B)(Paques and Haber, 1999).

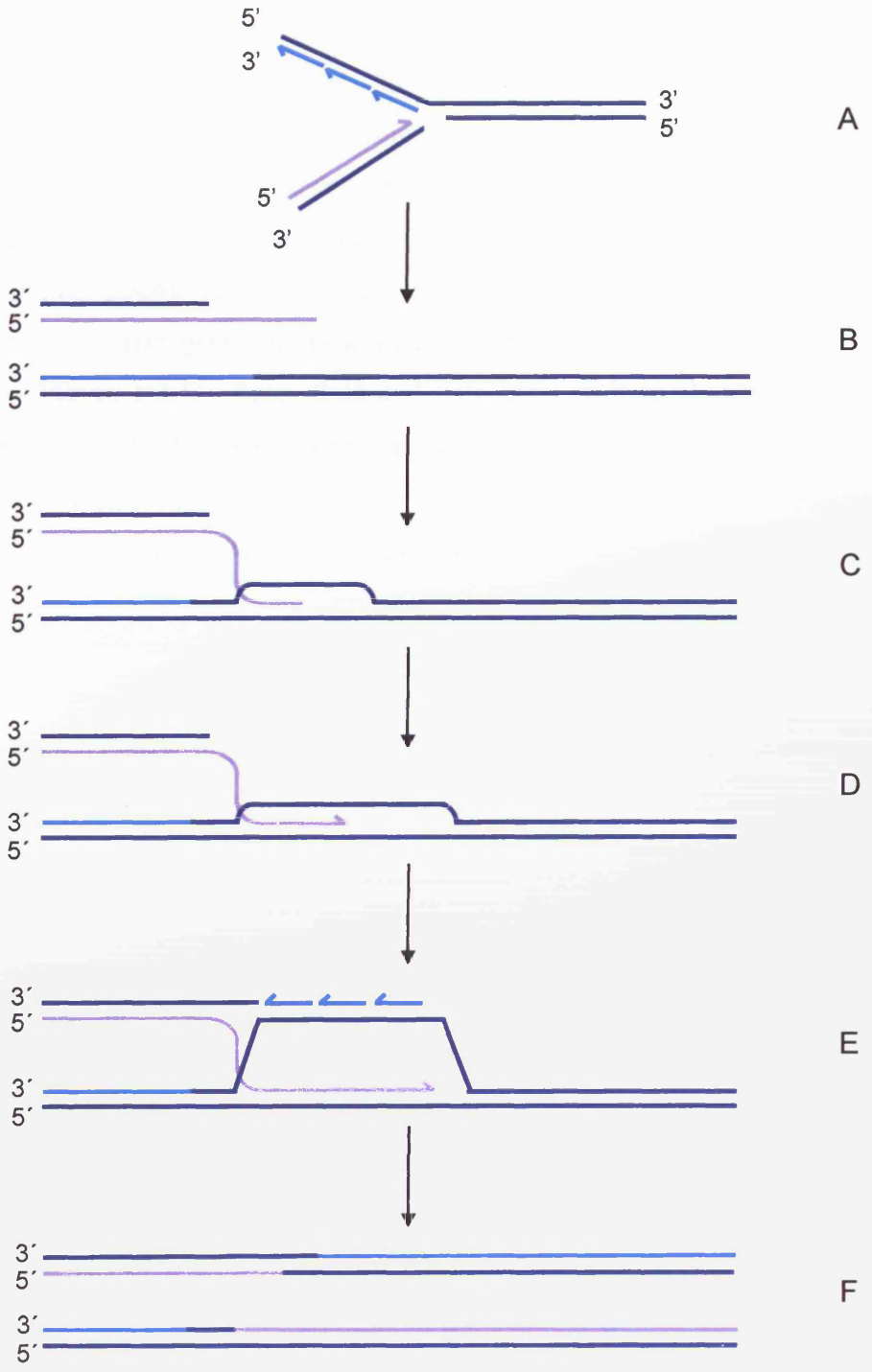


Figure 1.2: Break Induced Replication pathway for DNA double strand break repair following a replication fork collapsed. A: Single strand gap ahead of replication fork cause the fork to collapse B: Collapsed fork form a DSB where 5' end resection leaves 3' overhang. C: Single end invasion of homologous sequence. D: Leading strand DNA synthesis and D-loop displacement. E: Initiation of lagging strand DNA synthesis with continued leading strand synthesis. F: DNA replication can proceed for the entire length of the molecule (Paques and Haber, 1999).

Resection of the single-end of the DSB is MRX dependent (Mre11/Rad50/Xrs2 protein complex) (Trujillo and Sung, 2001) and possibly also involved the Sae2p (Rattray *et al.*, 2001) and Exo1p (Lewis *et al.*, 2002) exonucleases, leaving a single stranded 3′ tail (Davis and Symington, 2003; Krishna *et al.*, 2007; Signon *et al.*, 2001). As in the DSB repair model, the single stranded tail invades the unbroken molecule (Figure 1.2 C). 97% of BIR events involved Rad51-dependent single-end invasion intermediates (Davis and Symington, 2004), suggesting that the major pathway to repair a chromosomal DSB is Rad51-dependent gene conversion (BIR). Upon strand exchange, DNA synthesis takes place (Figure 1.2 D). Polα, Polδ and Polε have all been shown to play a role in BIR, taking part in leading and lagging strand synthesis (Figure 1.2 F) (Lydeard *et al.*, 2007). Since BIR does not happen when a second DSB-end is present, suggests that the second DSB-end might regulate DNA synthesis or prevents BIR (Aguilera, 2001).

1.2.2. Repair of stalled forks via homologous recombination:

Temporarily stalled replication forks are not deleterious *per se*. However, if they do not restart, the replisome fails off and the fork collapses. In fission yeast, natural replication pausing sites, mainly replication termination sequences (*RTS1*) can be used to block replication. In these studies, *RTS1*-blocked forks have been shown to be repaired via homologous recombination (Ahn *et al.*, 2005; Lambert *et al.*, 2005). Likewise, DNA lesions such as single-strand gaps and DNA adducts need to be repaired before replication can proceed. Recent evidence has shown that single strand gaps and nicks also trigger homologous recombination (Smith, 2004). However, because un-repaired stalled replication forks can be processed by homologous recombination, they can lead to deleterious events such as gross chromosomal rearrangements and deletions if recombination takes place between ectopic sites (Lambert *et al.*, 2005). The repair of a stalled fork will depend on where the lesion is *i.e.* lagging (Figure 1.8) or leading strand (Figures 1.9-10). In both cases, although the damaged DNA molecule will differ, the repair will involve the canonical double-strand break repair model.

The current models of DSB repair are primarily based on meiotic studies from yeast where DSBs are endogenously induced by the meiosis specific trans-esterase Spo11 protein (see § 1.5). However, these models have also been validated in mitosis where many of the proteins involved in meiotic DSBs repair are also involved in mitotic recombination (Symington, 2002). Upon creation of a mitotic DSB (Figure 1.3 A),

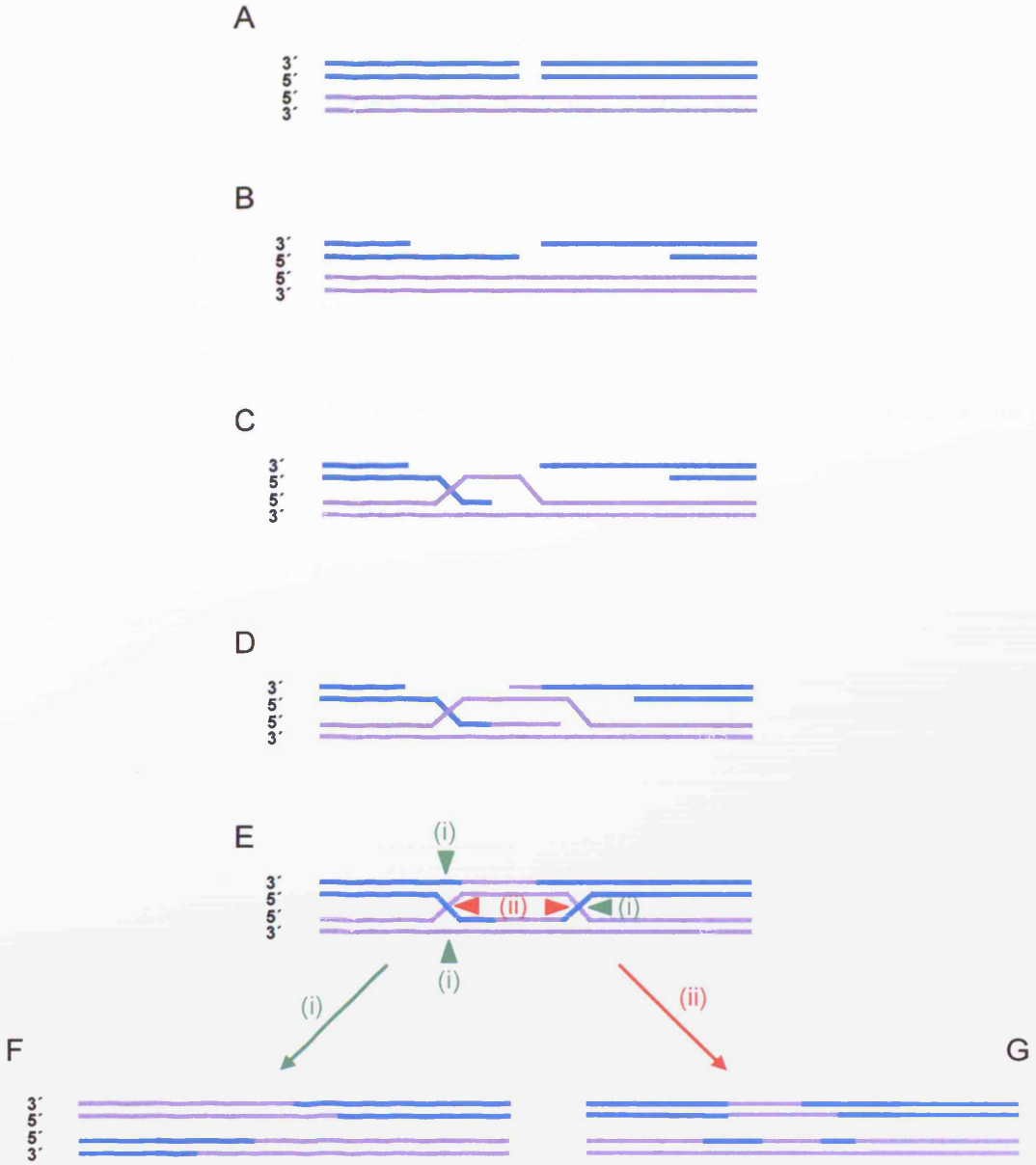


Figure 1.3: Double strand break repair model (Szostak *et al.*, 1983).
Blue and purple duplex DNA represent sister chromatids.
A: DSB occurs in one strand. B: MRX complex (Mre11p, Rad50p, Xrs2p), Sae2p and Exo1p contribute to the resection of the 5´-ends due to their nuclease activity.
C: 3´-single-ends invasion is catalysed by Rad51p.
D: DNA synthesis, D-loop displacement and 3´-end strand capture.
E: Upon DNA ligation, formation of a double Holliday junction. Resolution can give rise to crossovers molecules (F) by cutting in opposed plans (i) or non-crossovers (G) by cutting in the same plan (ii).

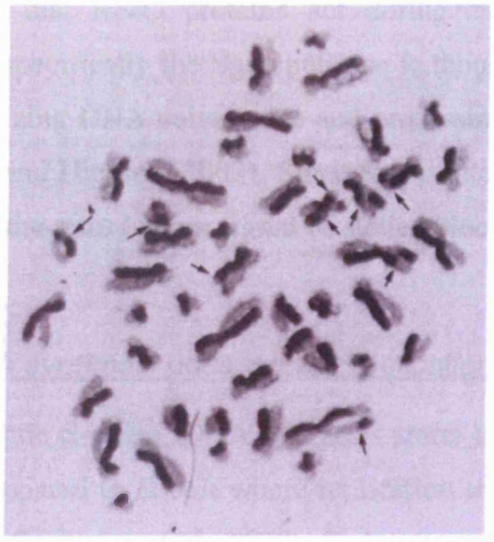
the 5′-ends of the double-strand break are resected (Figure 1.3 B). Mre11p, the endonuclease component of the MRX complex (Mre11p/Rad50p/Xrs2p) may not be the sole nuclease involved in this resection since *MRE11* mutant strains have hardly any effects on HO-induced DSB repair (Moreau *et al.*, 1999). Other proteins that might participate in this resection are Sae2p (Clerici *et al.*, 2005; Rattray *et al.*, 2001) and the exonuclease protein ExoI (Lewis *et al.*, 2002; Moreau *et al.*, 2001). Upon resection, replication protein A (RPA) loads onto 3′-end tails and promotes the binding of Rad52p (Shinohara *et al.*, 1998) by removing secondary structures in the ssDNA (Sung *et al.*, 2000). Rad52p and Rad51p have been associated with the homology search in the entire genome (Song and Sung, 2000). Rad51p also catalyses strand exchange reactions *in vitro* (Sung, 1994; Sung and Robberson, 1995) as a single-end invasion. The Rad51p-strand invasion is facilitated by Rad52, Rad55, Rad57, Rad54 and Rdh54/Tid1 proteins (Prado and Aguilera, 2003; Raoul Tan *et al.*, 2003; Sugawara *et al.*, 2003; Sugiyama and Kowalczykowski, 2002; Sung, 1997; Symington, 2002). These proteins have been shown to interact *in vitro* (Krejci *et al.*, 2001) and form a polymeric complex that stimulates strand exchange *in vitro* (Sung, 1997). In mitosis, strand exchange preferentially takes place between sister chromatids (Figure 1.3 C) as shown by the prevalence of sister chromatid exchange (SCE) in mammalian cells (Johnson and Jasin, 2000) and in yeast (Gonzalez-Barrera *et al.*, 2003; Kadyk and Hartwell, 1992). Upon single-end invasion and strand exchange, the Rad51-loaded nucleofilament creates a displacement loop (D-loop) in the invaded DNA molecule (Figure 1.3 C). This D-loop structure has been shown to be promoted by Rdh54p/Tid1p (Petukhova *et al.*, 2000). The invading 3′-end is used as a primer for DNA synthesis further stabilising this joint molecule. The extended D-loop structure is captured by the second end of the DSB (Figure 1.3 D) and upon DNA synthesis and ligation joint molecules are formed containing two Holliday junctions (dHJ – Figure 1.3 E). In mitosis, most dHJs are resolved as non-crossovers. Evidence for this comes from mammalian and yeast cells where most DSBs are repaired by gene conversion that are not associated with the exchange of flanking markers (Aguilera and Klein, 1989; Jackson and Fink, 1981; Johnson and Jasin, 2000; Strathern *et al.*, 1982). In the Szostak double-strand break model of recombination, crossover and non-crossover molecules both result from the resolution of dHJs (Figure 1.3 F and G) (Szostak *et al.*, 1983). Although this is no longer thought to be the case (see § 1.5).

Homologous recombination in mitosis needs to be tightly regulated as it can potentially lead to deleterious rearrangements if recombination takes place between ectopic loci or non-homologous chromosomes. The occurrence of deleterious rearrangements in the genome such as loss of heterozygosity, deletions, duplications, translocations and chromosome loss are often referred to as genomic instability.

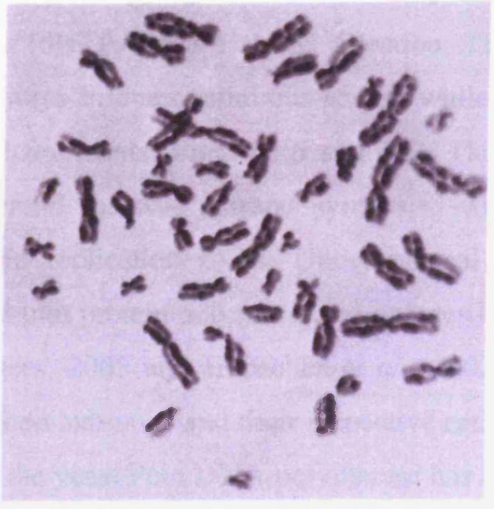
1.3. Roles of RecQ helicases in mitosis:

Mitosis is the mechanism by which a cell divides to give rise to two daughter cells carrying the same genetic information. The replication of DNA during S-phase, prior to cell division, is crucial, as any error or damage occurring during the replication stage could lead to cell cycle arrest and potentially cell death. Therefore, the mechanism by which a cell replicates its DNA is well regulated to maintain genomic integrity through mitotic generations. Genome instability is a characteristic of cancer cells. Different studies have reported that genes involved in mitosis surveillance mechanisms lead to increase instability when deleted (Myung and Kolodner, 2002, 2003). This instability is often characterised by increased gross chromosomal rearrangements and loss of heterozygosity, both of which are thought to arise via recombination events.

The BLM protein, as well as Sgs1p, has been shown to have a peak of expression during S-phase (Frei and Gasser, 2000). In *S. cerevisiae*, *SGS1* has been reported to play a role in chromosome segregation as well as in genomic stability (Gangloff *et al.*, 1994; Watt *et al.*, 1995; Watt *et al.*, 1996). *sgs1*Δ mutants display high levels of both mitotic homologous recombination and illegitimate recombination with increased gross chromosomal rearrangements and loss of heterozygosity (Ajima *et al.*, 2002; Miyajima *et al.*, 2000a; Myung *et al.*, 2001; Myung and Kolodner, 2003). In mice defective for *mBLM*, the elevated mitotic recombination and chromosome loss in embryonic stem cells has been shown to increase tumorigenicity *in vivo* (Chester *et al.*, 2006; Luo *et al.*, 2000). Interestingly, human RecQ helicase mutants have also been associated with genomic instability. For instance, Bloom's syndrome cells are characterised by an elevated number of sister chromatid exchanges (Chaganti *et al.*, 1974) to the point that this phenotype is used to diagnose Bloom's patients (Figure 1.4). The genomic instability of Werner's syndrome cells has been linked to an increased frequency of genomic rearrangements such as translocations, inversions and extensive deletions (Fukuchi *et al.*, 1989; Salk *et al.*, 1981). Analyses of karyotypes of Rothmund-Thompson's syndrome have also revealed increased chromosomal rearrangements and mis-segregations (Orstavik *et al.*, 1994; Ying *et al.*, 1990). Thus, Bloom's, Werner's and Rothmund-Thompson's patients are all predisposed to a wide range of cancers (Ellis *et al.*, 1995; Kitao *et al.*, 1999a; Yu *et al.*, 1996). The peak expression of RecQ helicase



A. Chromosomes of a normal lymphocyte at the second metaphase after growth in bromodeoxyuridine, fluorodeoxyuridine, and uridine, stained first with 33258 Hoechst and a day later with Giemsa. Arrows are at points of exchange between sister chromatids (SCE). Enlarged 1800X.



B. Chromosomes of a Bloom's syndrome lymphocyte cultured and stained as in A showing many more SCEs than in wild type cells. Enlarged 1800X.

Figure 1.4: From R.S. Chaganti, S. Schonberg, J..German, A manyfold increase in sister-chromatid exchanges in Bloom's syndrome lymphocytes. Proc. Natl. Acad. Sci. U.S.A. 71 (1974) 4508-4512.

during S-phase, their involvement in genomic stability and cancer predisposition syndromes, suggest that RecQ proteins act during during mitotic S-phase. RecQ helicases and more specifically the Sgs1 helicase is thought to act in the intra-S phase checkpoint by stabilizing DNA polymerase and processing stalled replication forks (for review see (Oakley and Hickson, 2002). Since Sgs1p interacts with Rad53p, the former might play a role in the transduction signal of stalled/blocked replication forks (Frei and Gasser, 2000).

1.3.1. *When DNA synthesis goes wrong: replication forks arrest or collapse:*

During mitotic S-phase, DNA synthesis starts at replication origins along the DNA molecule. Compared to *E. coli* where replication starts from a single origin, *OriC* (Meijer *et al.*, 1979; Sugimoto *et al.*, 1979), *S. cerevisiae* DNA replication is initiated at discrete sites along the chromosomes. For example, chromosome IV of *S. cerevisiae* possesses 9 origins of replication that differ in terms of initiation times and replicate sequentially during S-phase (Friedman *et al.*, 1997; Yamashita *et al.*, 1997). Polymerases are responsible for the copying of DNA using small RNA primers and add deoxyribonucleotides (dNTPs) in a 5′ to 3′ direction. Therefore, the leading strand of DNA synthesis is always in one continuous stretch while the lagging strand is made of ~2 kb 5′-3′ Okazaki fragments (Figure 1.5 and 1.6). Thus, the replication of the 3′-5′ strand is always behind the 5′-3′ strand synthesis. Multiple DNA polymerases are present at eukaryotic replication forks. Under normal conditions, DNA replication requires the multi-subunit protein polymerase complexes Polα, Polδ and Polε (for review see (Garg and Burgers, 2005 and Hubscher *et al.*, 2002). The different *S. cerevisiae* DNA polymerases, their sub-units and their respective activities are summarised in Table 1.2. The function of the yeast Polα DNA polymerase has been limited to the initiation of replication and repeated priming of Okazaki fragments during lagging strand DNA synthesis (Figure 1.5) (Harrington and Perrino, 1995). Therefore, it is often referred to as a primase (Bell and Dutta, 2002; Garg and Burgers, 2005; Hubscher *et al.*, 2002). Polα synthesises short RNA/DNA hybrid oligonucleotides of 40 to 50 nucleotides in length used by both Polδ and Polε for processive elongation (Waga *et al.*, 1994) (Figure 1.5). However, it remains unclear which DNA polymerase is responsible for leading or lagging strand DNA synthesis. DNA Polδ complex possesses an active proofreading 3′-5′exonuclease activity and its processivity is dependent on the binding of Proliferating

Table 1.2: Summary of subunit structures and activities of Polymerase α, δ and ε.

DNA Polymerase	Subunits and functions				Main function
Polα	*POL1* Catalytic subunit	*POL12* Protein interactions	*PRI1* Primase	*PRI2* Primase	Synthesises RNA/DNA primer hybrids
Polδ	*POL3* Catalytic subunit	*POL31* Structural	*POL32* PCNA interaction	n.a	Lagging strand DNA synthesis
Polε	*POL2* Catalytic subunit	*DPB2* Multi-merisation	*DPB3* Structural, protein interactions	*PDB4* Structural, protein interactions	Leading strand DNA synthesis

Adapted from Hubscher et *al.*, 2002.

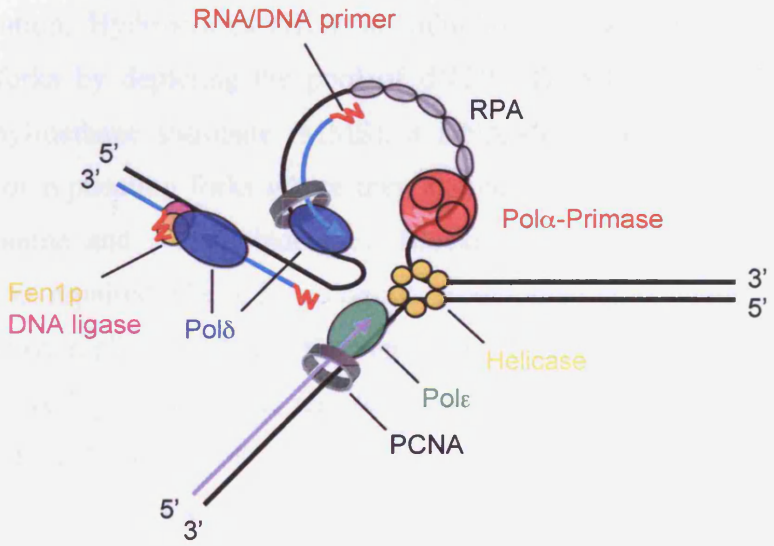


Figure 1.5: Structural model of DNA polymerase α, δ and ε at replication forks. RPA is the eukaryotic single-stranded DNA binding protein. Polα synthesises small RNA/DNA primers where Polδ loads and synthesises streaches of DNA on the lagging strand *i.e.* small Okazaki fragments. Polε may be the polymerase implicated in the leading strand DNA replication (adapted from Grag and Burgers, 2005).

Cell Nuclear Antigen (PCNA) (Prelich *et al.*, 1987). Polδ synthesises stretches of DNA by loading onto the small RNA/DNA primers synthesised by Polα. Recent evidence suggests that Polδ might be the lagging strand DNA polymerase since it is involved in the maintenance of telomeres in the absence of telomerase (Lydeard *et al.*, 2007), a mechanism that resembles lagging strand DNA synthesis. A mutant strain for the catalytic subunit of Polε, *pol2,* is viable but replicates at a slower rate than wild-type cells (Dua *et al.*, 1999). This phenotype is consistent with overlapping function between Polε and Polδ (Kesti *et al.*, 1999) and the plasticity of the replication forks seems to be greater than previously thought. *In vitro* experiments have shown that compared to Polδ, Polε is highly processive in absence of PCNA (Burgers, 1991). Polε may be responsible for leading strand DNA synthesis rather than lagging strand synthesis but firm evidence is still lacking (for review see Garg and Burgers, 2005 and Figure 1.5).

While the DNA is being replicated, replication forks may encounter several types of obstacles or lesions, the repair of which can lead to genomic instability. Obstacles such as DNA adducts, hairpins and G-quatruplex on the DNA lead to fork arrest while a single-stranded DNA break or gap can lead to fork collapse (Figure 1.6). Different types of chemicals can mimic the effects of obstacles or DNA lesions during DNA replication. Hydroxyurea (HU), an inhibitor of ribonucleotide reductase, stalls replication forks by depleting the pool of dNTPs (Bianchi *et al.*, 1986; Zhao *et al.*, 1998). Methylmethane sulfonate (MMS), a DNA-alkylation agent, slows the rate of progression of replication forks where they encounter alkylated guanines and adenines (7-methylguanine and 3-methyladenine). Blocked forks due to base methylation are thought to be repaired via a homologous recombination (Lundin *et al.*, 2005). In arrested/blocked replication forks, the replication machinery must stay loaded on the fork, while in collapsed forks, the replisome is destabilised and fork progression cannot be resumed. Destabilisation of the replication machinery can be critical and collapsed forks are often repaired via homologous recombination (§ 1.2). In the presence of DNA damaging agents, replication must stop or be delayed (while the replisome must remain loaded on the fork) to allow enough time to repair the damage so that the replication can restart.

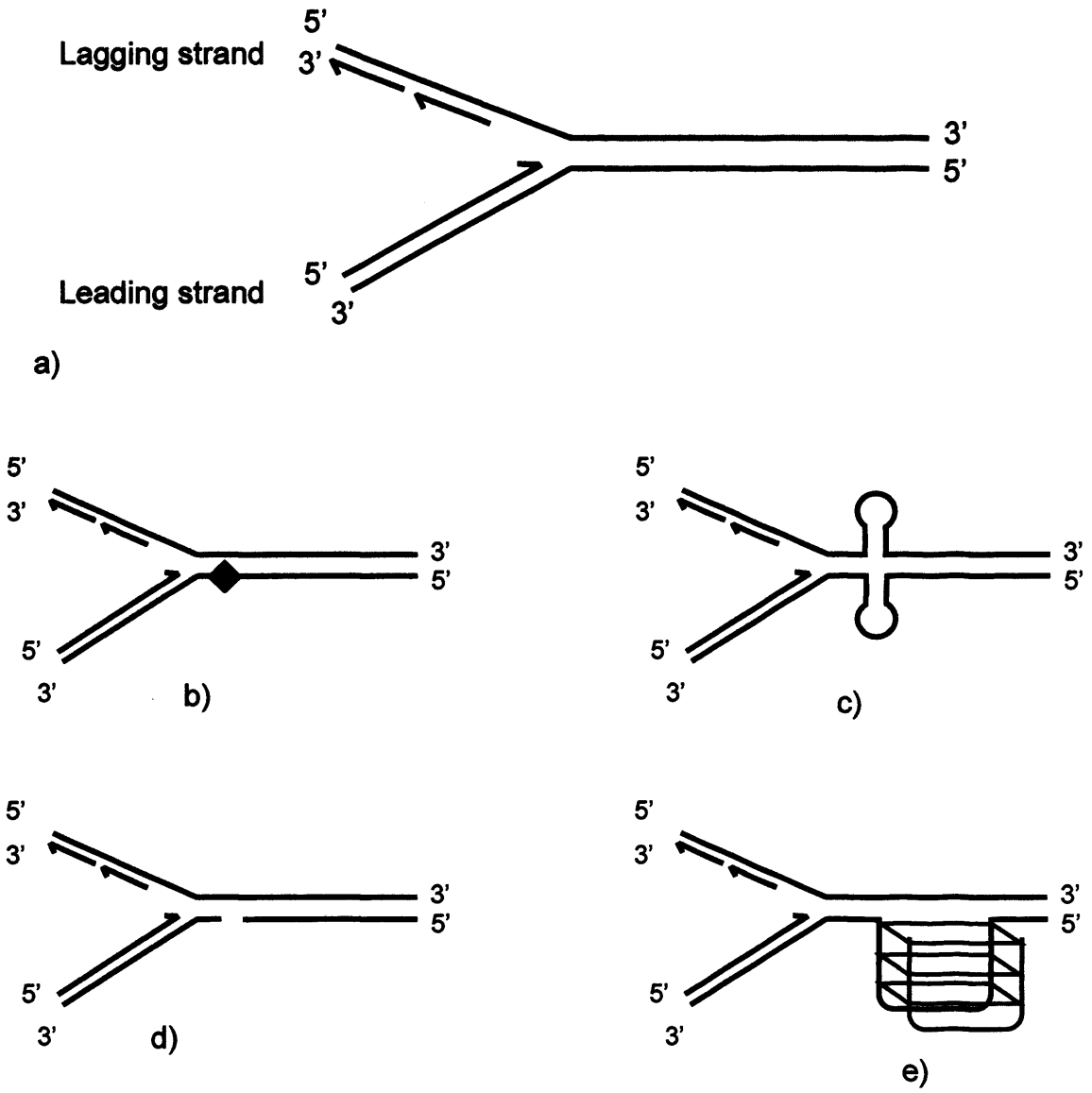


Figure 1.6: (a) Replication is asymmetric with the synthesis of small Okazaki fragments on the lagging strand. Possible causes of replication fork stall or collapse on the leading strand: (b) DNA adduct, (c) hairpin and (e) G-quadruplex, which occurs naturally in arrays of guanine residues, might force the fork to arrest while (d) single strand break or a DNA gap will cause the replication fork to collapse.

1.3.2. Sgs1p stabilises replication forks:

In the absence of DNA damaging agents replication fork progression is not impaired in *sgs1*Δ cells compared to wild type (Frei and Gasser, 2000; Gangloff *et al.*, 1994). This absence of defects shows that Sgs1p does not play an essential role in normal DNA replication. However, in the presence of DNA damaging agents, the role of Sgs1p is more substantial. Mutant *sgs1*Δ cells exposed to HU have a 50% viability compared to wild type (Frei and Gasser, 2000). This decreased viability of HU-exposed *sgs1* cells is epistatic to a temperature-sensitive mutation in the catalytic domain of the *POL2* gene, *pol2-11*, a subunit of the Polε polymerase complex. The observed viability of the double mutant *sgs1*Δ *pol2-11* is not different from that of the *sgs1* single mutant. This epistatic relationship between *sgs1* and *pol2* (Polε) suggests that both proteins play a role during DNA replication in presence of HU. Resumption of fork progression, after HU has been removed, requires stabilisation of the replisome during fork arrest. Consistent with this is the stabilisation of Polα and Polε at replication forks by Sgs1p in the presence of DNA damaging agents (Cobb *et al.*, 2003; Cobb *et al.*, 2005). Chromatin immuno-precipitation (ChIP) experiments on Polα and Polε, in HU-complemented medium, have shown that the amount of polymerase recovered at an early-firing origin (ARS607), stalled under those conditions, is dependent on the presence Sgs1p and Mec1p. Interestingly, this stabilisation of DNA polymerase ε at stalled replication forks also requires Top3p and Rad51p (Bjergbaek *et al.*, 2005).

The lack of stabilisation of DNA polymerases at stalled replication forks in *SGS1* mutant strains might account for their hyper-recombination phenotype. In *sgs1*Δ cells, the replisome is destabilised by DNA damage and replication forks collapse. Such collapsed forks need to be repaired via a Rad52-dependent recombination pathway.

1.3.3. Sgs1p activates intra-S phase checkpoint:

In addition to polymerase stabilisation, the presence of Sgs1 at replication forks might recruit Rad53p to facilitate activation of the intra S-phase checkpoint. This is consistent with the *in vivo* interaction between the two proteins (Bjergbaek *et al.*, 2005).

Surveillance mechanisms, called mitotic checkpoints, occur during the DNA synthesis stage and prior to cell division to detect damaged DNA and stalled/collapsed

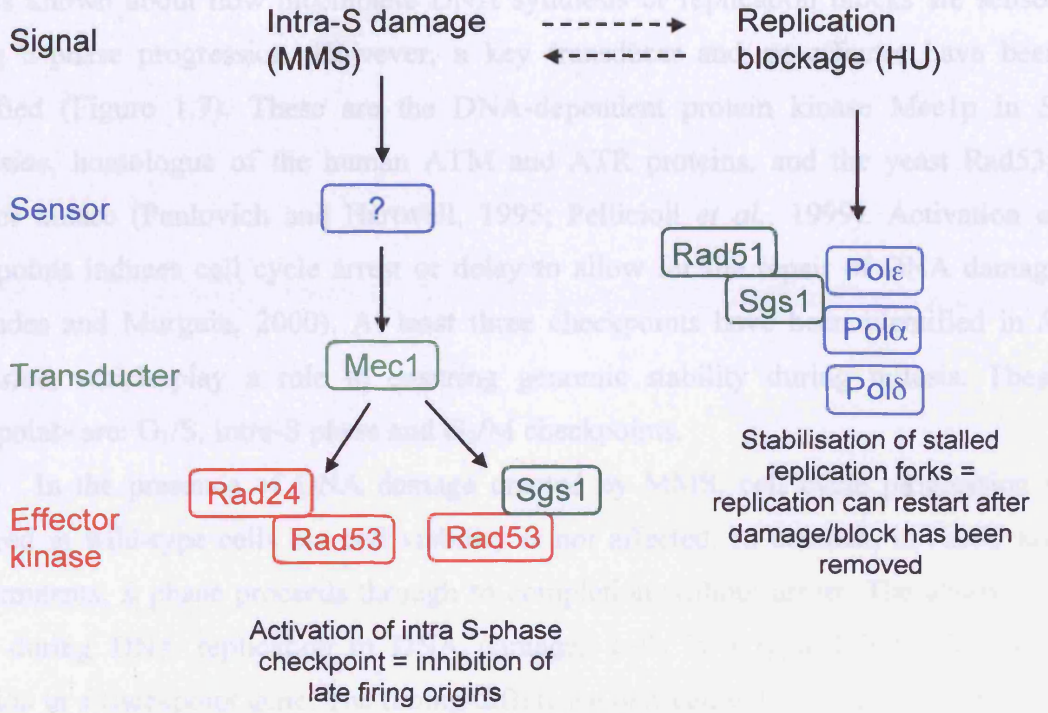


Figure 1.7: Cascade of activation of the intra S-phase checkpoint in *S. cerevisiae*. Mec1p and Rad53p are essential for the checkpoint activation. Sgs1p stabilises DNA polymerase at stalled replication forks but also activates the checkpoint independently of Rad24.

replication forks. Checkpoints are characterised by three essential components: sensor proteins, which detect the damage or lesions, transducer proteins that activate effector kinases by phosphorylation; and effector proteins, which act upon the damage. To date little is known about how incomplete DNA synthesis or replication blocks are sensed during S-phase progression. However, a key transducer and an effector have been identified (Figure 1.7). These are the DNA-dependent protein kinase Mec1p in *S. cerevisiae*, homologue of the human ATM and ATR proteins, and the yeast Rad53p effector kinase (Paulovich and Hartwell, 1995; Pellicioli *et al.*, 1999). Activation of checkpoints induces cell cycle arrest or delay to allow for the repair of DNA damage (Lowndes and Murguia, 2000). At least three checkpoints have been identified in *S. cerevisiae*, which play a role in ensuring genomic stability during mitosis. These checkpoints are: G_1/S, intra-S phase and G_2/M checkpoints.

In the presence of DNA damage created by MMS, cell cycle progression is impaired in wild-type cells but cell viability is not affected. In contrast, in *rad53* and *mec1* mutants, S phase proceeds through to completion without arrest. The absence of arrest during DNA replication in DNA damaged cells is a typical indication of a mutation in a checkpoint gene. The timing difference between wild type and *rad53/mec1* cells is due to the activation of intra S-phase replication checkpoints that trigger the inhibition of late firing origins in wild-type cells treated with MMS (Shirahige *et al.*, 1998).

Sgs1p has been reported to activate Rad53p-dependent checkpoints in the presence of DNA damaging agents such as HU and MMS (Bjergbaek *et al.*, 2005). In *S. cerevisiae*, the activation of the intra-S phase checkpoint is largely mediated by Mec1p phosphorylation of Rad53p (Figure 1.7). This activation occurs in response to forks stalling due to high concentration of HU, or collapsed forks due to the creation of secondary breaks to repair MMS induced alkylations (§ 1.3.1). When deleted, *RAD24* (an effector kinase) affects the integrity of the G_1/S and G_2/M and the intra-S phase checkpoints, while Sgs1p only plays a role during the intra S-phase checkpoint (Frei and Gasser, 2000). Double mutants for *SGS1* and *RAD24* have an additive effect (faster completion of S-phase) compared to *sgs1*Δ, suggesting that Sgs1p and Rad24p might be involved in two different branches of the intra-S phase checkpoint (Frei and Gasser, 2000 and Figure 1.7). Furthermore, the activation of the intra-S phase checkpoint by Sgs1p is independent of the helicase domain of the protein (Frei and Gasser, 2000) and

does not require Top3p or Rad51p as does stabilisation of DNA polymerases (§ 1.3.2). Therefore, intra-S phase checkpoint activation by Sgs1p seems to be a distinct mechanism from the stabilisation of stalled replication forks.

1.4. Sgs1p reduces recombination at damaged replication forks:

SGS1 was first identified as a suppressor of the slow growth phenotype of *top3Δ* cells (Gangloff *et al.*, 1994). The decreased growth rate associated with *TOP3* mutant is due to a cell cycle arrest in late S/G2 phase (Arthur, 1991; Chakraverty *et al.*, 2001; Gangloff *et al.*, 1994). *TOP3* encodes the sole *S. cerevisiae* type IA topoisomerase protein. This enzyme catalyses the decatenation of intertwined DNA molecules via an ATP-dependent strand-passage activity (for review see Champoux, 2001). In addition to its slow growth phenotype, deletion of *TOP3* causes hyper-recombination and sensitivity to DNA damaging agents (Kim and Wang, 1992; Wallis *et al.*, 1989), both phenotypes shared by *SGS1* mutants. Not surprisingly, Sgs1 and Top3 proteins physically interact (Bennett *et al.*, 2000; Fricke *et al.*, 2001; Ui *et al.*, 2001; Wu *et al.*, 2000) and mutations in the *SGS1* helicase domain rescue the slow growth phenotype associated with *top3Δ* (Mullen *et al.*, 2000). The *top3Δ* slow growth is thought to be due to the presence of intermediates created by Sgs1p that cannot be processed effectively in absence of Top3p (Mankouri and Hickson, 2006; Mullen *et al.*, 2000; Oakley *et al.*, 2002). Therefore, Sgs1p is thought to act before Top3p (Oakley and Hickson, 2002). *top3* and *sgs1* mutants have a hyper-recombination phenotype in mitosis and this suggests that both genes play a role in suppressing recombination events during mitosis (Ira *et al.*, 2003; Wu and Hickson, 2003).

Synthetic-lethal screen assays have revealed that mutations in *MUS81* and *MMS4* genes required *SGS1* for viability (Mullen *et al.*, 2001) i.e. *mus81/mms4* mutants are lethal in combination with *sgs1Δ*, *sgs1-hd* (helicase domain mutation) and *top3Δ* suggesting that these proteins have overlapping functions. Furthermore, the endonuclease Mus81p-Mms4p forms a heterodimeric complex *in vivo* (1:1 ratio) and their preferred substrate resembles replication forks (Kaliraman *et al.*, 2001). Kaliraman *et al.* (2001) therefore proposed that this complex might play a role during the repair of stalled replication forks. Since mutation in *MUS81/MMS4* and *SGS1* are synthetically lethal, Fabre *et al.* (2002) performed a genetic suppression test for this lethality. Mutations in homologous recombination genes such as *RAD51*, *RAD52*, *RAD54*, *RAD55* and *RAD57* rescued the *mus81Δsgs1Δ* and *mms4Δsgs1Δ* synthetic lethality. The same results were obtained with a *mus81Δtop3Δ* double mutant. Taken together these results

suggested that homologous recombination is the main cause of death in mutants for *MUS81-MMS4* and *SGS1-TOP3* since the death can be alleviated by mutation in *RAD* genes (Fabre *et al.*, 2002). As mentioned previously, (§ 1.2.2) Rad52p, Rad54p, Rad55p and Rad57p facilitate the Rad51-nucleofilament strand invasion during DSB repair. Hence, Fabre *et al.* (2002) suggested that the toxic recombination intermediates in *mus81-mms4* and *sgs1-top3* mutants are initiated by Rad51p.

1.4.1. Sgs1p plays a role in lagging-strand repair:

The intrinsic discontinuity of replication on the lagging strand (due to the annealing of Okazaki fragments) allows for the by-pass of DNA lesions (Figure 1.8 A-B) (Langston and O'Donnell, 2006). This by-pass leaves a single-strand gap, which can be repaired via homologous recombination, using the leading DNA strand as a template (Figure 1.8 C-D). Interestingly, microarray approaches have identified interaction between genes involved in lagging strand synthesis and *SGS1*. For example, mutation in *RAD27* or any of the subunit of the RNase H2 protein complex (*RNH201*, *RNH202* or *RNH203*) leads to synthetic sickness in *sgs1*Δ cells (Ooi *et al.*, 2003; Tong *et al.*, 2001). More direct evidence of the repair of damage on the lagging strand has been found recently in *S. cerevisiae*. Not only the strand exchange protein Rad51p, but also the Sgs1p and the Mus81-Mms4 endonuclease protein complex are required for the repair of lagging strand lesions. This evidence was found by Ii *et al.* (2005), who used triple mutants *rad51/mus81/sgs1* to screen for synthetic lethal genes in this background. One identified mutant in this screen contains a mutation in the *RNH202* gene, a subunit of the RNase H2 involved in the maturation of Okazaki fragments (Jeong *et al.*, 2004). Although, RNase H2 is not essential for Okazaki fragment maturation, as this function is normally performed by *FEN1/RAD27* (Kao and Bambara, 2003), RNase H2 seems to play a more prominent role in cells exposed to DNA damaging agents such as MMS, HU and UV irradiation (Ii and Brill, 2005). Multiple mutant studies, using epistasis assays, have confirmed that the function of Sgs1p lies downstream of Rad51p (Fabre *et al.*, 2002; Ii and Brill, 2005).

Furthermore, the requirement for Rad51p in the Sgs1-Top3 pathway highlights the involvement of recombination as a mean of repairing the damage (Figure 1.8 D). The presence of Rad51-dependent Holliday junction-like molecules has been detected *in vivo*

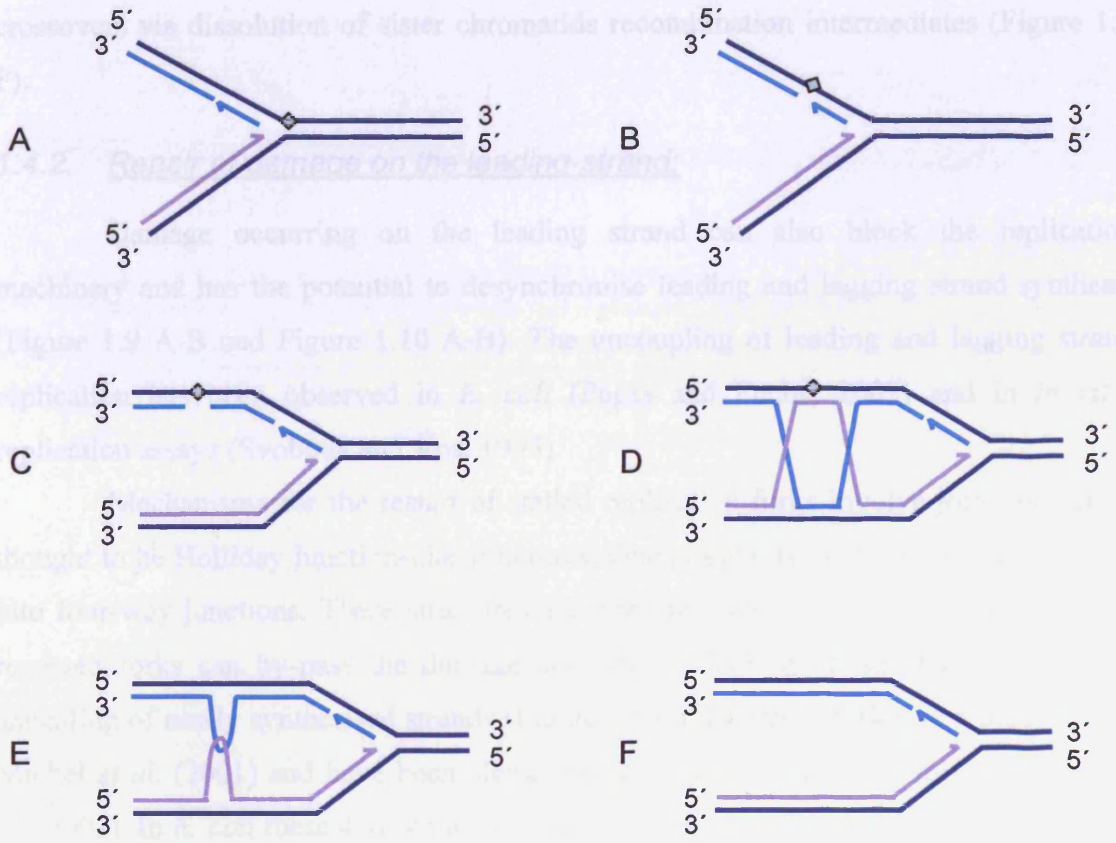


Figure 1.8: Lesions on the lagging strand are easily by-passed due to the continuous annealing of Okazaki fragments. A: lesion/block ahead of the fork on the lagging strand. B: Due to priming by Okazaki fragments, replication on the lagging strand is not blocked. C: The lesion is by-passed creating a single strand gap which does not impede fork progression. D: The single strand gap is repaired via homologous recombination using the leading strand as a template creating a double Holliday junction. The DNA lesion is repaired. E: Sgs1p can branch migrate the dHJs to form a hemicatenate. F: This hemi-catenate can be dissolve by the Top3 topoisomerase restoring a normal fork.

in *S. cerevisiae* (see next section). During mitosis, RecQ helicases in conjunction with Topoisomerase III have been shown to dissolve double Holliday junctions. This dissolution of double Holliday junctions, via a branch migration mechanism (Figure 1.8 E), suppresses crossovers in *S. cerevisiae* and humans (Ira *et al.*, 2003; Mankouri and Hickson, 2006; Seki *et al.*, 2006). Thus, in the presence of Sgs1p and Top3p, repair of the damage on the lagging strand by homologous recombination could lead to non-crossovers via dissolution of sister chromatids recombination intermediates (Figure 1.8 F).

1.4.2. Repair of damage on the leading-strand:

Damage occurring on the leading strand can also block the replication machinery and has the potential to desynchronise leading and lagging strand synthesis (Figure 1.9 A-B and Figure 1.10 A-B). The uncoupling of leading and lagging strand replication has been observed in *E. coli* (Pages and Fuchs, 2003) and in *in vitro* replication assays (Svoboda and Vos, 1995).

Mechanisms for the restart of stalled replication forks involve joint molecules thought to be Holliday junction-like structures. One possibility is to reverse stalled forks into four-way junctions. These structures are often referred to as "chicken feet". These reversed forks can by-pass the damage and restart DNA synthesis thanks to the re-annealing of newly synthesised strands (Figure 1.9 C). Reversed forks were proposed by Michel *et al.* (2001) and have been identified by electron microscopy in yeast (Sogo *et al.*, 2002). In *E. coli* these 4-way junctions are dependent on the strand exchange protein RecA or the RecG helicase (McGlynn and Lloyd, 2001; McGlynn *et al.*, 2001; Robu *et al.*, 2001). *In vitro* experiments have shown that the human RecQ orthologue, BLM, can promote the regression of stalled replication forks to generate chicken feet (Ralf *et al.*, 2006). This role is consistent with the presence of RecQ helicase at stalled forks (§ 1.3.2). Uncertainty remains as how those structures are resolved. Chicken feet structures could potentially be re-regressed by RecQ helicase to re-establish replication forks (Figure 1.9 D) and therefore by-pass the damage. They could also be cleaved by the Mus81-Mms4 endonuclease complex to be repaired via a Rad51-dependent mechanism.

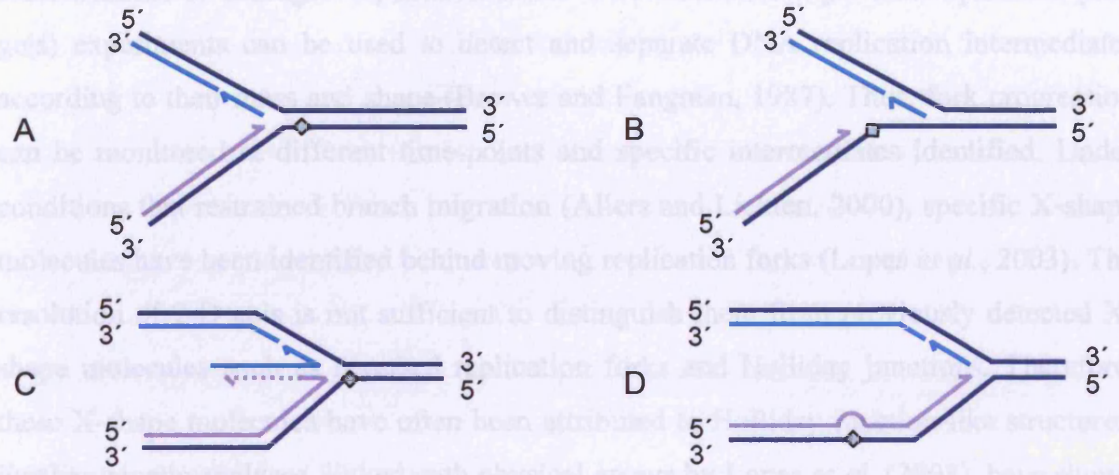


Figure 1.9: Schematic representation of "chicken feet".
A: lesion/block ahead of the fork on the leading strand. B: De-synchronisation of the replisome, the leading strand synthesis is blocked while lagging strand synthesis continues. C: Sgs1 (?) can regress the fork such that the leading strand can use the lagging as a template. D: Sgs1p/Top3p can unwind regressed replication fork to restore normal progression of the replisome and thus by-passing the lesion.

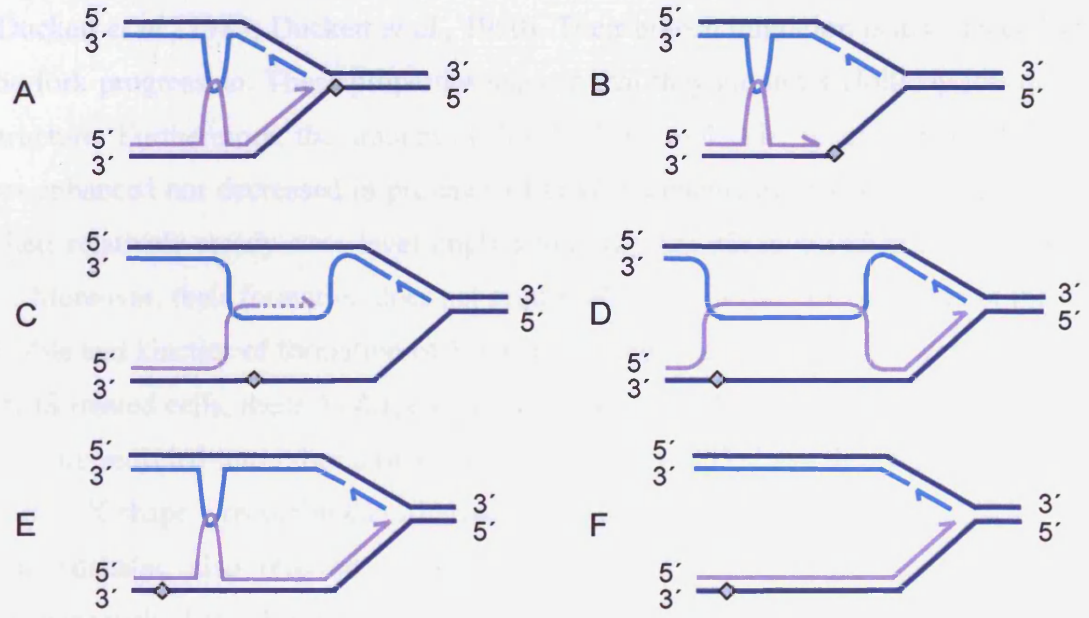


Figure 1.10: By-pass of lesion on the leading strand using Holliday junction-like structures. A: lesion/block ahead of the fork on the leading strand while Sister Chromatid Junction (SCJ) formed normally behind the fork. B: The fork becomes asynchronous where leading strand synthesis is blocked while lagging strand synthesis continues. C: The SCJ is a precursor for Rad51-dependent strand invasion allowing template switching. D: The replication continues off the lagging strand to form a Holliday junction-like molecule (rec-X structure). E: Sgs1p collapses the pseudo-Holliday junction to form hemicatenate. F: Top3p can dissolve hemicatenate and restore the replication fork. The DNA lesion has been by-passed.

Based on recent evidence, other mechanisms have been proposed for the re-establishment of damaged replication forks. Two-dimensional gel electrophoresis (2D-gels) experiments can be used to detect and separate DNA replication intermediates according to their mass and shape (Brewer and Fangman, 1987). Thus, fork progression can be monitored at different time-points and specific intermediates identified. Under conditions that restrained branch migration (Allers and Lichten, 2000), specific X-shape molecules have been identified behind moving replication forks (Lopes *et al.*, 2003). The resolution of 2-D gels is not sufficient to distinguish them from previously detected X-shape molecules such as reversed replication forks and Holliday junctions. Therefore, these X-shape molecules have often been attributed to Holliday junction-like structures. Further genetic analyses, linked with physical assays by Lopes *et al.* (2003), have shown that these X-shape molecules are different from reversed replication forks and Holliday junctions. They are not recombination intermediates as their formation is independent of the Rad51 and Rad52 proteins. Furthermore, they possess the ability to branch migrate in the presence of Mg^{2+}, which distinguish them from Holliday junctions that cannot (Duckett *et al.*, 1988; Duckett *et al.*, 1990). Their branch migration is also dependent on the fork progression. These properties suggest that they are not a Holliday junction-like structure. Furthermore, the amount of this X-shape molecule, recovered on 2D gels, is not enhanced nor decreased in presence of DNA damaging agents such as MMS or HU. Their relatively steady-state level implies that they are not involved in DNA repair *per se*. Moreover, their formation does not require Rad53p, as *rad53Δ* cells exhibit the same profile and kinetics of formation of X-shape molecules as wild type. However, in *rad53*-MMS treated cells, these X-shape molecules seem to fade more quickly, suggesting that they are recycled into other structures. Lopes *et al.* (2003) have therefore proposed that these X-shape recombination-independent molecules are hemicatenates. Such hemicatenates, also referred to as four-way sister chromatid junctions (SCJ), form spontaneously behind replication forks in either normal or under stress conditions (Figure 1.10 A). They can branch-migrate away from the fork, as their structure does not require base pairing. Their role might be to further strengthen interactions between sister chromatids. Although they are not involved in repair, they could be converted into structures resembling double Holliday junctions (Figure 1.10). Ii *et al.* (2005) further hypothesised that these hemicatenates might facilitate pairing between two newly synthesised DNA strands and favoured template switching.

Since Sgs1p and Top3p have been implicated in the dissolution of recombination events during replication (§ 1.4), the fate of SCJs was analysed in a *sgs1*Δ background (Liberi *et al.*, 2005). As in wild-type cells, SCJ Rad51-independent molecules were also detected in *sgs1*Δ under normal conditions. However, in MMS-treated *sgs1*Δ cells, SCJ molecules accumulate at a later stage compared to MMS-treated wild type. Liberi *et al.* (2005) have also shown that the accumulation of these later hemicatenates in *sgs1*Δ is dependent on Rad51 and Rad52 proteins and they are therefore not the same structures. To distinguish them from the former hemicatenates, these recombination-dependent sister chromatid junction molecules are referred to as rec-X molecules (Figure 1.10 D). As previously speculated, Rad51-independent hemicatenates could be precursors for Holliday junction-like structures and therefore precursors for the later rec-X molecules. In *rad53*Δ cells, hemicatenates degenerate progressively into other intermediates (Lopes *et al.*, 2003). This profile was compared to that of rec-X molecules in *sgs1*Δ*rad53*Δ MMS-treated cells (Liberi *et al.*, 2005). In *sgs1*Δ*rad53*Δ cells, the rec-X structures no longer accumulate and this absence of accumulation was not due to decrease stability due to the inactivation of *RAD53*. Therefore, Liberi *et al.* (2005) proposed that in the presence of DNA damage, Rad51-independent hemicatenates, that formed normally behind replication forks (Figure 1.10 A), are the precursors for a template switch when replication on the leading strand is blocked (Figure 1.10 B and C). Due to the uncoupling of leading and lagging strand synthesis, DNA can be replicated off the lagging strand following a Rad51p-mediated strand invasion (Figure 1.10 C). A template switch and replication off the lagging strand allow for the by-pass of the DNA damage on the leading strand. In theory, re-annealing to the leading strand will form Holliday junction-like molecules (rec-X – Figure 1.10 D), although intermediates involved in the second template switch have yet to be identified. The branches of these pseudo-Holliday junctions can be collapsed via the helicase activity of Sgs1p to form back hemicatenates (Figure 1.10 E). In yeast, an inducible, dominant-negative allele of *TOP3* has been shown to accumulate these rec-X molecules (Mankouri and Hickson, 2006). Dissolution of Holliday junctions between sister chromatids by topoisomerase Top3α and BLM has also been demonstrated in human (Seki *et al.*, 2006). In human tumour cells, anaphase bridges have been identified as DNA links between incompletely segregated daughter nuclei (Gisselsson *et al.*, 2000). One possibility is that these anaphase-bridges represent unresolved hemicatenates.

Recently, BLM along with Top3α have been shown to co-localise to anaphase bridges in fibroblast cells (Chan *et al.*, 2007). Therefore, it is likely that Sgs1p can collapsed double Holliday junction-like structure into hemicatenate. The topoisomerase activity of Top3p is required for their dissolution (Figure 1.10 F) to restore the progression of replication forks.

1.5. Meiotic double-strand break: crossover or non-crossover?

During meiosis, homologous recombination is initiated by genetically programmed DSBs catalysed by the meiosis specific trans-esterase Spo11 protein (Keeney *et al.*, 1997). Until recently, the accepted model for meiotic DSB repair was the Szostak model (Szostak *et al.*, 1983) where both crossover and non-crossover molecules where thought to occur from the resolution of double Holliday junctions.

In meiosis, the semi-stability of single-end invasion intermediates involved in crossovers compared to non-crossovers (Borner *et al.*, 2004) and the length of recombination-associated DNA synthesis (Terasawa *et al.*, 2007) have suggested that double Holliday junction resolution always give rise to crossovers while non-crossovers arise from the synthesis-dependent strand-annealing pathway (Allers and Lichten, 2001b; Borner *et al.*, 2004; Jessop *et al.*, 2006; Paques and Haber, 1999). The cleavage of both strands of the DNA helix probably requires coordination of Spo11p dimers (Figure 1.11A). Consistent with this is the identification of semi-dominant negative alleles of *spo11* (Diaz *et al.*, 2002) affecting the dimerisation of the protein. After DSB formation the Spo11 protein dimers remain covalently attached to each end of the DSB (Keeney and Kleckner, 1995). The DNA-Spo11 complexes are removed via nicking of oligonucleotides at opposite 5′-ends of the DSB by the MRX complex (Mre11p/Rad50p/Xrs2p) and Sae2 protein (Clerici *et al.*, 2005). Both the MRX complex and Sae2p are constitutively expressed and function both in mitosis and meiosis. Interestingly the two Spo11-oligonucleotide complexes at each 5′-end of the break might be cleaved asymmetrically leading to single-stranded tails of different length (Neale *et al.*, 2005). If correct, this early difference, in term of oligonucleotide length between the two ends of a DSB, might be of significance regarding the loading of Dmc1p and Rad51p at the opposite DSB-ends. Exonucleases then extend the 5′-digestion tracks by several hundred nucleotides, leaving 3′-overhang tails (Sun *et al.*, 1991) (Figure 1.11B). Different studies have suggested that Exo1 might be one of the exonuclease involved in this resection (Khazanehdari and Borts, 2000; Tsubouchi and Ogawa, 2000). Recent evidence also suggests that the length of the resection track influences the outcome of the repair where long resection tracks lead to crossovers while short resection tracks might preferentially lead to gene conversion associated with synthesis-dependent strand

annealing (Cotton, 2007). Upon resection of the 5′-end of the DSB, nucleofilament proteins load on the 3′-end single-stranded DNA tails. Both Rad51p and the meiosis specific Dmc1 protein load onto the 3′-overhang tails (Bishop *et al.*, 1992; Rockmill *et al.*, 1995). The assembly of Rad51p onto the ssDNA tails requires Rad52p, Rad54, Rad55, Rad57 and Rdh54/Tid1 proteins (see § 1.3.2 for details) while assembly of Dmc1p only requires Mei5 and Sae3 (Tsubouchi and Roeder, 2004). The presence of the meiosis-specific nucleofilament protein Dmc1 was thought to result in the bias toward the homologue during meiotic recombination, although this no longer seems to be correct (see § 5.1.2 for details). Both nucleofilament proteins catalyse a strand exchange reaction between one of the 3′-resected tail and the homologue. This strand exchange reaction was believed to involve only one end of the DSB in a so-called single-end invasion reaction (Hunter and Kleckner, 2001) (Figure 1.11C). Although, new evidence suggests that, both ends of a DSB might be involved independently in invading either the sister chromatids, or the homologue, or both (Oh *et al.*, 2007) (See discussion for details). The stability of these single-end invasions is thought to be dependent on the length of resection as well as degree of homology between the two chromosomes. This later aspect is controlled by mismatch repair proteins (§ 1.6 and Chapter 5). The invasion of the homologue by one 3′-end provides a primer for recombination-associated DNA synthesis by DNA polymerases (Figure 1.11 D and G). The length of DNA synthesis is also believed to play a part in the stability of the joint molecules (Terasawa *et al.*, 2007) where long DNA synthesis tracks are associated with crossovers (Figure 1.11 D), while shorter ones are found in non-crossovers (Figure 1.11 G). In the crossover pathway, extensive DNA synthesis displaces the invaded strand in a D-loop-like structure that can be captured by the other 3′-end of the resected DSB (Figure 1.11 D). The result of this branch invasion and DNA synthesis is a joint molecule (JM) containing two Holliday junctions (Figure 1.11E). Little is know about the proteins involved in the resolution of dHJs although their resolution seems to always give rise to crossover products (Figure 1.11 F).

As mentioned earlier, non-crossover products are believed to come from a parallel pathway, which branches from the crossover pathway prior to or at the single end invasion stage. Although the crossover/non-crossover decision itself might be taken at an even earlier stage (Allers and Lichten, 2001b; Bishop and Zickler, 2004; Borner *et al.*, 2004; Hunter and Kleckner, 2001). The synthesis-dependent strand-annealing

pathway (SDSA), from which non-crossovers are believed to derive (Figure 1.11G-I), was first described by Pâques and Haber (1999). Although the stability of SEIs leading to dHJs (Borner *et al.*, 2004) suggests that crossovers/non-crossovers are coming from different pathways, physical intermediates remain to be identified for the synthesis-dependent strand-annealing pathway. In this pathway, the recombination-dependent DNA synthesis, as detected by incorporation of bromodeoxyuridine by Terasawa *et al.* (2007) occurs earlier than in the crossover pathway. Such evidence is consistent with the differences between crossovers and non-crossovers as observed and Börner et *al.* (2004). Furthermore, in SDSA, recombination-dependent DNA synthesis is not associated with long synthesis tracks supporting the idea that shorter DNA synthesis does not provide enough D-loop displacement to permit the capture by the second end of the DSB (Figure 1.11 F). This joint molecule, comprising the newly extended single-end invasion DNA, is destabilised and ejected from the D-loop prior to the strand recapture. Annealing of the newly synthesised DNA extension with the second end of the DSB (Figure 1.11H) allows for the repair of the DSB by gap-filling DNA-synthesis, creating non-crossover DNA molecules (Figure 1.11 I).

In both the crossover pathway and the SDSA pathway, strand invasion and strand capture create heteroduplex DNA (hDNA) *i.e.* the invading end and the recipient molecule are coming from different parental DNA molecules. hDNA is potentially misspaired due to sequence divergence between the parental molecules. When divergence is low, mismatch repair proteins can act on the hDNA to correct mismatches, leading to gene conversion events. However, when mismatches are extensive, such as between homeologous sequences, the mismatch repair proteins act to prevent recombination (§ 1.6 and Chapter 4 and 6).

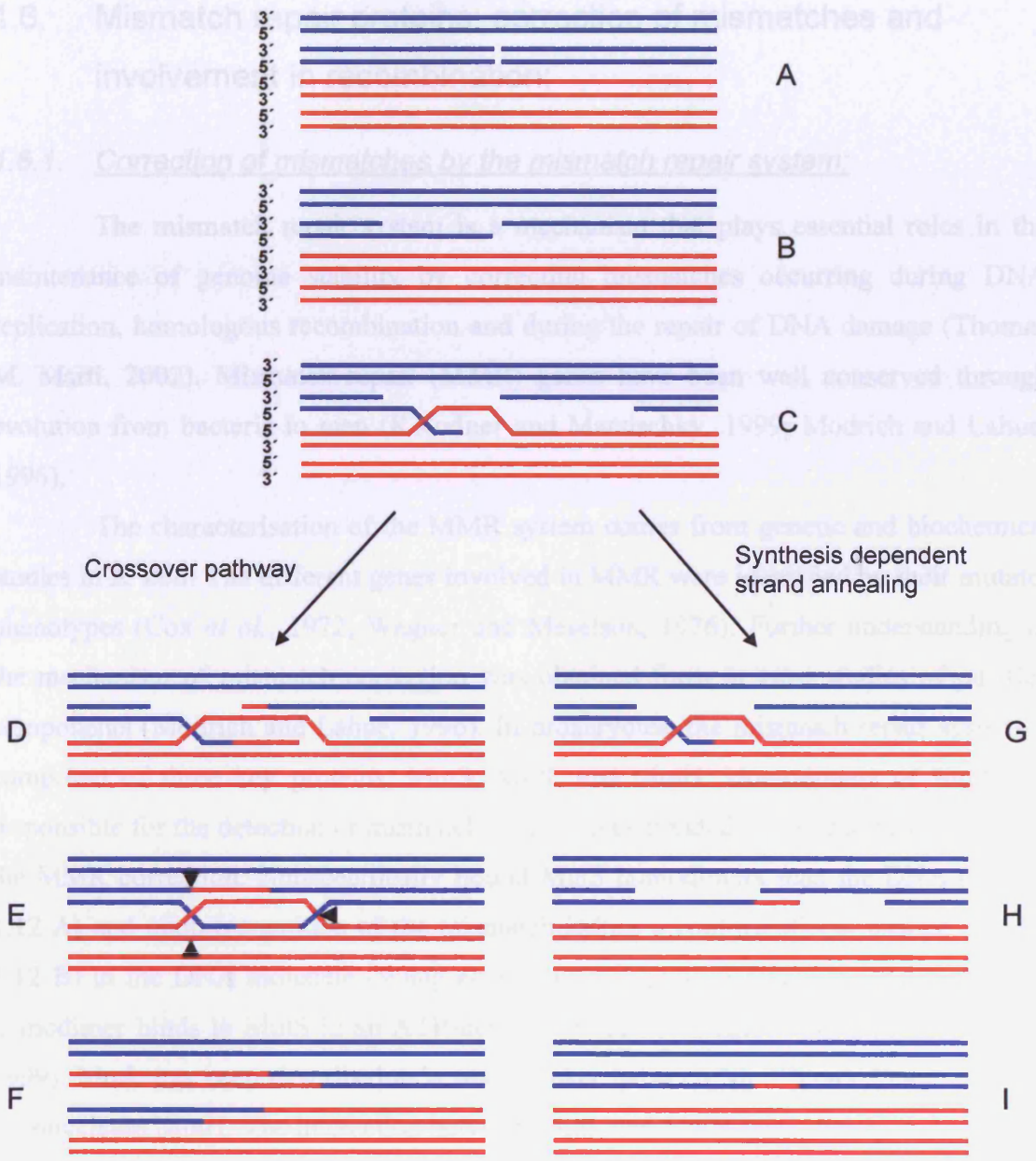


Figure 1.11: Meiotic double-strand break repair model.
A: DSB occurs in one chromatid catalysed by Spo11p. B: 5´-end resection via MRX complex (Mre11p, Rad50p, Xrs2p), Sae2p and Exo1p. C: Single-end invasion (SEI) catalysed by Rad51p/Dmc1p. Depending on the length of resection, the stability of the SEI and the length of newly synthesised DNA, the DSB is repaired by either the crossover pathway (D-F) or the non-crossover pathway (synthesis dependent strand annealing, G-I). D: extensive DNA synthesis, D-loop displacement and 3´-end strand capture. E: Upon DNA synthesis and ligation, formation of double Holliday junction. The resolution, marked by the black triangles, gives rise to crossover molecules (F). G: shorter DNA synthesis does not allowed for strand capture. The newly extended 3´-end is ejected from the D-loop and annealed to the opposite end of the break (H). DNA synthesis and ligation give rise to non-crossover molecules.

1.6. Mismatch repair proteins: correction of mismatches and involvement in recombination:

1.6.1. Correction of mismatches by the mismatch repair system:

The mismatch repair system is a mechanism that plays essential roles in the maintenance of genome stability by correcting mismatches occurring during DNA replication, homologous recombination and during the repair of DNA damage (Thomas M. Marti, 2002). Mismatch repair (MMR) genes have been well conserved through evolution from bacteria to men (Kolodner and Marsischky, 1999; Modrich and Lahue, 1996).

The characterisation of the MMR system comes from genetic and biochemical studies in *E. coli*. The different genes involved in MMR were identified by their mutator phenotypes (Cox *et al.*, 1972; Wagner and Meselson, 1976). Further understanding of the mechanism of mismatch correction was obtained form *in vitro* studies of purified components (Modrich and Lahue, 1996). In prokaryotes, the mismatch repair system is composed of three key proteins, MutS, MutL and MutH. Homodimers of MutS are responsible for the detection of mismatches in double-stranded DNA and the initiation of the MMR correction. Nonspecifically bound MutS homodimers scan the DNA (Figure 1.12 A) and upon recognition of the mismatch induce a conformational change (Figure 1.12 B) in the DNA molecule (Wang *et al.*, 2003). Upon mismatch recognition, MutL homodimer binds to MutS in an ATP-dependent manner (Figure 1.12 B) (Galio *et al.*, 1999). MutL has been described as a matchmaker between MutS homodimers and the endonuclease MutH. The interaction between MutL and MutH homodimers is dependent on ATP hydrolysis and stimulates the activity of MutH (Hall and Matson, 1999). MutL is also responsible for the loading of the DNA helicase UvrD (Hall *et al.*, 1998), which unwinds DNA duplex from nicks generated by MutH (Figure 1.12 C) (Dao and Modrich, 1998). Hemi-methylated GATC sites are the substrate for the endonclease MutH. This activity is dependent on the presence of mismatches and is stimulated by MutS, MutL and ATP (Au *et al.*, 1992). Depending on the position of the mismatches compared to the single-strand gap, the excision of the newly synthesised DNA (i.e. un-methylated) is performed by the 3′-5′ exonucleases ExoI and ExoX (Figure 1.12 C) or the 5′-3′

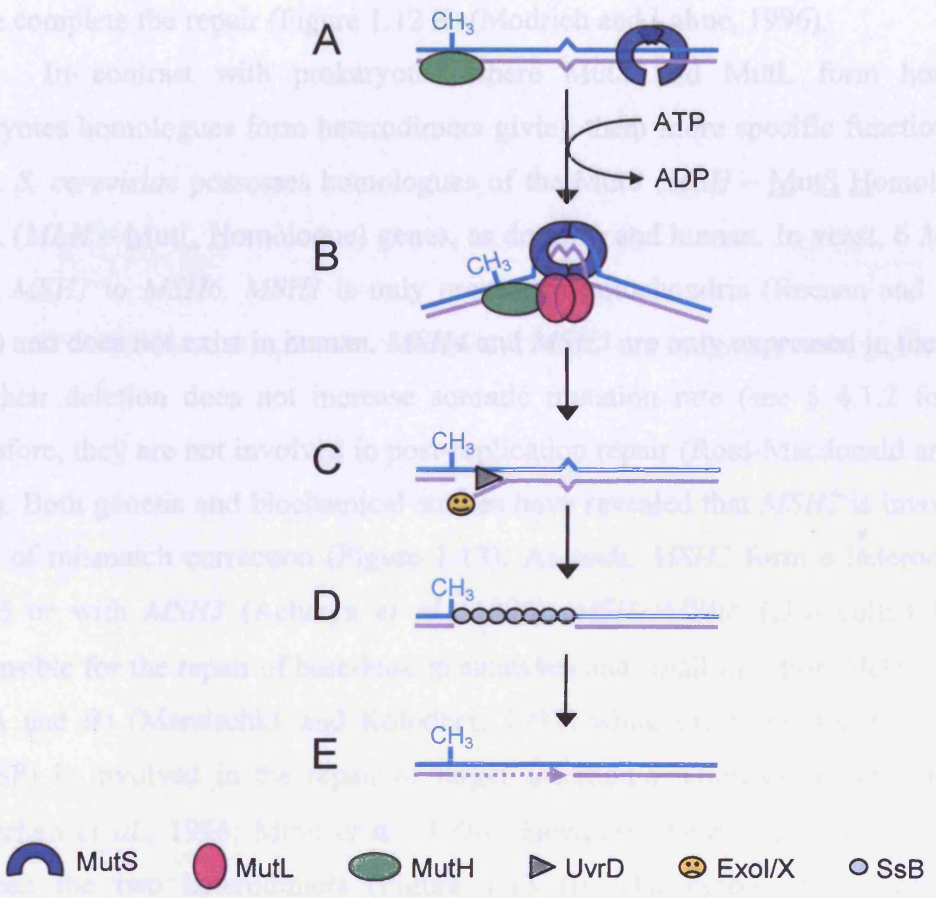


Figure 1.12: Mismatch repair correction in E.coli. A: MutS homodimers scan the DNA for mismatches. B: At mismatch site MutS induces a change in DNA conformation and recruits MutL homodimers and MutH. MutH catalyses a nick in the non-metylated DNA strand. C: Helicase UvrD unwinds the DNA duplex while the exonucleases ExoI and ExoX digest the 3´-5´ tail and remove the mismatch. D: Single-strand binding proteins localise to the ssDNA. E: Polymerase III and ligase restore the DNA duplex.

exonucleases RecJ and ExoVII (Burdett *et al.*, 2001). Upon removal of the mismatches, DNA polymerase III, single-stranded-DNA binding protein (Figure 1.12 D) and DNA ligase complete the repair (Figure 1.12 E) (Modrich and Lahue, 1996).

In contrast with prokaryotes where MutS and MutL form homodimers, eukaryotes homologues form heterodimers giving them more specific functions (Figure 1.13). *S. cerevisiae* possesses homologues of the MutS (*MSH* – MutS Homologue) and MutL (*MLH* – MutL Homologue) genes, as do mice and human. In yeast, 6 *MSH* genes exist, *MSH1* to *MSH6*. *MSH1* is only present in mitochondria (Reenan and Kolodner, 1992) and does not exist in human. *MSH4* and *MSH5* are only expressed in the germ line and their deletion does not increase somatic mutation rate (see § 4.1.2 for details). Therefore, they are not involved in post-replication repair (Ross-Macdonald and Roeder, 1994). Both genetic and biochemical studies have revealed that *MSH2* is involved in all types of mismatch correction (Figure 1.13). As such, *MSH2* form a heterodimer with *MSH6* or with *MSH3* (Acharya *et al.*, 1996). *MSH2/MSH6* (also called MutSα) is responsible for the repair of base-base mismatches and small insertions/deletions (Figure 1.13A and B) (Marsischky and Kolodner, 1999) while the heterodimer *MSH2/MSH3* (MutSβ) is involved in the repair of larger insertion/deletion events (Figure 1.13 C) (Habraken *et al.*, 1996; Miret *et al.*, 1996). However, there is an overlap of function between the two heterodimers (Figure 1.13 B) (Harrington and Kolodner, 2007; Marsischky *et al.*, 1996).

The different MutL homologues in yeast were identified by sequence homology to the *E. coli MutL* gene or genetically as mutants who increase post-meiotic segregation (Kramer *et al.*, 1989; Prolla *et al.*, 1994). There are four *MLH* genes in yeast, *MLH1*, *PMS1*, *MLH2* and *MLH3*. The different MutL homologues also form heterodimers. Like *MSH2*, *MLH1* is found in all heterodimers in association with either *PMS1*, *MLH2* or *MLH3* (Wang *et al.*, 1999). The major role in mismatch repair correction is played by the *MLH1/PMS1* heterodimer (Figure 1.13) while the other two *MLH1/MLH2* and *MLH1/MLH3* take part in the suppression of some types of frameshift mutations (Harfe *et al.*, 2000).

The different *MSH* and *MLH* heterodimers can interacts with one another as shown in Figure 1.13 to correct specific kind of mispairs. Genetic studies in yeast have failed to identify homologues of the *E. coli* MutH protein. Sgs1p was first thought to be the helicase involved in mismatch correction, although this hypothesis was not

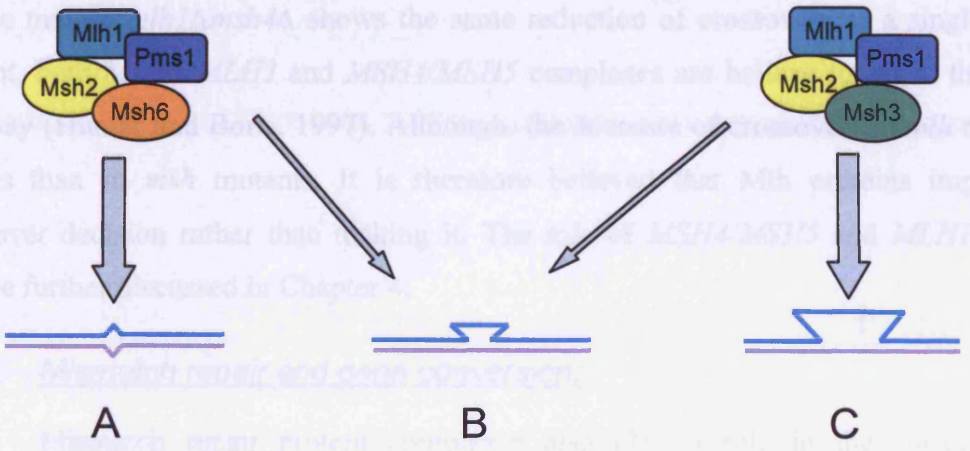


Figure 1.13: Different heterodimers of *MSH* and *MLH* paralogues provide different specificity for the repair of mismatches. Thick arrows indicate preferred substrates while thin arrows indicate redundancy in *S. cerevisiae*. Msh2-Msh6/Mlh1-Pms1 proteins complex repair preferentially base-base mismatches (A) and small insertion/deletion (B). Msh2p-Msh3p/Mlh1p-Pms1p repair larger deletion/insertion events (C) but also smaller events (B).

confirmed, as *sgs1* mutants do not have a mutator phenotype (Ajima *et al.*, 2002). Because eukaryotic genome might not carry *MutH* homologue, the stand discrimination and the nicking process must differ between prokaryotes and eukaryotes.

Apart from their functions in mismatch repair, *MSH4/MSH5* and *MLH1/MLH3* heterodimers also play a role in meiotic recombination (Hollingsworth *et al.*, 1995; Hunter and Borts, 1997; Khazanehdari and Borts, 2000; Wang *et al.*, 1999). Since the double mutant *mlh1Δmsh4Δ* shows the same reduction of crossovers as a single *msh4* mutant, both *MLH1/MLH3* and *MSH4/MSH5* complexes are believe to act in the same pathway (Hunter and Borts, 1997). Although, the decrease of crossovers in *mlh* mutants is less than in *msh* mutants. It is therefore believed that Mlh proteins implement crossover decision rather than making it. The role of *MSH4/MSH5* and *MLH1/MLH3* will be further discussed in Chapter 4.

1.6.2. Mismatch repair and gene conversion:

Mismatch repair protein complexes also play a role in the correction of mismatches in heteroduplex DNA during meiotic recombination. Such correction leads to gene conversion events (Figure 1.14 and Chapter 4 § 4.3.5). Gene conversions are non-reciprocal transfer of genetic material from one DNA molecule to its homologue. They usually occur between two alleles of a given gene. Gene conversions arise when mismatched DNA in heteroduplex joint-molecules are corrected by the mismatch repair system (for review see (Surtees *et al.*, 2004). In *S. cerevisiae* gene conversions for auxotrophic markers (or drug resistance markers), such as *his4*, can be detected genetically in the four viable spore tetrads class as their outcome in meiosis give rise to a 3:1 (or 6:2) segregation pattern instead of the expected 2:2 (or 4:4) Mendelian segregation. *S. cerevisiae* strains deficient for *MSH2*, *PMS1* or *MLH1* have an increased frequency of non-Mendelian or aberrant segregation events of a particular type referred to as post-meiotic segregation. In yeast, PMS events lead to sector spore colonies and indicate failure in the repair of heteroduplex DNA (Alani *et al.*, 1994; Hunter and Borts, 1997; Williamson *et al.*, 1985).

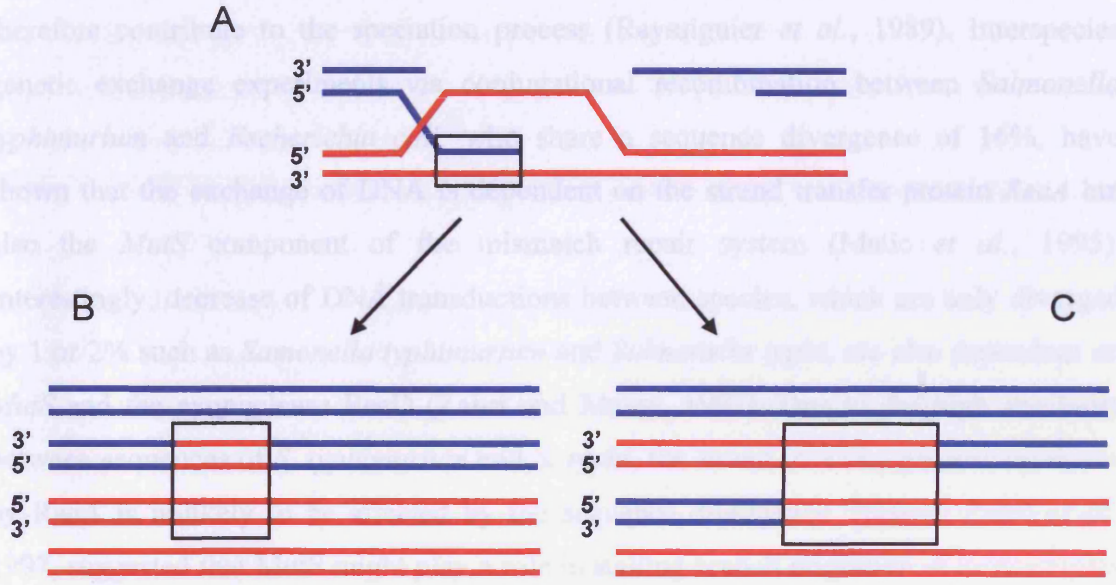


Figure 1.14: Schematic representation of gene conversion mechanisms in *S.cerevisiae*. A: Single-end invasion intermediate contains heteroduplex DNA on which mismatch repair proteins can act. B: A gene conversion can be associated with non-crossover via synthesis dependent strand annealing pathway. C: A gene conversion can be associated with crossovers if the repair occurs in a double Holliday junction intermediate .

1.6.3. MMR genes and Sgs1p decrease homeologous recombination during mitosis:

Several studies in bacteria have shown that the post-replicative mismatch repair system plays an important role in regulating interspecies gene exchanges and could therefore contribute to the speciation process (Rayssiguier *et al.*, 1989). Interspecies genetic exchange experiments via conjugational recombination between *Salmonella typhimurium* and *Escherichia coli*, who share a sequence divergence of 16%, have shown that the exchange of DNA is dependent on the strand transfer protein *RecA* but also the *MutS* component of the mismatch repair system (Matic *et al.*, 1995). Interestingly, decrease of DNA transductions between species, which are only diverged by 1 or 2% such as *Samonella typhimurium* and *Salmonella typhi*, are also dependent on *MutS* and the exonuclease RecD (Zahrt and Maloy, 1997). Due to the high similarity between sequences of *S. typhimurium* and *S. typhi*, the strand invasion process catalysed by RecA is unlikely to be affected by the sequence divergence. Instead, Zahrt *et al.*, 1997, suggested that MutS might play a role in stalling branch migration of heteroduplex DNA. Stalled branch migration might be a substrate for helicase(s), which could unwind heteroduplex DNA and expulse the donor strand. The expelled DNA could be a subsequent substrate for the RecD exonuclease-dependent activity of RecBCD. Studies on *E. coli* have shown that these bacteria carry large duplications of genomic segments thought to arise via unequal recombination between those dispersed sequences. An example of such duplicated sequence is the *rhs* sequences (Petes and Hill, 1988). The different *rhs* sequences are slightly diverged. The mismatch repair proteins MutS and MutL are responsible for the regulation of large duplication due to homeologous recombination between two *rhs* sequences *rhsA* and *rhsB* (Petit *et al.*, 1991). As hypothesised by Petit *et al.*, 1991, the intervention of the MutS and MutL proteins might occur in heteroduplex DNA after strand exchange catalysed by RecA. Experiments on the formation of λ transducing phages as a means to study illegitimate recombination in *RecQ* helicase mutants of *E. coli* have shown that this helicase plays a role in suppressing recombination between homeologous sequences (Hanada *et al.*, 1997).

As seen in section 1.6, an intermediate in the formation of double Holliday junction during homologous recombination contains heteroduplex DNA molecules. Interacting homeologous sequences will form heteroduplex intermediates containing various degrees of mismatches according to their extent of divergence. It has been shown previously that recombination (both during mitosis and meiosis) is very sensitive to DNA divergence (Borts and Haber, 1987; Datta *et al.*, 1996).

In mitosis, deletion of the *RECQ* gene, *SGS1*, in *S. cerevisiae* leads to an increase in ectopic recombination via both intra and inter-chromatids events (Watt *et al.*, 1996), loss of heterozygosity via intra-chromosomal deletion, chromosome loss and hyper-recombinations (Ajima *et al.*, 2002). Sgs1p also suppresses homeologous recombination and might be partially redundant in this function with Msh2p (Myung *et al.*, 2001). Furthermore, retrotransposition of *Ty1* elements in rDNA is increased in *sgs1*Δ cells in a *RAD52*-dependent manner (Bryk *et al.*, 2001). The mismatch repair system and particularly the Msh2 and Msh3 proteins seem to play a role in the regulation of recombination between diverged sequences in mitosis (Datta *et al.*, 1996; Selva *et al.*, 1995). Datta *et al.*, 1996, have shown, in an assay using inverted repeats within the intron of a chromosomal selectable gene (*HIS4*), that the degree of homeology plays a key role in the rate of mitotic recombination in wild-type cells. A 50-fold reduction in mitotic recombination was observed between sequences sharing 91% homology while a 1000-fold decrease was observed between sequences sharing 77% homology compared to homologous sequences (100% homology). In mutants for component of the mismatch repair system (*msh2*Δ, *msh3*Δ and *pms1*Δ) the rate of recombination was increased compared to wild-type homeologous strains depending on the degree of divergence. For example, *pms1*Δ did not affect the rate of homeologous recombination in sequences sharing 77% homology while it did increase homeologous recombination by 10-fold in a 91% homologous strain. In *msh2*Δ and *msh3*Δ strains, the rate of homeologous recombination was increase in both homeologous strains (77% and 91% identity, respectively) but the increase in the 91% identical strains was greater than in the strains sharing only 77% of homology suggesting once more that the degree of divergence greatly affects the rate of recombination and cannot be fully complemented by mutations in mismatch repair genes. Assays differentiating the types of recombinant molecules in Selva *et al.* (1995) shown that all kinds of recombination events (intra-chromatid crossovers, single strand annealing and sister chromatid exchanges) were increased in

msh2 and *msh3* mutants. Co-immunoprecipitations and two hybrids experiments have shown that the RecQ helicase BLM and Sgs1 proteins interact with mismatch repair proteins Mlh1p and Msh6p (Pedrazzi *et al.*, 2001; Pedrazzi *et al.*, 2003). Furthermore, Sgs1p, Msh2p and Msh6p are required for heteroduplex rejection during single strand annealing (Sugawara *et al.*, 2004) in mitosis. Taken together those different studies suggest that the Sgs1 protein, in collaboration with the mismatch repair proteins Msh2 and Msh6, might play a role in the regulation of ectopic and homeologous recombination during mitosis.

1.7. Aims of this study:

The aim of this thesis is to further characterise the role of the *SGS1* gene during meiosis in *S. cerevisiae*. Although *SGS1* has been studied extensively in mitosis, little was known about the role of this protein during the meiotic division when we first started this work. Since, Bloom's patients, specific mice models of *BLM* and *Dmblm* flies are infertile, RecQ helicases might serve an important function during the meiotic division (§ 1.1). Yeast meiosis is also perturbed in *SGS1* mutants since *sgs1* cells do not sporulate with great efficiency and the spore viability is decreased (Miyajima *et al.*, 2000a; Miyajima *et al.*, 2000b; Rockmill *et al.*, 2003; Watt *et al.*, 1995). Sgs1p and Top3p have also been shown to play a role in the regulation of recombination events during mitosis (§ 1.4) but no apparent defect in *SGS1* mutants was detected during meiotic recombination (Watt *et al.*, 1995).

Sgs1p and its RecQ homologues are essential for genomic stability during mitosis (§ 1.3 and § 1.4). Our first aim was then to determine if this defect contributed to decrease meiotic viability in *SGS1* mutants. To avoid mitotic problems, we tried to create a meiosis-specific null mutant of the *SGS1* gene.

Our third aim was to study homologous recombination in *sgs1* strains and to reconcile recent conflicting data that have emerged since this work started (Rockmill *et al.*, 2003; Rockmill *et al.*, 2006).

The mismatch repair system plays a role in heteroduplex rejection during mitosis and meiosis (Borts and Haber, 1987; Chambers *et al.*, 1996; Goldfarb and Alani, 2004; Hunter *et al.*, 1996). Since, Sgs1p is an helicase that have been shown to interact with MMR genes such as *MLH1* and *MSH6* in mitosis (Pedrazzi *et al.*, 2001; Pedrazzi *et al.*, 2003; Sugawara *et al.*, 2004; Wang and Kung, 2002), our fourth aim was to investigate if Sgs1p was the helicase involved in heteroduplex rejection during meiotic recombination.

Chapter 2: Materials and Methods

2.1. Materials:

2.1.1. Yeast strains:

All *Saccharomyces cerevisiae* strains used in this study are derived from Y55 strains unless otherwise specified. Diploids were made by crossing a *MAT a* strain to a *MAT α* strain on YEPD plates. Mating was performed at 30°C for 12 to 16 hours. When required, diploid selection was performed by streaking for single colonies on minimum plates supplemented with the required amino acids. All strains used in this study were *ade1-1*, *ura3::nco* and *HOΔ*.

Homeologous diploids were obtained by mating an Y55 *S. cerevisiae* strain to an engineered strain containing a chromosome III from *S. paradoxus* (partial hybrid) (Chambers *et al.*, 1996).

The different strains used in this study are detailed in the experimental procedures relevant to each chapter.

2.1.2. Yeast media:

All yeast media were made using the following core ingredients supplied by Difco: Bacto-Agar, yeast extract, Bactopeptone, yeast nitrogen base and dextrose (D-glucose). The rich medium, YEPD (Yeast Extract, Peptone, Dextrose) is made of yeast extract (1%, w/v), Bactopeptone (2%, w/v) and dextrose (D-glucose) (2%, w/v). pH was adjusted between 6-6.5 by adding 1M HCl. For plates, 2.5% of solid agar was added to the liquid medium. After autoclaving one hour, 10 ml/l of 0.5% adenine was added.

Synthetic medium was used to study the segregation of auxotrophic markers after tetrad dissection. Such medium consists of minimal media supplemented with a required nutritional supplement (nutrient mixture – Table 2.1). Minimal medium is made of yeast nitrogen base (2.7% w/v) and dextrose (2% w/v). pH was adjusted to 6-6.5 by adding 2.5M NaOH. The mixtures missing the required supplement were referred to as "drop-out media". 870 mg/L of the nutrient mixture, missing the appropriate amino acid, were added to minimal medium.

Table 2.1: Composition of nutrient mixture:

Nutrient	Amount (mg)
Adenine	800
Arginine	800
Aspartic acid	4000
Histidine	800
Leucine	800
Lysine	1200
Methionine	800
Phenylalanine	2000
Threonine	800
Tryptophane	800
Tyrosine	1200
Uracil	800

Drugs such as antibiotics, 5-fluororotic acid (5-FOA) or Methyl Methane Sulfonate (MMS) were added in the required amount to the specific medium after autoclaving. Details of the different drugs used can be found in Table 2.2.

Table 2.2: Drug concentration per plate.

Drugs	Concentration (%)	Supplier
Ampicillin - Luria Broth	0.1%	Sigma
Canavanine	2%	Sigma
Cycloheximide	1%	Sigma
G-418 (Geneticin)	0.8% or 1.6%	Invitrogen
Hygromycine B	0.6%	Invitrogen
Nourseothricin	0.5%	D-Jena
Methylmethane sulfonate (MMS)	0.005% or 0.0075%	Sigma
5-FOA - uracyl droup-out	0.1%	Melford labs
Doxycycline	10%	Sigma

2.1.3. Bacterial medium:

Luria-Broth (LB) medium (1% bactopeptone (w/v), 0.5% bacto-yeast extract (w/v), 1% NaCl (w/v), pH 7) was used to grow *Escherichia coli* and was supplemented with ampicillin (100 µg/ml, Sigma) when selection for a plasmid was required.

2.1.4. Competent bacterial cells:

Chemically competent *Escherichia coli* cells were obtained from Invitrogen and Amersham (pMOS-Blue transformation kit). Such cells were transformed according to the instructions from the suppliers.

2.1.5. Oligonucleotides:

Oligonucleotides were order on-line from Invitrogen. The different primers used are listed in Table 2.3.

Table 2.3: list of PCR primers as used in this study:

Primers	Sequences 5' to 3'	Purpose
sgs1 Fwd flanking	GCCATATTTCGTGTTGGGC	PCR *sgs1::KANMX4* (Hickson strain) and diagnostic
sgs1 Rev flanking	GTCGCTAGACTGGATGACAC	
SGS1 internal1 Fwd	CCTGCATCTGGACCAACGAA	*SGS1* internal primers
SGS1 internal1 Rev	CTCATCTTCTTCTCTTTCGAC	
SGS1 internal2 Fwd	CATCTCCCACAGCACAGAGC	
SGS1 internal2 Rev	AGGCCTGAAATCGTGGCCCC	
SGS1 internal3 Fwd	CGGGTATAATATATTGCCAC	
SGS1 internal3 Rev	GTTCCTTCGCACTTATAAAG	
sgs1::Gfp-KANMX4 Fwd	AGGGACGATTATCCGGATGTCGAAAGAGAAGAA GATGAGATGTCTAAAGGTGAAGAATT	*sgs1-408* deletion
sgs1::Gfp-KANMX4 Rev	TGTCGTAGTTATAAGTAACACTATTTATTTTTCTA CTCTCGTACGCTGCAGGTCGAC	
sgs1::NATMX4 Fwd	CGATTTTTCATTAAGTGATATAGTGAGTAAATCC AATTTATCTCGTACGCTGCAGGTCGAC	*sgs1-652* deletion
sgs1::NATMX4 Rev	TGTCGTAGTTATAAGTAACACTATTTATTTTTCTA CTCTATCGATGAATTCGAGCTCG	

Primers	Sequences 5' to 3'	Purpose
K2	TTCAGAAACAACTCTGGCGCA	Junction PCR *KANMX4* insertions
K3	CATCCTATGGAACTGCCTCGG	
H2	CGGCGGGAGATGCAATAGG	Junction PCR *HYGMX4* insertions
H3	TCGCCCGCAGAAGCGCGGCC	
N2	GATTCGTCGTCCGATTCGTC	Junction PCR *NATMX4* insertions
N3	AGGTTCACCAACGTCAACGCA	
RRP7-HIS4::HYG-CYH Fwd flanking	GCTACTACCTCTCTTTTAATCCAAAATTACAATTT TTACGTTACCGTACGCTGCAGGTCGAC	*HYG-CYH* insertion
RRP7-HIS4::HYG-CYH Rev flanking	CAAAATACAGTCTTGAATGAATAGAGATACACTA TGTAATGAATGGGATCCACTAGTTCTAGAGC	
LEU2-NFS1::HYG Fwd flanking	GAAAAAGAATTGCACTTTAACATTAATATTGACAA GGAGGAGGCGTACGCTGCAGGTCGAC	*HYGMX4* insertion
LEU2-NFS1::HYG Rev flanking	TGACAAGGAGGAGGGCACCACACAAAAAGTTAG GTGTAACATCGATGAATTCGAGCTCG	
SGS1-Degron Fwd	GCCAGCTTGGAGTCATTGGCTAGAGGAAAGGAA AAAATACAGATTAAGGCGCGCCAGATCTG	Degron insertion
SGS1-Degron Rev	CCATTTGTGCTCCCTTCTTAAGTTATGTGACGGC TTCGTCACCATGGCACCCGCTCCAGCGCCTG	
Degron D2	CGCTCCAGCGCCTGCACCAG	Junction PCR Degron insertion
Degron D3	CTGGTGCAGGCGCTGGAGCG	Junction PCR Degron insertion

Underlined nucleotide sequences are homologous to plasmid drug resistance cassettes.

2.1.6. Plasmids:

The different plasmids used in this study are listed below.

The *Degron* and the *UBR1* plasmids were given by Dr. Pedro San-Segundo.

Table 2.4: List of plasmids used in the study:

Plasmids	General Description	References
pFA6kanMX4	*KANMX4*	Wach *et al.*, 1994
pAG25	*NATMX4*	Goldstein *et al.*, 1999
pAG32	*HYGMX4*	Goldstein *et al.*, 1999
pRS306	*SacI-SacII* fragment of *CYH2* ORF in pBluescript	Szent-Giorgy unpublished, provided by Michael Lichten
pGR28	*Gfp-KANMX4*	Griffin, thesis
pRED548	*HYG-CYH2*	Chaix, this study.
pKL183	*N-Degron*	San-Segundo, unpublished
pSS126	*2μ UBR1; TRP1* (derived from pRS424)	San-Segundo, unpublished
pYES2	*2μ; URA3*	Invitrogen
pRED688	*2μ UBR1; URA3* (derived from pYES2)	Chaix, this study

2.1.7. DNA molecular weight markers:

Bacteriophage λ DNA, digested by either HindIII or BstEII, was purchased from New England Biolabs as a standard DNA marker. 100bp and 1kb DNA ladders were purchased from Invitrogen. DNA standards were used at 25 ng/μl or 50 ng/μl. For accurate DNA quantification and southern blotting, 500 ng of DNA markers were loaded onto agarose gel. Picture were taken using Kodak Gel Logic 200 Imaging System camera and Kodak GL 200 Acquire Software. The Kodak 1D Image analysis software allowed the quantification (molecular weight and mass) of DNA fragments by comparison with the pre-defined size markers.

2.1.8. Concentration of enzymes:

When needed enzymes were used in the following concentrations:

Table 2.5: Different enzymes used in this study:

Enzymes	Concentration	Supplier
Klenow DNA polymerase fragment I	5 units/reaction	Roche
Lysozyme	10 mg/ml	Sigma
Pfu DNA polymerase	5 units/reaction	Stratagene
Proteinase K	Varying	Roche
Restriction enzymes	1-5 units/reactions	New England Biolab
RNase A	1mg/ml	Sigma
Taq DNA polymerase	0.1-1 unit/reaction	ABgene
T4 DNA ligase	20 units/reaction	Roche
Zymolyase 20T	10mg/ml	Sigma

2.1.9. Microscopes:

Cells and spores counting experiments were conducted using an improved Neubauer or Fuchs-Rosenthal haemocytometer under a Zeiss phase contrast microscope. Tetrad dissection experiments were carried under a Zeiss phase contrast microscope. Dissecting needles were supplied by Singer Instruments.

2.2. Methods:

2.2.1. Agarose gel electrophoresis:

Agarose from Roche was used at 0.8%, 1% or 1.2% concentration, depending on the size discrimination of DNA fragments required. 1 M TBE (Tris-Borate EDTA: 90 mM Tris base, 90 mM Boric acid, 2 mM EDTA, pH 8.3) was used to dissolve the agarose to the required concentration and also as an electrophoresis running buffer (Sambrook *et al.*, 1989). DNA was stained using 1 µl of ethidium bromide (10mg/ml, Sigma) per 100 ml of agarose solution before polymerisation. Visualisation of DNA molecules was performed under U.V. light (wavelength λ = 302 nm) and pictures were taken using a Kodak 200L dark chamber and camera. Pictures were then processed using Kodak 1D Image analysis software.

2.2.2. DNA purification by gel extraction:

Once DNA fragments were separated on an agarose gel, the required DNA band was cut out of the gel under blue light (wavelength λ = 450 nm). The use of blue light instead of U.V. light minimised the nicking of double stranded DNA. The DNA was then extracted from the gel using a QIAquick Gel Extraction Kit from QIAGEN, following instructions by the supplier.

2.2.3. Genomic DNA Extraction:

DNA extractions were performed according to Borts *et al.*, 1986. Yeast strains were cultured from 12 to 16 hours at 30°C. Cells were harvested by centrifugation at 3600 rpm for 5 minutes then re-suspended in 0.5 ml of 1M sorbitol, 0.2M tris-HCl pH 8.5, 0.02M EDTA and 0.1% 5-ß-mercaptoethanol. 50 µl T20 zymolyase (10 mg/ml; Sigma) was added and the cells were incubated at 37°C until they became spheroplasted. The supernatant was removed after centrifugation at 13000 rpm for 1 minute. The spheroblasts were gently resuspended in 50 µl of 1M sorbitol. A solution of 50mM Tris-HCl pH 7.5, 100mM NaCl, 100mM EDTA, 0.5% SDS (w/v) and H_2O was then added to 50 ml final volume. The cells were resuspended in this solution very gently. 0.2 mg of proteinase K + 50 µl of RNase (1mg/ml) was added to each tube. Tubes were then incubated for 12 hours. The tubes were chilled on ice for 5 minutes and 0.5ml of phenol-

chloroform was added. Tubes were centrifuged for 10 minutes at 13000 rpm and the aqueous phase was removed. Phenol-chloroform extraction was performed twice for each tube. 1ml of 100% ethanol was added and each tube was centrifuged for 10 minutes. Ethanol was removed. The DNA was washed by adding 1ml of 70% ethanol, air dried and then dissolved in 100-200 µl of 1X TE (0.1 M Tris-HCl pH 8.0; 1 mM EDTA).

2.2.4. Plasmid DNA extraction:

Plasmids from *Escherichia coli* were extracted via two methods depending on the purpose required. The first method, based on a boiling extraction, was used to screen large numbers of clones. *E. coli* cells were grown for 12 to 16 hours at 37°C in a 5 ml culture. Cells were spun for 5 minutes in a table top centrifuge and resuspended in 0.35 ml of STET (8% sucrose (w/v), 5% Triton X1009 (w/v), 50 mM Tris-HCl pH 8.0, 50 mM EDTA, H_2O). 25 µl of lysozyme (10 mg/ml) was added. The solution was boiled for 3 minutes and then spun in a centrifuge at 13000 rpm for 15 minutes. The pellet was removed with a toothpick and 0.35 ml of isopropanol was added. The tubes were centrifuged for another 10 minutes and the supernatants were poured off. The tubes with the plasmid DNA were air-dried for a few minutes and the plasmid DNA was resuspended in 25-100 µl TE. Identified positive clones were re-extracted using QIAGEN MiniPrep extraction kit following the supplier's instructions. The DNA was cut by restriction enzymes following the supplier's recommendations and electrophoresed through a 0.8% (w/v) agarose gel.

2.2.5. DNA modification and restriction:

Ligation, phosphatase treatment and restriction digests were used to clone and/or modify DNA fragment. T4 DNA ligase (New England Biolab) was used to ligate blunt or cohesive ends generated by restriction enzymes. Shrimp alkaline phosphatase (Roche) was used to remove phosphate residues at the 5′ end of DNA molecule to facilitate ligation of blunt end molecules. The molecular ratio between plasmid and insert was as recommended by suppliers (1:2.5). Restriction enzymes were purchased from New England Biolabs or Roche. Reactions were performed in 10µl final volume reaction for normal plasmid digestion and control. When needed larger volumes were used as for partial digest (150µl) and cloning (50µl).

2.2.6. *Clamped Homogenous Electric Field analysis:*

Clamped Homogenous Electric Field (CHEF) analysis requires intact chromosomal DNA. For this purpose, DNA extractions were performed in agarose plugs as described by (Louis and Haber, 1990). Cells were inoculated in 3 ml YEPD liquid and grown for 12 to 16 hours at 30°C. The cells were then harvested as for genomic DNA extraction and washed in 1 ml of 50 mM EDTA, pelleted as above and resuspended in 200 µl 50 mM EDTA. 100 µl SCE/Zymolase solution was added (1M sorbitol, 0.1M sodium citrate, 10mM EDTA, 5-ß-mercaptoethanol, 10 mg/ml zymolase), quickly followed by 0.5 ml low melting point (LMP) agarose (1% LMP agarose in 0.125 M EDTA, melted and cooled to 45°C). After mixing by pipetting, the solution containing the yeast cells and the agarose was transferred into a clean 75µl plug former (BIORAD) on ice. After setting, the polymerased plugs were transferred to clean Eppendorf tubes containing 0.5 ml of EDTA solution (0.5 M EDTA, 0.1 M Tris-HCl pH 7.5, 5% ß-mercaptoethanol (w/v)). The plugs were incubated in this solution for 12 hours at 37°C. The solution was then pipetted out and replaced by a solution of Proteinase K/RNase (0.5 M EDTA, 1% sodium sarkosyl (w/v), 10 mg/ml Proteinase K, 0.1 mg/ml RNase). Plugs were incubated for 12 hours at 37°C to allow complete digestion of proteins and RNA. Following this second incubation, plugs were washed once in 50mM EDTA and then stored at 4°C in 1ml 0.5 M EDTA, 0.1 M Tris-HCl, pH 7.5. Each plug was cut in half and loaded in 1% agarose gel wells. CHEF gel variable angle electrophoreses were run in 0.5 X TBE for 24 hours.

The programme used to separate yeast chromosomes was set with two different switching times whereby the current going through the electrodes alternated at a 120° angle. This programme was as followed:

Stage 1: Initial switch time: 60 seconds.
Final switch time: 60 seconds.
Run time: 15 hours.
Stage 2: Initial switch time: 90 seconds.
Final switch time: 90 seconds.
Run time: 9 hours.
Voltage: 6 volts/cm.
Angle: 120°.
Pump speed setting: 80.

2.2.7. Southern Blotting:

Southern blot were carried out as described in (Sambrook *et al.*, 1989).

DNA fragments on agarose gels were blotted for 12 hours on Hybond-N^+ nitrocellulose membranes (Amersham Pharmacia Biotech). After transfer, the DNA was cross-linked to the membrane using U.V. exposure (energy: 100μJ/cm^2 for 20 seconds).

DNA probes were prepared using Gene Images Random Labelling Module (Amersham) following the instructions provided by the supplier. 0.5μg to 1μg of unrestricted plasmid DNA was diluted in 50μl volume and denatured at 95°C for 10 minutes. After snap cooling on ice for 5 minutes, the plasmid DNA was labelled by adding a mix of nucleotides, fluorescently labelled dUTP, random primers and Klenow DNA polymerase. The solution was incubated at 37°C overnight for better incorporation of the labelled nucleotides. The quality of the probe, i.e. the incorporation of fluorescently labelled dUTP, was determined by comparing 5μl of labelled probe and 5μl of nucleotide mix dotted on a Hybond N^+ membrane to a reference strip containing known quantities of nucleotide mix.

Generally 5μl of probe were used to detect DNA blotted on Hybond N^+ membranes.

2.2.8. Polymerase Chain Reaction (PCR):

PCR were performed using an Eppendorf Master Cycler Gradient PCR machines. The reactions were performed according to literature (Mullis *et al.*, 1986). The PCR mix used was developed by A.J. Jeffreys (Jeffreys *et al.*, 1990). PCR amplifications were used for gene specific amplifications, cloning, site directed knockouts and diagnostics of such modifications. When the product of amplification was to be used for cloning, *Pfu* polymerase (Stratagene) was used for amplification instead of *Taq* polymerase (ABgene) to minimize the mis-incorporation of nucleotides during the DNA synthesis stage. The volumes of PCR reactions ranged from 10μl to 50μl. The PCR cycle parameters varied, depending on the type of amplification. Usually, a pre-denaturation step of two minutes at 94°C was followed by a cycle of denaturation at 94°C for 45 seconds. The optimum annealing temperature, specific to each primer, was determined by their A-T and G-C contents. Annealing was performed for one minute while the elongation time varied according to the length of the PCR product. As a guideline an

elongation time of 1 minute per kilo-base was used when using *Taq* polymerase. The cyle of denaturation-annealing-elongation was repeated 30 times. Using *Pfu* polymerase, the elongation time was extended to two minutes per kilo-base, as this enzyme, having a proof-reading activity, synthesises DNA at a slower rate.

0.2 μmM of primers was used in both PCR reactions, while enzyme concentration varied from 1 unit (*Taq* polymerase) to 2.5 units (*Pfu* polymerase).

2.2.9. Colony PCR:

When possible, PCR were carried directly on yeast cells rather than extracted DNA. For such purpose, *S. cerevisiae* cells were taken from the top of the colony with a pipetman® tip and denatured in 20μl 0.02M NaOH for 5 minutes. 2μl of this solution were added directly to the PCR reaction mix containing buffer, oligonucleotide primers and *Taq* polymerase. The pre-denaturation sep was increased to 3 minutes and PCR cycling was performed as described above.

2.2.10. DNA sequencing:

DNA sequencing was either done by myself or sent to the sequencing facility at the University of Leicester (PNACL) by providing PCR product or plasmid DNA with the required primers. In either case, the DNA was first quantified using Kodak 1D Image analysis software prior to any reaction and the concentration adjusted to PNACL recommendations. Sequencing reactions were done using cycle sequencing (BigDye 1.1). Prior to the sequencing reaction, PCR products or plasmid DNA were treated with exonuclease I (exoI) and shrimp alkaline phosphatase (SAP) and incubated at 37°C for an hour. Enzymes were then denaturated at 80°C for 15 minutes to stop the reaction. Once the reactions were stopped, the sequencing reactions were performed as follows: 4 μl of Big Dye terminator, 1 μl of primers at 3.2 μM, 50 ng of DNA, water was then added to a final volume of 10 μl per reaction. The DNA was then ethanol precipitated to discard any unincorporated nucleotides. Once dry, pellets were resuspended in formamide prior being run on an ABI 3100 sequencer.

2.2.11. Gene disruption in Saccharomyces cerevisiae:

All gene disruptions were performed using a one-step PCR mediated transplacement using short sequence homology to the target gene (Wach *et al.*, 1994). Primers were designed, matching plasmid sequences containing drug resistance genes.

To such primers 5′-tails homologous to the 5′ and 3′ regions of the gene of interested were added. The drug resistant genes were then amplified by PCR from the plasmids. The products of PCR were checked on 0.8% agarose gel and then transformed into *Saccharomyces cerevisiae*. The resistance gene was inserted in the yeast genome by homologous recombination due to the homology of the 5′ tails. The primers used for such reactions are listed in Table 2.5.

2.2.12. Yeast transformation:

Yeast cells were grown overnight at 30°C in 5ml YEPD and then diluted 1:10 in fresh YEPD and grown for 4 hours until log phase. The cells were harvested by centrifugation at 3600 rpm for 5 minutes, resuspended and washed twice in distilled water. Two rounds of washes in 0.1M lithium acetate pH 7.5 followed and then the yeast solution was split into two tubes. After centrifugation and removing the supernatant, the following components were added: 240 µl of 50% PolyEthylene Glycol 3350, 36 µl of 1M LiAc, 25 µl of denatured salmon sperm DNA (2mg/ml) and transforming DNA or water were added to the pellet. *URA3* Plasmid DNA was used for transformation as a positive control. After resuspension by vortexing, the solution was incubated at 30°C for 30 minutes and heat shocked at 42°C for 20 minutes. The cells were washed once in distilled water and then grown in YEPD for three hours before being plated onto selective medium.

Transformants were selected for their drug resistance phenotypes. They were all tested by junction-PCR for insertion of the drug resistant cassette after DNA extraction. The different *SGS1* mutants used in this study were also checked genetically by crossing them to a *sgs1::LEU2* strain. The segregation of the right tranformants is 2:2 Geneticin resistant and the two spores Geneticin sensitive are prototroph for leucine.

2.2.13. Junction PCR:

Junction PCRs were used to confirm the correct insertion and orientation of drug resistance cassettes in the genome. The first primer pair was designed to amplify the 5′ junction between the endogenous DNA and the inserted drug resistance gene. The second primer pair was design to amplify the 3′ junction. For amplification of the 5′ junction, an oligonucleotide was design to hybridize on the Watson strand (5′-3′ direction) upstream of the inserted cassette while the reverse oligonucleotide hybridized

inside the drug resistance gene on the Crick strand (3′-5′ direction). For amplification of the 3′ junction, the first oligonucleotide hybridized inside the drug resistance gene on the Watson strand (5′-3′ direction) while the reverse primer hybridized on the Crick strand (3′-5′ direction) downstream of the insertion, on the endogenous genomic DNA.

2.2.14. Saccharomyces cerevisiae mutants:

Four different *SGS1* mutants were made by gene replacements and confirmed as described above. The complete deletion of the *SGS1* gene (*sgs1*Δ) was made by replacing the open reading frame of the gene by the *KANMX4* cassette (Figure 3.1), confering resistance to Geneticin (G418). This replacement left 90 base pairs upstream and downstream of the start and stop codons. Two partial deletion of *SGS1* were also made. The first leaves 408 amino acids at the N-terminal of the protein while deleting the last 1039 amino acids at the C-terminal (*sgs1*Δ*-C1089*). It is therefore a deletion of 3117 bp at the 3′ end of the gene. The deleted fragment has been replaced by *KANM4* and the remaining nucleotides are tagged in frame with Green Fluorescent Protein – Gfp (Figure 3.1). The second partial deletion leaves 652 amino acids at the N-terminal of the Sgs1 protein while deleting 795 amino acids at the C-terminal (*sgs1*Δ*-C795*). The deletion of 2385 base pairs (795 amino acids) at the 3′ end of the gene by replacement with the *NATMX4* gene confers resistance to Nourseothricin (Figure 3.1). We also made another *SGS1* mutant (*Degron-SGS1*), which targeted the protein for degradation during meiosis. The construction of this strain and the theory behind the targeted degradation are further explained in chapter 4.

*msh6*Δ strains were made by crossing using *msh6::KANMX4* strains already available (Chambers, 1999).

2.2.15. Sporulation:

Sporulation was induced by nitrogen starvation in yeast diploid cells by replication onto sporulation medium containing potassium acetate (2%), yeast extract (0.22%), dextrose (0.5%) and complete amino acid and nucleotides solution (0.0875%). Sporulation was performed on plates at 23° C for 5 days (Gangloff *et al.*, 1999).

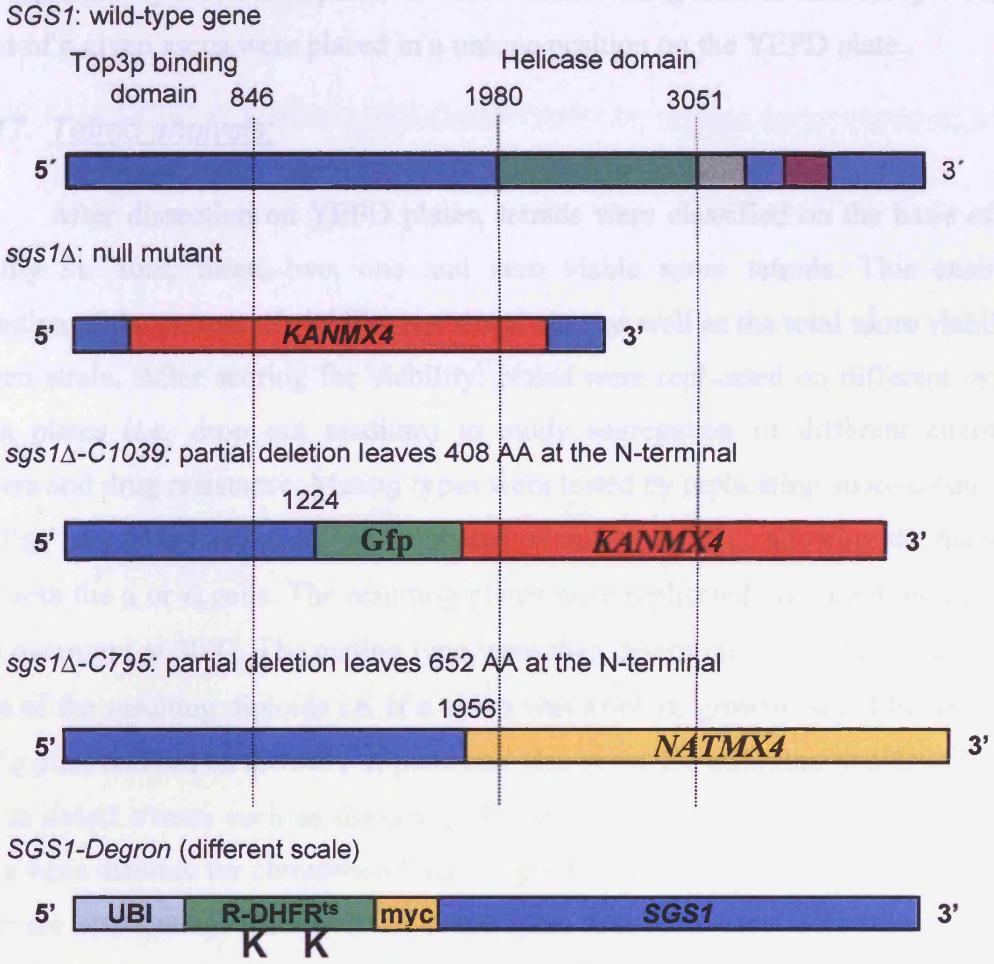


Figure 2.1: schematic representation of various *SGS1* mutants. The deleted sequences were created by single step PCR replacement methods (Wach et al, 1994).

2.2.16. Tetrad dissection:

After sporulation, asci were incubated at 37°C for 30 minutes with 5 µl zymolyase (5mg/ml) diluted in 100 µl Dissection Buffer (1M sorbitol, 10mM EDTA, 10mM NaPO4 ph 7.2). 400 µl of Dissection Buffer was added after incubation. Asci were separated by micromanipulation with a needle using a Zeiss microscope. The four spores of a given ascus were placed in a unique position on the YEPD plate.

2.2.17. Tetrad analysis:

After dissection on YEPD plates, tetrads were classified on the basis of spore viability *i.e.* four, three, two, one and zero viable spore tetrads. This enables an estimation of the pattern of viability per tetrad class as well as the total spore viability for a given strain. After scoring for viability, plates were replicated on different synthetic media plates (*i.e.* drop out medium) to study segregation of different auxotrophic markers and drug resistance. Mating types were tested by replicating spore colonies on a "*MAT* a" or a "*MAT* α" plate and incubated overnight at 30°C, allowing the haploids to mate with the a or α cells. The resulting plates were replicated on minimum medium to grow overnight at 30°C. The mating type were then determined by growth on minimum plates of the resulting diploids i.e. if a spore was *MAT* α, growth would be seen on the *MAT a* plate and not on the *MAT* α plate and vice versa. Determining mating type can be used to detect events such as disomy and loss of chromosome III. As an example, if spores were disomic for chromosome III, no growth would be seen on either plate. Such spores are non-mating. The results of auxotrophy, drug resistance and mating types were entered into an excel spreadsheet for analysis by MacTetrad 6.9 software. The software used is able to calculate the percentage of recombination between loci and expresses it in centiMorgan (cM) using the formula of Perkins (Perkins, 1949). The Perkins formula is given in Chapter 4 § 4.2.2. The map distance between loci is only calculable in the four viable spore tetrads class. In the three viable spore tetrads the genotype of the dead spore can be deduced according to the genotype of the remaining spores following the first law of Mendel. It can then be used to calculate the percentage of recombination. The centromere marker, *TRP1*, can be used to identify sisters or non-sisters spores and can give valuable information on the events leading to death. Mis-segregation events in meiosis are described in more details in Chapter 4 § 4.1.5.

2.2.18. Statistical analysis:

Statistical analyses were performed on two or more data sets to accept or reject the null hypothesis, H_0: "the two data sets are different".

Following Sokal and Rohlf's (1969) recommendations (Sokal and Rohlf, 1969), the G-test was used at a 95% confidence level to accept or reject H_0. G-tests were calculated using the formula:

$G = 2 \sum_i A_i \ln(A_i/B_i)$ where A_i = Observed frequency in class (i) in population A.

B_i = Observed frequency in class (i) in population B.

ln = natural logarithm (log to the base *e*).

Depending on the degree of freedom, the value of G was attributed a probability "p". If $p > 0.05$ the null hypothesis was rejected, *i.e.* the two populations are the same. When comparing more than two data sets, the α factor was adjusted using the Bonferroni correction procedure to minimise the chance of type II errors (accepting the null hypothesis while it is false).

The Bonferroni adjustment of the α factor is available online at: http://www.quantitativeskills.com/sisa/calculations/bonfer.htm.

When necessary, other statistical tests were used. These tests are described in the relevant chapters.

Chapter 3: Decrease in sporulation efficiency and spore viability in *SGS1* mutant strains

3.1. Introduction:

3.1.1. Regulation of meiosis:

Gametogenesis in *Saccharomyces cerevisiae* is a complex developmental programme because interrelated events such as genetic exchanges, haploidization and cellular differentiation must be coordinated. Therefore, the sporulation process, *i.e.* the formation of haploid ascospores, is closely linked with the initiation, progression and completion of meiotic divisions. Both processes need the integration of genetic, biochemical and morphological signals, which are regulated to ensure the correct partitioning of genetic material between each spore (Honigberg and Purnapatre, 2003).

The sporulation process and meiotic divisions, although tightly linked, can be divided into three stages (Pringle *et al.*, 1997). The first stage, meiotic initiation, ensures that early key meiotic genes, such as *IME1* (Initiation of Meiosis) and *IME2*, are expressed after mitotic G1 arrest and allow entry into meiosis. In the second stage, the DNA is replicated and pairing of homologues and recombination can take place. During the third stage, or late meiosis, the cell goes through two sequential rounds of chromosome segregation to produce four haploid daughter cells. In *Saccharomyces cerevisiae*, gamete (or spore) development immediately follows the meiotic divisions by formation of the spore walls (Honigberg and Purnapatre, 2003). The entry into meiosis, in *Saccharomyces cerevisiae*, requires particular conditions be met. These can be easily produced in the laboratory by nitrogen (N_2) starvation. N_2 starvation induces a mitotic cell cycle arrest in G1 from which the cell can then enter meiosis. A non-fermentable carbon source such as potassium acetate provides energy *via* respiration producing CO_2, which is indispensable for the activation of meiosis (Ohkuni and Yamashita, 2000).

The two principal regulators of meiotic initiation, and therefore sporulation, are *IME1* and *IME2* (Mitchell, 1994) (Figure 3.1). *IME1* encodes a transcription factor responsible for the transcription of several genes, including the Serine/Threonine protein kinase *IME2* (Rubin-Bejerano *et al.*, 1996). The promoter of *IME1* (Granot *et al.*, 1989)

is suppressed by glucose (Figure 3.1), while the presence of acetate activates the transcription (Sagee *et al.*, 1998). *IME1* transcription is also down-regulated in haploid cells via the repressor Rme1p (Figure3.1). Such regulation ensures that no division other than mitosis can occur in haploid cells (Covitz and Mitchell, 1993). In addition to *IME2*, Ime1p activates several other genes involved in early meiosis. For example, *HOP1,* which is involved in synapsis (Hollingsworth *et al.*, 1990) and *SPO13,* which one function is to prevent the segregation of sister chromatids during meiosis I (Wang *et al.*, 1987), are both activated by the transcription factor Ime1p. Interestingly, Ime2p kinase can phosphorylate Ime1p and therefore acts as a negative feedback loop by targeting Ime1p for degradation (Guttmann-Raviv *et al.*, 2002).

In response to the activation by nitrogen starvation and expression of Ime1 and Ime2 proteins, the mid-meiosis phase starts with DNA replication. In *ime1*Δ diploid cells, pre-meiotic DNA replication does not occur while in *ime2*Δ diploids, replication is delayed by 8 to 9 hours (Foiani *et al.*, 1996). The regulation of meiosis is tightly linked to the processivity of DNA replication. Treatment of early meiotic cells with hydroxyurea, which blocks the progression of replication forks, can repress the transcription of Ime2p (Lamb and Mitchell, 2001). As a kinase, Ime2p has multiple targets. It indirectly activates the initiation of DNA replication by phosphorylation of the Sic1p protein, which is an inhibitor of the Cdc28p kinase (Dirick *et al.*, 1998). Cdc28p, in a complex with Clb5p and Clb6p kinase, is required for meiotic S-phase initiation (Benjamin *et al.*, 2003). Ime2p also triggers the transcription of other genes such as *NDT80*. Ndt80p is a transcription factor playing a role in the activation of gene expression during the middle stage of meiosis (Hepworth *et al.*, 1998). As an activator of transcription, Ime2p suppresses the repression of the promoter-specific protein Sum1p. Sum1p inhibits transcription of the *NDT80* gene during mitosis and early meiosis (Lynch *et al.*, 2005; Pak and Segall, 2002). Ime2p also phosphorylates Ndt80p (Sopko *et al.*, 2002). The phosphorylated Ndt80 protein is responsible for the activation of the transcription of the B-type cyclin Clb proteins, encoded by *CLB1*, *CLB3*, *CLB4*, *CLB5* and *CLB6* (Sopko *et al.*, 2002). These cyclins are activators of the cyclin-dependent kinase Cdc28, which plays a role during meiotic chromosome segregation. Cells lacking Clb1p, Clb3p and Clb4p or a temperature-sensitive mutant of Cdc28p, arrest at meiosis I (Dahmann and Futcher, 1995). Ime2p has also been proposed to play a late role in meiosis by negatively regulating Cdh1p, a targeting subunit of the anaphase-promoting

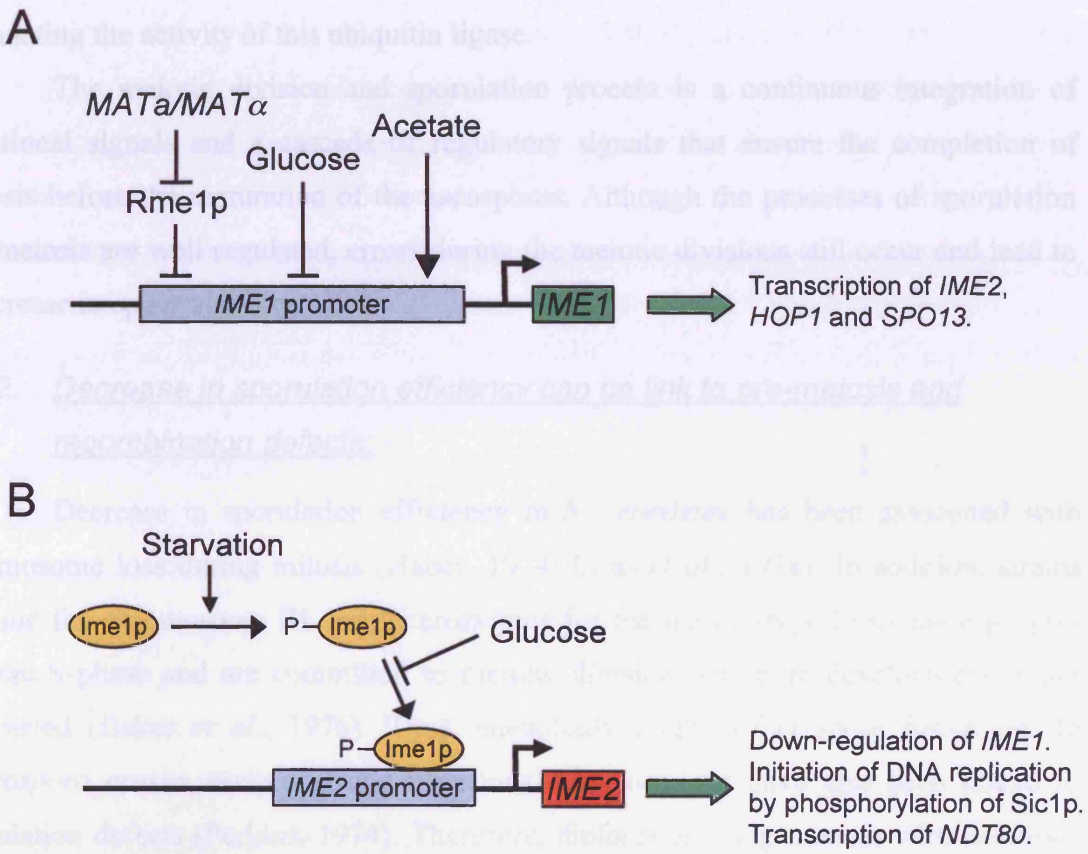


Figure 3.1: **A**: regulation of IME1 transcription. In haploid cells, Rme1p repress *IME1* transcription while in diploids, absence of glucose and presence of acetate enhance *IME1* transcription. **B**: Ime1p regulates transcription of *IME2*. Under starvation conditions, Ime1p is phosphorylated. In absence of glucose, phosphorylated Ime1p binds to IME2 promoter and activates its transcription.
(Adapted from Honigberg *et al.* (2003)).

complex/cyclosome, APC/C (Bolte *et al.*, 2002). The anaphase-promoting complex/cyclosome regulates the metaphase-anaphase transition (for review see (Acquaviva and Pines, 2006). Thus Ime2p might regulate chromosome segregation by modulating the activity of this ubiquitin ligase.

The meiotic division and sporulation process is a continuous integration of nutritional signals and a cascade of regulatory signals that ensure the completion of meiosis before the maturation of the ascospores. Although the processes of sporulation and meiosis are well regulated, errors during the meiotic divisions still occur and lead to a decrease in spore viability.

3.1.2. Decrease in sporulation efficiency can be link to pre-meiosis and recombination defects:

Decrease in sporulation efficiency in *S. cerevisiae* has been associated with chromosome loss during mitosis (Haber, 1974; Liras *et al.*, 1978). In addition, strains disomic for chromosome III and heterozygous for the mating-type locus undergo pre-meiotic S-phase and are committed to meiotic division but spore development is not completed (Baker *et al.*, 1976). Thus, aneuploidy might affect spore formation. In *Neurospora crassa*, reciprocal and insertional translocations have also been linked to sporulation defects (Perkins, 1974). Therefore, diploids entering meiosis with deletions or duplications of a partial, or entire chromosome could potentially have a decrease in spore formation if these deletions occur in middle-late meiotic genes involved in spore development. Likewise, diploids having lost chromosome III cannot enter meiosis because Rme1p will down-regulate the expression of Ime1p in the absence of either *MATa* or *MATα* genetic information. Several kinds of rearrangements can account for loss of heterozygosity such as chromosome non-disjunctions, chromosome losses, deletions (due to ectopic crossovers or unequal crossovers), gene conversions and/or combinations of all the above. Loss of heterozygosity in *sgs1* diploids has been assessed both qualitatively and quantitatively in mitotic-dividing cells (Ajima *et al.*, 2002) although their assay could not distinguish between chromosome missegregation and chromsome loss (1:0 segregation events). Diploids deleted for *SGS1* have a 13-fold increase in loss of chromosome III compared to wild type. Additionally, loss of heterozygosity arising from gene conversion events associated with allelic crossovers were increased 12-fold compared to wild type, while non-crossover gene conversions

accounted for a 18-fold increase. Interestingly, the contribution to loss of heterozygosity of ectopic crossovers was far greater. Ectopic recombination was first defined by Lichten *et al.* (1987) as recombination between sequences present at different genomic positions (Lichten *et al.*, 1987). Here, Ajima *et al.* (2002) defined ectopic crossovers as events occurring by interchromosomal recombination either between two chromosomes III at non-allelic sites or between a chromosome III and another chromosome. Such events were increased 46-fold in *sgs1*Δ compared to wild-type diploids. More surprisingly, allelic crossovers and gene conversions were often associated with chromosome loss. Crossovers associated with chromosome loss accounted for a 40-fold increase in loss of heterozygosity while gene conversions associated with chromosome loss was more than 50 times that observed in wild type. The relatively high frequency of both events could not be solely attributed to a random association between crossovers or genes conversions and chromosome losses. Thus, Ajima *et al.* (2002) attributed the chromosome loss defect to a concerted mechanism with homologous recombination. At last, intrachromosomal deletions between the *MAT* locus and *HMR* on chromosome III were also assessed in the same assay. These events showed a 2.9-fold increase compared to wild-type diploids. Ajima *et al.* defined intrachromosomal deletions as events occurring by homologous recombination either within a chromatid between ectopic sites or via unequal crossovers between sister chromatids. These events did not occur as frequently as the previously described interchromosomal deletions, showing that the former might be more stringently regulated.

More importantly, the use of an ochre suppressor (*SUP11*) of an *ade2* mutation can distinguish between chromosome loss and chromosome non-disjunction (Spencer *et al.*, 1990). *SUP11* is located on a single-copy extra chromosome in an otherwise *S. cerevisiae* diploid. Effective mitotic segregation of the extra *SUP11* chromosome gives rise to two pink daughter cells. Non-disjunction of the chromatids of the extra chromosome produces adjacent red and white spore colonies while chromosome loss only gives rise to red spore colonies without adjacent white spore colonies. Using this technique, Watt *et al.* (1996) have shown that 72% of the total events of mitotic missegregation in *sgs1* mutants were due to chromosome non-disjunction rather than chromosome loss (Watt *et al.*, 1996).

As in mitosis, checkpoints have been identified in meiosis that ensured the proper sequence of events during this cell cycle. A pre-meiotic replication checkpoint has been described in both *S. cerevisiae* and *S. pombe* ensuring completion of meiotic S-phase prior to recombination (Murakami and Nurse, 2000; Stuart and Wittenberg, 1998). A key step in meiosis is the establishment of homologous pairing and meiotic recombination. Not surprisingly, these events are also controlled by a checkpoint. The meiotic recombination checkpoint prevents exit from the pachytene stage of meiotic prophase when meiotic recombination and chromosome synapsis are incomplete (Roeder, 1997). Thus, this checkpoint is also referred to as the pachytene checkpoint. This cell cycle arrest is only activated when diploids initiate meiotic recombination but fail to complete this process (Roeder and Bailis, 2000). Several *S. cerevisiae* meiosis defective mutants trigger the pachytene checkpoint. For example mutants for *DMC1* and *ZIP1* are arrested at pachytene (Rockmill *et al.*, 1995; Sym *et al.*, 1993). Since initiation of recombination is a prerequisite for activation of the pachytene checkpoint, the arrest can be by-passed in mutant defective for DSB formation such as *spo11* and *mer2* (Gardiner *et al.*, 1997; Keeney *et al.*, 1997; Li *et al.*, 2006a). Furthermore, the pachytene checkpoint requires the meiotic chromosomal proteins Red1p, Mek1p and Hop1p, and DNA damage checkpoint proteins such as Rad24p, Rad17p, Mec3p and Ddc1p (for review see (Roeder and Bailis, 2000)). For example, a *rad24Δ dmc1Δ* mutant will go through nuclear meiotic division even though the repair of DSBs is incomplete (Lydall *et al.*, 1996), suggesting that the in absence of Rad24p, the pachytene checkpoint is not activated.

sgs1Δ and *top3Δ* diploids have been shown to arrest in pachytene (Gangloff *et al.*, 1999; Rockmill *et al.*, 2003). Although, this cell cycle arrest does not seem to be attributable to the pachytene checkpoint. First, double mutant *sgs1Δ spo11Δ* cannot by-pass the arrest (Rockmill *et al.*, 2003). Secondly, *top3Δ rad24Δ* were not able to complete meiosis I (Gangloff *et al.*, 1999). This absence of by-pass suggests that the arrest in both *sgs1Δ* and *top3Δ* is not due to the pachytene checkpoint.

3.2. Experimental procedures:

3.2.1. Materials:

S. cerevisiae strains:

Diploid strains of *S. cerevisiae* used in this chapter are detailed in Table 3.1. Diploid cells were obtained by mating haploid strains for 12 hours. Mutant strains were constructed as specified in Chapter 2.

Table 3.1: Diploid strain genotypes.

Diploids	Genotypes
ACD 75 *SGS1* WT control	*ade1Δ HML::ADE1 his4-r1 leu2-r1 MATa HOΔ trp1::bsu36 lys2-d ura3::nco cyh2-1* *ade1-1 HML HIS4 LEU2 MATα HOΔ TRP1 LYS2 ura3::nco cyh2-1*
ACD 97 *SGS1* WT control	*ade1Δ HML::ADE1 HYG-CYH:his4-r1 leu2-r1:HYG MATa HOΔ trp1::bsu36 lys2-d* *ade1-1 HML HIS4 LEU2 MATα HOΔ TRP1 LYS2* *ura3::nco met13-2 cyh2-1* *ura3::nco met13-2 cyh2-1*
ACD 73 homozygote *sgs1Δ*	*ade1Δ HML::ADE1 his4-r1 leu2-r1 MATa HOΔ trp1::bsu36 lys2-d ura3::nco cyh2-1* *ade1-1 HML HIS4 LEU2 MATα HOΔ TRP1 LYS2 ura3::nco cyh2-1* *sgs1::KANMX4* *sgs1::KANMX4*
ACD 95 homozygote *sgs1Δ*	*ade1Δ HML::ADE1 HYG-CYH:his4-r1 leu2-r1:HYG MATa HOΔ trp1::bsu36 lys2-d* *ade1-1 HML HIS4 LEU2 MATα HOΔ TRP1 LYS2* *ura3::nco met13-2 cyh2-1 sgs1::KANMX4* *ura3::nco met13-2 cyh2-1 sgs1::KANMX4*
ACD 92 homozygote *sgs1Δ-C795*	*ade1Δ HML::ADE1 his4-r1 leu2-r1 MATa HOΔ trp1::bsu36 lys2-d ura3::nco cyh2-1* *ade1-1 HML HIS4 LEU2 MATα HOΔ TRP1 LYS2 ura3::nco cyh2-1* *sgs1::NATMX4* *sgs1::NATMX4*
ACD 107 homozygote *sgs1Δ-C795*	*ade1Δ HML::ADE1 HYG-CYH:his4-r1 leu2-r1:HYG MATa HOΔ trp1::bsu36 lys2-d* *ade1-1 HML HIS4 LEU2 MATα HOΔ TRP1 LYS2* *ura3::nco met13-2 cyh2-1 sgs1::NATMX4* *ura3::nco met13-2 cyh2-1 sgs1::NATMX4*

Table 3.1 (continue): Diploid strain genotypes.

Diploids	Genotypes
ACD 89 homozygote *sgs1Δ-C1039*	*ade1Δ* *HML::ADE1 his4-r1 leu2-r1 MATa* *HOΔ trp1::bsu36* *lys2-d* *ura3::nco* *cyh2-1* *ade1-1* *HML* *HIS4* *LEU2* *MATα* *HOΔ* *TRP1* *LYS2* *ura3::nco* *cyh2-1* *sgs1::GFP-KANMX4* *sgs1::GFP-KANMX4*

Media:

The different media used were described in Chapter 2. Sporulation was induced by nitrogen starvation as explained above. When required, diploids were selected on minimal medium supplemented with uracil (0.03%).

Sporulation experiments:

The counting of ascospores was performed under a Zeiss microscope using a Neybauer haemocytometer.

Tetrad dissection:

Tetrad dissection was performed on a Zeiss microscope coupled with a micromanipulator plate. After dissection and 3 days growth at 30°C, the number of viable spore colonies per tetrads was recorded.

3.2.2. Methods:

Sporulation efficiency:

Sporulation efficiency was measured using "pure" diploid cell colonies. After isolation of three different clones for each diploid, cells were incubated at 23°C for 5 days on sporulation medium plates. To measure sporulation efficiency, yeast tetrads (asci) were counted on an hæmocytometer. After cells were diluted in 1 ml distilled water, 10 µl of cell solution (either *SGS1*, *sgs1Δ*, *sgs1Δ-C795* or *sgs1Δ-C1039* mutants) was spread on the haemocytometer. For each cross, the total number of tetrads containing four or three spores (completion of meiosis II) was counted on squares of $4x10^6$ μm^3 each. The number of cells contained in 5 independent squares was counted for each cross. The total number of cells or the number of tetrads were calculated according to the formula:

$$1/100 \times [(\text{sum \# cell}) / 5] \times (4x10^6) = \text{\# cells/ml}$$

Independent sporulation experiments were performed at least 3 times for each cross. The ratio between the total number of cells and the number of tetrads gives the sporulation efficiency.

Pre-meiotic divisions:

To assess the effect of multiple mitotic divisions prior to entry in meiosis (*i.e.* the mitotic defect of *sgs1* mutants) on spore viability, diploids were selected on minimal medium supplemented with uracil and left to grow for 12 hours at 30°C. Single colonies were streaked for single on the same medium. After selection, these single diploid colonies were patched on YEPD medium for another 12 hours prior to replication onto sporulation medium. The selection of diploids from single colonies and the patching of selected diploids on YEPD medium is believed to account for ~50 mitotic divisions. Controls were directly replicated on sporulation medium after 12 hours mating.

Viability analysis:

After dissection, the numbers of four, three, two, one and zero viable spores per tetrads were counted for each diploid. The total viability for each diploid was calculated according to the formula:

Total viability (%) = (Total # spore colonies) ÷ (4x # of tetrads dissected) x100

The viability per tetrad class was calculated as a percentage of the number of tetrads containing a given number of spores (4, 3, 2, 1 or 0), divided by the total number of tetrads dissected.

Statistics:

As explained in Chapter 2, a G-test was used to determine if two distributions were statistically different or belonged to the same population. Tests were conducted in 95% confidence level (unless stated otherwise). If $p < 0.05$, the two distributions were considered statistically different.

For sporulation efficiency, standard deviations were calculated for each diploid to measure the dispersion of the data. The formula used to calculate the standard deviation is as follow:

$$SD = \sqrt{[\sum (x - X)^2 / (n-1)]}$$ where

x = sample value
X = mean value
n = sample size.

A Student T-test was used to compare the sporulation efficiency data between the two partial mutants *sgs1Δ-C795* and *sgs1Δ-C1039* (Three independent experiments). Due to large sample sizes ($n > 30$), Z-tests were used to compare total viability data.

3.3. Results:

3.3.1. *Sporulation efficiency decreases in SGS1 mutant strains:*

The efficiency of sporulation was measured as described in experimental procedures. The different *SGS1* diploid mutants (Figure 2.1), *sgs1*Δ (null deletion), *sgs1*Δ-*C1039* (partial deletion leaving 408 amino acids at the N-terminal) and *sgs1*Δ-*C795* (leaving 652 amino acids at the N-terminal) were assessed for ascospore formation. The *SGS1* WT diploid was used as a control. Previous studies have reported a decrease sporulation in *SGS1* mutants. Such experiments were been done in either the "BR" (Rockmill *et al.*, 2003), "SK1" or "S288C" strain background (Miyajima *et al.*, 2000a; Miyajima *et al.*, 2000b). As explained in Chapter 2, our study uses the *S. cerevisiae* Y55 strain. To determine if the sporulation process was not affected by strain differences, sporulation efficiency was re-tested in Y55 strains.

Table 3.2: Decrease in sporulation efficiency in *SGS1* mutants.

Strains	Sporulation efficiency [a] (% of asci containing 4 or 3 spores)
SGS1 WT control	67.34 ± 5.55 (837/1243)
*sgs1*Δ	18.57 ± 7.51 (270/1454)
*sgs1*Δ-*C1039*	26.39 ± 2.21 (252/955)
*sgs1*Δ-*C795*	34.54 ± 4.97 (450/1303)

[a] ± Standard errors.

In wild type 67.34% of cells contain asci with 4 or 3 spores (Table 3.2). Compared to other *S. cerevisiae* strain backgrounds (BR and S288C) sporulation of wild-type Y55 is more efficient. Rockmill *et al.* (2003) reported 59% of asci formed in the BR strain while Miyajima *et al.* (2000) only saw an 11.0% sporulation efficiency in the S288C strain background. In Table 3.2, the sporulation efficiency in *sgs1*Δ cells is decreased by more than 3.6 fold (to 18.57%) compared to *SGS1* cells. The partial mutants, *sgs1*Δ-*C795* and *sgs1*Δ-*C1039*, have a decreased sporulation efficiency compared to *SGS1* cells, by 2.5-fold and 1.9-fold respectively. The sporulation defect of

the *sgs1*Δ strain is partially restored by the two terminal truncation mutants. The increase in sporulation efficiency in both *sgs1*Δ-*C1039* and *sgs1*Δ-*C795* compared to *sgs1*Δ suggests that a domain in the N-terminal part of the Sgs1 protein is important for sporulation (Miyajima *et al.*, 2000b). The difference in sporulation efficiency between the two partial mutants is significant (T-test, $p = 0.003$) suggesting that a domain in the Sgs1 protein between the amino acids 408 and 795 is important for full function during sporulation. Interestingly, Rockmill *et al.* (2003) did not report a defect in sporulation in the *sgs1*Δ-*C795* mutant compared to wild type in the BR strains. However, in Y55 strains, the sporulation defect could not be restored to a *SGS1* wild-type level in this mutant, suggesting that the C-terminal part of the Sgs1 protein is required during sporulation in Y55.

3.3.2. SGS1 mutant strains have a decrease in spore viability:

Several experiments have already shown a decrease of viability in meiosis in *SGS1* mutant strains (Jessop *et al.*, 2006; Oh *et al.*, 2007; Rockmill *et al.*, 2003; Watt *et al.*, 1995). The study by Watt *et al.* (1995) reported a decrease in spore viability in a *SGS1* mutant that was thought to be a null mutant (*sgs1::LEU2*). Preliminary results, using the full deletion of the *SGS1* gene (*sgs1*Δ made by insertion of the *KANMX4* cassette, see Chapter 2 for details) made in this laboratory, revealed that the mutant used in Watt's study was not comparable to the total deletion of the gene as previously reported. The deletion made by Watt *et al.* (1995) used a one-step replacement method using the *LEU2* gene to delete the C-terminal 1039 amino acids of *SGS1*. The difference in viability between the two mutants (*sgs1::LEU2* and *sgs1*Δ) could be due to an effect of the leucine insertion on the rate of recombination and possibly the viability (Abdullah and Borts, 2001). Therefore, the *LEU2* disruption of the carboxy-terminal 1039 amino acids of *SGS1* was replaced by a geneticin drug resistant cassette and tagged with Green fluorescent protein (*sgs1-Gfp::KANMX4*) creating a new *sgs1*Δ-*C1039* mutant.

Spore viability in the different mutants was measured and compared to wild type as explained in the experimental procedures.

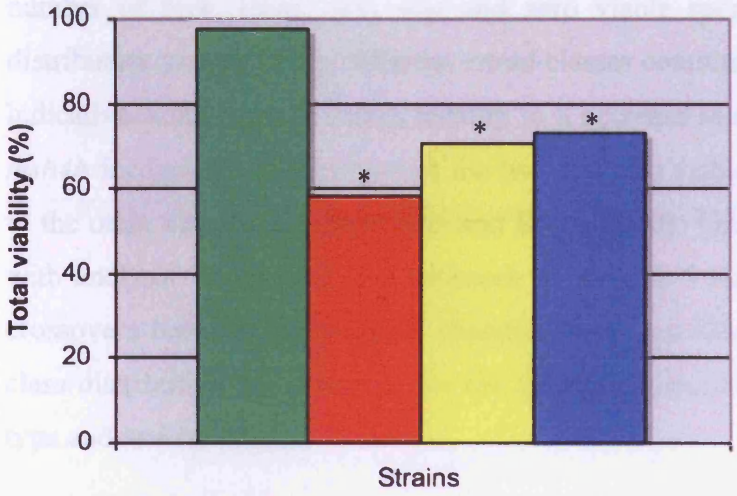


Figure 3.2: Total spore viability is reduced in *SGS1* mutants compared to *SGS1* WT cells.
SGS1; *sgs1Δ*; *sgs1Δ-C1039*; *sgs1Δ-C795*.
* Statistical analysis: Z-tests, $p < 0.01$ compared to WT.

The *SGS1* strain has a spore viability of 97.85% (Figure 3.2), which is similar to that previously reported in Y55 and other wild-type strains such as SK1 and BR (Jessop *et al.*, 2006; Oh *et al.*, 2007; Rockmill *et al.*, 2003; Watt *et al.*, 1995). In the *sgs1Δ* cross, the viability decreased to 58.27% ($p < 0.01$, Z-test). The differences in spore viability between wild type and *SGS1* partial mutants were also analysed. In both partial mutants, *sgs1Δ-C795* (73.2%) and *sgs1Δ-C1039* (70.83%), the viability is increased compared to *sgs1Δ* but not to a SGS1 WT level ($p < 0.01$ compared to *sgs1Δ*, Figure 3.2). Interestingly, the difference between the two partial mutants is not statistically significant ($p > 0.1$). Thus, the N-terminal part of the Sgs1 protein, upstream of amino acid 408, contains a (some) domain(s) with a function necessary for viability. However, the terminal truncation mutants of Sgs1p were unable to restore viability to a *SGS1* WT level. Therefore, the C-terminal part of the Sgs1 protein is also required for spore viability in Y55 strains.

3.3.3. SGS1 mutants have an unusual tetrad class distribution:

The distribution of viable spore tetrads can be monitored by counting the number of four, three, two, one and zero viable spore tetrads for each cross. The distribution pattern of the different tetrad classes combined with genetic analysis can be indicative of the type of defect leading to a decrease in spore viability. As an example, *msh4*Δ increases the proportion of the two and zero viable spore tetrad classes compared to the other classes (Khazanehdari and Borts, 2000). This distribution pattern combined with analysis for disomy is a hallmark of meiosis I non-disjunction due to a lack of crossovers between homologous chromosomes (see Chapter 5 for details). The tetrad class distribution was assessed for the different diploids and compared to that of wild type and *msh4*Δ mutant.

Table 3.3: Tetrad class distribution.

	Number of viable spores per tetrads					
Strains	4 (%)[c]	3 (%)	2 (%)	1 (%)	0 (%)	G-test [b]
SGS1	617 (92.92%)	39 (5.87%)	6 (0.9%)	2 (0.3%)	0 (0%)	n.a
*msh4*Δ[a]	58 (30.5%)	23 (11.85%)	36 (18.65%)	20 (10%)	56 (29%)	<< 0.0125
*sgs1*Δ	253 (20.82%)	323 (26.58%)	329 (27.08%)	193 (15.88%)	117 (9.63%)	<< 0.0125
*sgs1*Δ-*C795*	249 (37.28%)	206 (30.84%)	149 (22.31%)	44 (6.59%)	20 (2.99%)	<< 0.0125
*sgs1*Δ-*C1039*	43 (35.83%)	36 (30%)	24 (20%)	12 (10%)	5 (4.17%)	<< 0.0125

[a] data from Khazanehdari and Borts (2000).
[b] compared to wild type distribution, $p < 0.0125$ (4 comparisons).
[c] percentage for each tetrad class are given in bracket.

The tetrad distributions in mutant strains are different from that of wild type (Table 3.3 – G-test, $p < 0.0125$). The tetrad distribution in *sgs1*Δ is also different from that of *msh4*Δ ($p < 0.01$) suggesting that the source of death in *sgs1*Δ mutant differs from the source of death in *msh4*Δ. While the distributions in both partial mutants are not different from one another (G-test, p value 0.689) they differ from the tetrad distribution

in *sgs1*Δ and wild type (G-test, $p < 0.0125$). The distribution of spore viability in the different tetrad class (Table 3.3) can also be represented by the percentage of viable spores in each class (Figure 3.3).

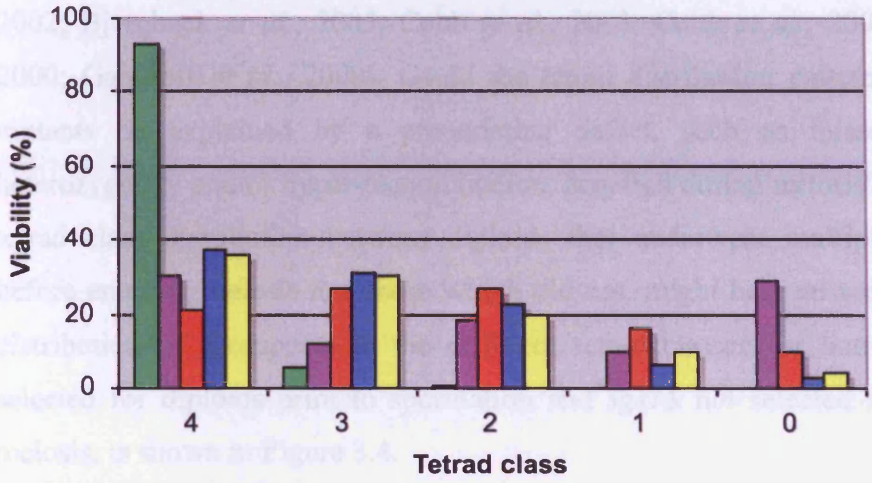


Figure 3.3: Spore viability distribution in *SGS1* mutants compared to *SGS1* WT cells and *msh4*Δ. *SGS1*; *msh4*Δ (Khazanedari and Borts, 2000); *sgs1*Δ; *sgs1*Δ-*C795*; *sgs1*Δ-*C1039*.

The tetrad distributions in the *SGS1* mutants do not match the profile indicative of meiosis I non-disjunction (*msh4*Δ). In *sgs1*Δ, higher proportions of three and two viable spore tetrads are recovered with substantially fewer one and zero viable spore tetrads compared to *msh4*Δ. Although both C-terminal truncation mutants are able to partially restore the *sgs1*Δ defect (higher proportion of four viable spore tetrads and better total viability), they also display this unusual distribution pattern. Recently, the increase in three viable spore tetrads in an *sgs1* mutant has been attributed to an increase in precocious separation of sister chromatids (PSSC) during meiosis I (Rockmill *et al.*, 2006). Such an increase in PSSC was not detected in our strains, neither genetically by mean of non-maters (chromosome III non-disjunction) nor physically by CHEF gel analysis. This phenotype is further discussed in Chapter 4.

3.3.4. Mitotic divisions affect the outcome of meiosis:

As already discussed in the introduction of this thesis, various experiments have shown that Sgs1p plays a role in genomic stability during mitotic divisions (Ajima *et al.*, 2002; Bjergbaek *et al.*, 2005; Cobb *et al.*, 2003; Cobb *et al.*, 2005; Frei and Gasser, 2000; Gangloff *et al.*, 2000). Could the tetrad distribution pattern observed in *SGS1* mutants be explained by a pre-existing defect, such as missegregation, loss of heterozygosity and/or hyper-recombination, acquired during mitosis? The comparison of tetrad class distribution between diploids that underwent multiple mitotic divisions before entering meiosis and those which did not, might help answer this question. The distribution of ascospores in the different tetrad classes for both crosses *i.e.* *sgs1*Δ selected for diploids prior to sporulation and *sgs1*Δ not selected for diploids prior to meiosis, is shown in Figure 3.4.

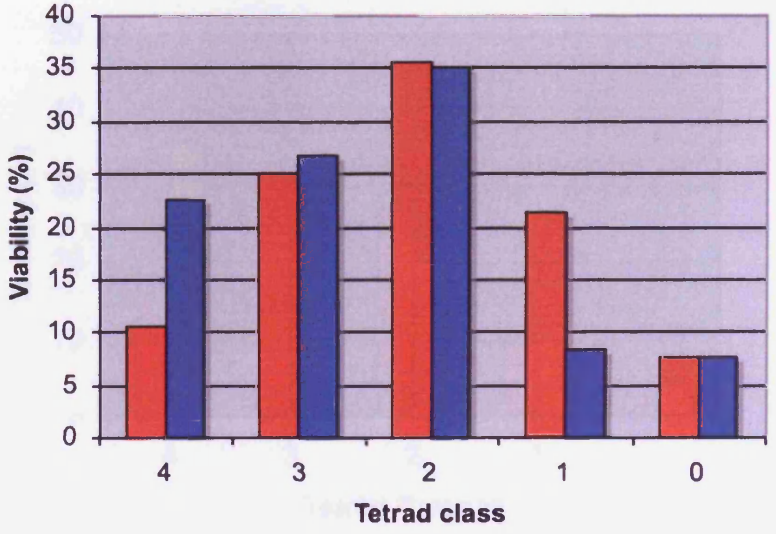


Figure 3.4: The process of selecting for diploids affects meiotic viability in *sgs1*Δ cells. *sgs1*Δ selected for diploids prior to meiosis. *sgs1*Δ not selected for diploids prior to meiosis. Statistical analysis: G-test ($p < 0.05$)

*sgs1*Δ cells, which have been selected for diploids, have a significant decrease of four viable spore tetrads (22.5% to 10.62%) and an increase in one viable spore tetrads (8.34% to 21.25%) after meiosis compared to the unselected diploids. This decrease in four viable spore tetrad and increase in one viable spore tetrad classes is

solely due to an *sgs1*Δ defect as the distribution of viable spore tetrads in the SGS1 WT cells selected for diploid is not statistically different from the unselected SGS1 WT cross (p value = 0.575, data not shown). Cells lacking the Sgs1 protein might accumulate damage, rearrangement and/or chromosome loss prior to meiosis, during the ~50 mitotic divisions occurring while selecting for diploids. Such defects can then been seen after meiosis as a decrease in spores viability.

3.3.5. Sgs1p contains domains essential for mitosis and meiosis:

We have shown previously that *sgs1*Δ strains have decreased sporulation efficiency and decreased spore viability. As described above, cells lacking Sgs1p have a further decrease in viability if they undergo several mitotic divisions prior to entering meiosis. In order to determine if the N-terminal domain is responsible for this phenotype, we compared the distribution of ascospores in the presence or absence of mitotic division prior to meiosis in both *sgs1*Δ-*C1039* and *sgs1*Δ-*C795*.

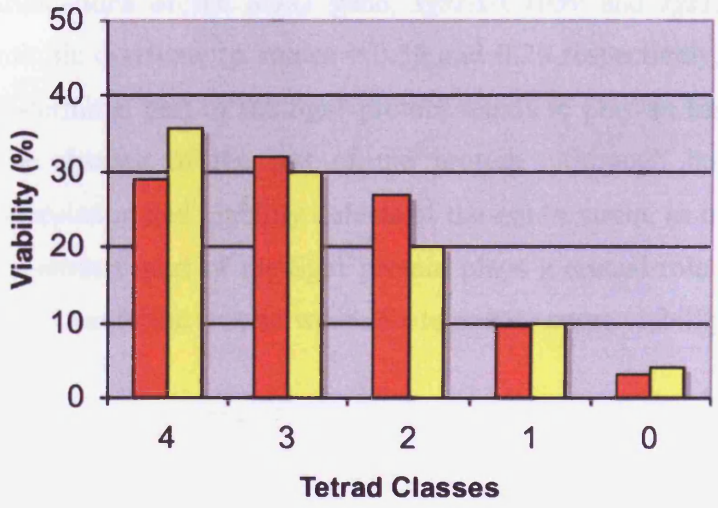


Figure 3.5: Pre-meiotic divisions have no effect on spore viability in the *sgs1*Δ-*C1039* diploids.
*sgs1*Δ-*C1039* selected for diploids prior to meiosis.
*sgs1*Δ-*C1039* not selected for diploids prior to meiosis.

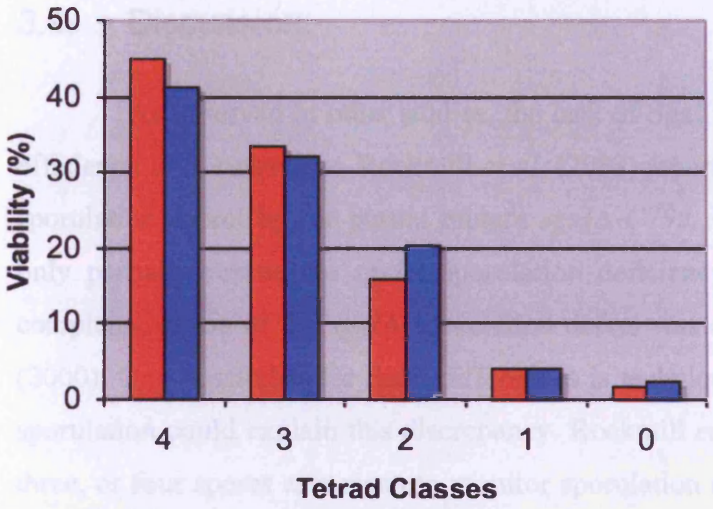


Figure 3.6: Mitotic divisions, prior entering meiosis, have no effect on viability in the *sgs1Δ-C795* diploids. ■ *sgs1Δ-C795* selected for diploids prior to entry in meiosis.
■ *sgs1Δ-C795* not selected for diploids prior to meiosis.

Interestingly, the distributions of ascospores for the two partial C-terminal truncations of the *SGS1* gene, *sgs1Δ-C1039* and *sgs1Δ-C795*, were not affected by mitotic divisions (p values = 0.59 and 0.29 respectively, Figure 3.5 and 3.6). Thus, the N-terminal part of the Sgs1 protein seems to play an important role in mitosis, even in the absence of the rest of the protein. Although both partial mutants restore the sporulation and viability defects of the *sgs1Δ* strain, as mentioned in section § 3.3.2, the C-terminal part of the Sgs1 protein plays a crucial role in meiosis since neither partial deletions of the protein were able to restore spore viability to a *SGS1* WT level.

3.4. Discussion:

As observed in other studies, the lack of Sgs1 protein decreases the sporulation efficiency in *S. cerevisiae.* Rockmill *et al.* (2003) reported a full restoration of the *sgs1*Δ sporulation defect by the partial mutant *sgs1*Δ*-C795.* In our strains *sgs1*Δ*-C795* could only partially rescue the *sgs1*Δ sporulation deficiency. Partial, rather than complete, complementation of the *sgs1*Δ sporulation defect was also observed by Miyajima *et al.* (2000). One possibility for these differences is technical as the method used to monitor sporulation could explain this discrepancy. Rockmill *et al.* counted asci containing two, three, or four spores as a mean to monitor sporulation efficiency while we only counted asci with three and four spores that had completed the meiosis II division. If in a mutant lacking the C-terminal 795 amino acid of the Sgs1 protein, a substantial fraction of cells cannot complete meiosis II and are arrested after meiosis I, monitoring sporulation by including asci containing two spores will appear to increase sporulation efficiency. As mentioned in the introduction to this chapter, *sgs1*Δ diploids have an increased loss of heterozygosity, preferentially by mean of chromosomes loss (either linked with crossovers and/or gene conversion) (Ajima *et al.*, 2002; Watt *et al.*, 1995). Mitotic chromosome loss has been associated with decreased sporulation efficiency (Baker *et al.*, 1976). Thus, loss of a chromosome in *SGS1* mutants prior to entering meiosis might lead to an increase in asci with two spores. Counting ascospores with four and three spores may therefore be a more accurate way of estimating the sporulation efficiency in *SGS1* mutant strains.

The difference in sporulation efficiency in *sgs1* cells could also be dependent on the differences between the various strains used. Indeed, a recent study analysing sporulation efficiency between various strains of *S. cerevisiae* such as SK1 and S288C, has shown that sporulation is dependent upon a combination of different polymorphic genes in the various strains (Ben-Ari *et al.*, 2006; Deutschbauer and Davis, 2005). Such polymorphism could also account for the variation in sporulation efficiency between the Y55 and SK1 mutants. In addition, *sgs1*Δ cells have been shown to arrest in pachytene via a checkpoint different from the meiotic recombination checkpoint (§ 3.1.2) (Gangloff *et al.*, 1999; Miyajima *et al.*, 2000a; Rockmill *et al.*, 2003). As seen in the introduction of this thesis, Sgs1p plays a role in the intra S-phase checkpoint in mitosis (Frei and Gasser, 2000). Lydall *et al.* (1996) have shown that proteins involved in the mitotic S-

phase checkpoint could also control meiotic progression. Both arrests, in mitosis and in meiosis, might be triggered by the same mechanistic events such as stalled replication forks. Rockmill *et al.* (2003) have also suggested this idea whereby *sgs1*Δ mutant might accumulate stalled forks, which may trigger a meiotic S-phase checkpoint that prevents meiotic progression. The robustness of this checkpoint might also differ depending on the strain background and could account for the discrepancies between the various strains studied.

Thirdly, the partial deletion *sgs1*Δ*-C795* made by us is an endogenous deletion of the 795 amino acids of the Sgs1 protein while Rockmill *et al.* (2003) intergrated the *sgs1*Δ*-C795* allele at a *LEU2* or *TRP1* locus in an *sgs1*Δ strain. Therefore, the exogenous expression of the *sgs1*Δ*-C795* in the Rockmill experiement could account for the difference in sporulation efficiency if the expression of the partial mutant was up-regulated at the *LEU2* or *TRP1* loci.

The decrease of viability in the different *SGS1* mutants could be attributable to both mitotic and meiotic specific defects. The difference in viability between *sgs1*Δ and both *sgs1*Δ*-C795* and *sgs1*Δ*-C1039* is mainly due to a mitotic defect of *sgs1*Δ, as shown by the greater decrease in viability in *sgs1*Δ diploids having completed approximatively 50 mitotic divisions before entering meiosis compared to *sgs1*Δ diploids that have not (§ 3.3.4). This increased reduction in spore viability could not be detected in the partial mutant of the *SGS1* gene (§ 3.3.5). The mitosis-specific defect of *sgs1*Δ has also been shown by Rockmill *et al.* (2003) in experiments described as follow. *SPO13* is required for successful completion of meiosis I by preventing the degradation of cohesin (Lee *et al.*, 2002). One of the functions of Spo13p is therefore to hold sister chromatids together until anaphase II (Wang *et al.*, 1987). Meiotic division in *spo13* diploids results in a single meiosis II-like segregation of chromatids, producing dyad asci containing (2n) chromosomes (McCarroll and Esposito, 1994). *SPO13* mutants can therefore rescue the meiotic lethality of early-stage meiotic recombination-deficient mutants. For example, *spo11*Δ, *mer2*Δ and *mre11*Δ do not produce viable spore colonies due to their inability to either form (*spo11*Δ) or subsequently repair (*mer2*Δ, *mre11*Δ) DSBs (Keeney *et al.*, 1997; Li *et al.*, 2006a; Moreau *et al.*, 1999). The by-pass of meiosis I in *SPO13* mutant is able to rescue the lethality associated with *spo11*Δ, *mer2*Δ or *mre11*Δ since the formation and repair of DSBs in no longer a prerequisite for completion of meiosis.

Rockmill *et al.* (2003) analysed the spore viability of the triple mutants *sgs1Δ mer2Δ spo13Δ* and *sgs1Δ-C795 mer2Δ spo13Δ* and compared them to *mer2Δ spo13Δ*. The *sgs1Δ* triple mutant had a decreased spore viability compared to *mer2Δ spo13Δ* while the *sgs1Δ-C795* triple mutant did not. Thus, Rockmill *et al.* inferred that the decreased spore viability in *sgs1Δ* was due to a mitotic defect in the absence of Sgs1 protein. Our result confirms theirs whereby mitotic divisions in *sgs1Δ* diploids further reduce the spore viability in meiosis. This defect is dependent on the absence of the N-terminal part of the Sgs1 protein, as it is not observed in either of the two partial mutants. Therefore, the Sgs1 protein might contain domains, in the N-terminal part of the protein that are essential for mitotic functions. In light of previous studies, such essential domain could be the Top3 interacting domain of the Sgs1 protein. This domain of the Sgs1 protein has been mapped to the N-terminal part of the helicase (Bjergbaek *et al.*, 2005; Miyajima *et al.*, 2000a; Miyajima *et al.*, 2000b; Mullen *et al.*, 2000; Onodera *et al.*, 2002; Ui *et al.*, 2001). Thus, the Top3 interacting domain of Sgs1p is included in the partial mutants *sgs1Δ-C795* and *sgs1Δ-C1039*. As discussed in Chapter 1 § 1.3.2, the stabilisation of the polymerases Polα and Polε requires Sgs1p and also Top3p (Bjergbaek *et al.*, 2005). Therefore, the better spore viability of *SGS1* partial mutants compared to the full deletion of the gene could be due to the stabilisation of the replisome during mitotic S-phase.

A specific meiotic defect in *SGS1* mutants also contributes to the decrease in spore viability. Since the C-terminal truncations *sgs1Δ-C795* and *sgs1Δ-C1039* cannot complement the sporulation and viability defects of *sgs1Δ* to a SGS1 WT level, the C-terminal part of the Sgs1 protein must play a part during the meiotic division. Therefore, the helicase domain contained between amino acids 698 and 995 could play an essential role during meiotic division. The decreased viability in *sgs1Δ*, *sgs1Δ-C795* and *sgs1Δ-C1039* might not be attributable to a classical meiosis I non-disjunction phenotype as their spore viability distributions differ from that of *msh4Δ* (Khazanehdari and Borts, 2000). As mentioned previously, *sgs1Δ-C795* did not affect the viability of the double mutant *mer2Δ spo13Δ* (Rockmill *et al.*, 2003). This result could be interpreted as *sgs1Δ-C795* being epistatic to *mer2Δ*. More specifically, the Sgs1 protein might require initiation of recombination to play a role during meiosis. Rockmill *et al.* have therefore argued that *sgs1Δ-C795* reduces spore viability only in diploids proficient in meiotic recombination *i.e.* the death associated with *sgs1Δ-C795* is due to a specialised meiotic

function. Gangloff *et al.* (1999) have also studied *sgs1*Δ and *top3*Δ mutants in conjunction with *spo11*Δ *spo13*Δ. They also concluded that, since both triple mutants *sgs1*Δ *spo11*Δ *spo13*Δ and *top3*Δ *spo11*Δ *spo13*Δ sporulated and created viable spores, the meiotic defect in *sgs1*Δ and *top3*Δ was associated with meiotic recombination (Gangloff *et al.*, 1999). As seen in the introduction of this thesis (§ 1.4), Sgs1p reduces recombination in mitosis by collapsing recombination intermediates into hemicatenates (Liberi *et al.*, 2005), which are themselves dissolved by Top3p (§ 1.4.2) (Mankouri and Hickson, 2006). The combined activities of Sgs1p and Top3p offer an alternative to the resolution of mitotic crossover by endonucleases, by dissolving Holliday junction-like structures to form non-crossover products. These activities of both Sgs1p and Top3p could be conserved during the meiotic division to dissolve recombination intermediates. *In vitro* studies on the human orthologue BLM protein have shown that this human RecQ helicase in conjunction with the topoisomerase I, Top3α, can dissolve double Holliday junctions (Wu and Hickson, 2003). If this function is conserved in the *S. cerevisiae* RecQ protein homologue, Sgs1p, it would contribute toward explaining the deficit in spore viability in partial mutants of the Sgs1 gene. This aspect of the reduced viability of *sgs1* in meiosis will be further discussed in Chapter 6.

Chapter 4: Meiotic recombination in *SGS1* mutants.

4.1. Introduction:

Meiosis is the process by which diploid cells give rise to four haploid gametes (spores). The meiotic division is different from mitosis as two rounds of cellular divisions follow a single round of DNA replication (Figure 4.1 A – B). The genetic material is therefore reduced from a diploid cell of 2n = 4x chromosomes into four haploid gametes (spores) of 1n = 1x chromosomes (Figure 4.1). The first division, or meiosis I, sees a reduction of the number of chromosomes as homologues segregate to the opposite poles of the meiotic spindle (Figure 4.1 C – D). Many interactions and regulatory events take place in prophase of meiosis I. Therefore, this stage is divided into four sub-stages, Leptotene, Pachytene, Zygotene and Diplotene (Table 4.1).

Table 4.1: Time course of meiosis in *Saccharomyces cerevisiae*.

Stage:	**DNA rep.**	**Pre-leptotene**	**Leptotene**	**Zygotene**	**Pachytene**	**Diplotene**
"DNA"		Pairing	DSBs with single end invasions 15-30'	→	dHJs ≥ 40' →	CR, NCR
EM			Axial Element	Axial Element + Synaptonemal Complex forming	SC full Length	Stretched SCs
LM	Diffuse chromatin	Compact Nucleoid	Thin Lines	Thick Lines > Knot > Bouquet	Sausages	Stretched chromosomes
Time (hrs)	1	2	3	4	5	6

Note: "DNA" events include interstitial pairing interactions and recombination; EM and LM events are those detected by electron (EM) and light microscopy (LM). DNA rep: DNA replication. SC: synaptonemal complex, dHJs: double Holliday Junctions; CR: crossovers; NCR: non-crossovers; ': minutes. Table redrawn from Kleckner (1996).

The second division or meiosis II sees the segregation of sister chromatids to opposite poles (Figure 4.1 E – F) and, in that respect, is similar to a mitotic division. In order for the first meiotic division to segregate chromosomes appropriately, the homologous pairs of chromosomes tightly pair and interact during prophase. This pairing, starting during the pre-leptoptene stage of meiotic prophase I, takes place just after DNA synthesis until the diplotene stage (Table 4.1). The alignment of homologues on the equator of the meiotic spindle, followed by their accurate segregation to opposite poles, is made possible by counteracting forces. Forces pulling toward the poles of the cell are created by the microtubules anchored on kinetochores while resisting forces, created by chiasmata (physical manifestation of crossovers) between homologues, are responsible for holding homologous chromosomes together (Figure 4.1 C). During the first division, (reductional division) the sister chromatids remain attached together (Figure 4.1 D) by cohesin complex protected from degradation by the shugoshin protein (see § 4.1.4 for details). It is only at the second division (equational) that the sister chromatids segregate from one another (Figure 4.1 F). The segregation of homologues in meiosis I, followed by the segregation of sister chromatids in meiosis II, are crucial events for the outcome of meiosis and such aspects will be discussed later in this chapter.

4.1.1. Homologous pairing and the synaptonemal complex:

Meiotic homologous chromosomes need to be tightly associated until the end of the pachytene stage (prophase of meiosis I). It is believed that interaction between homologous chromosomes starts to take place as early as pre-meiotic S-phase (Kleckner and Weiner, 1993). Evidence for such early interactions also comes from the extended length of the meiotic S-phase compared to DNA replication during mitosis. The establishment of specialized chromosome features needed for later pairing has been proposed as an explanation of this time difference (Holm, 1977). Furthermore, proteins, involved in interactions between homologous chromosomes (Spo11p) and inter-sister interactions (Rec8p), have been shown to regulate the progression of meiotic DNA replication (Cha *et al.*, 2000). Such interactions might facilitate the presynaptic alignment of homologues, then their pairing and finally synaptonemal complex formation.

The full synapsis of homologous chromosomes is achieved with the formation of a tripartite zipper-like protein structure, the synaptonemal complex. The synaptonemal complex connects homologous chromosomes along their entire length (Figure 4.2 A).

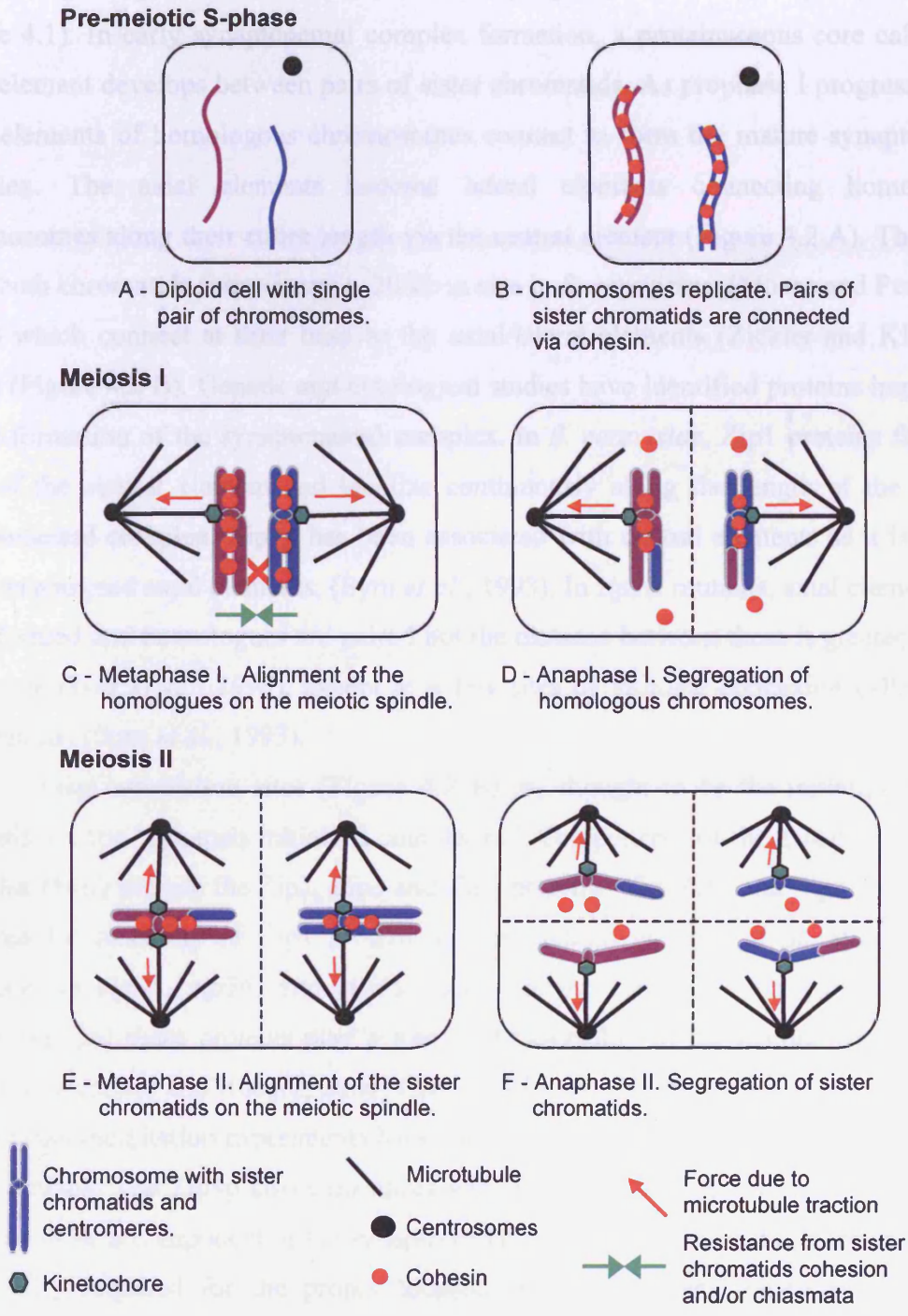


Figure 4.1: Overview of meiosis. A - B: Pre-meiotic S-phase, sister chromatids are connected via cohesin complex along their entire length. C: Crossovers ensure the proper orientation of homologous chromosomes on the meiotic spindle by physically connecting homologues together. D: During segregation of homologues centromere proximal cohesion ensures that sister chromatids remain attached together. E -F: Equational mitosis-like division. Cohesin complexes are relived from sister chromatids at anaphase II.

Its synthesis takes place throughout the first meiotic prophase, starting at the leptoptene stage after pairing of the homologues, with full synapsis achieved by the pachytene stage (Table 4.1). In early synaptonemal complex formation, a proteinaceous core called the axial element develops between pairs of sister chromatids. As prophase I progresses, the axial elements of homologous chromosomes connect to form the mature synaptonemal complex. The axial elements become lateral elements connecting homologous chromosomes along their entire length via the central element (Figure 4.2 A). The DNA from both chromatids forms loops (~20 kb in size in *S. cerevisiae,* (Moens and Pearlman, 1988) which connect at their base to the axial/lateral elements (Zickler and Kleckner, 1999) (Figure 4.2 B). Genetic and cytological studies have identified proteins implicated in the formation of the synaptonemal complex. In *S. cerevisiae*, Zip1 proteins form the core of the central element and localize continuously along the length of the mature synaptonemal complex. Zip1p has been associated with central elements as it is absent from unsynapsed axial elements, (Sym *et al.*, 1993). In *zip1*Δ mutants, axial elements are fully formed and homologues are paired but the distance between them is greater than in wild type (Nag *et al.*, 1995), except at a few sites of intimate connexion called axial associations (Sym *et al.*, 1993).

Axial association sites (Figure 4.2 B) are thought to be the initiation sites of synapsis via the "synapsis initiation complexes". Components of the synapsis initiation complex (SIC) include the Zip2, Zip3 and Zip4 proteins. The Zip2 and Zip3 proteins are required for assembly of Zip1 protein into the central element of the synaptonemal complex. In *zip2*Δ, *zip3*Δ, and *zip4*Δ single mutants timing of synapsis is delayed suggesting that these proteins play a role in the assembly of the mature synaptonemal complex (Agarwal and Roeder, 2000; Chua and Roeder, 1998; Tsubouchi *et al.*, 2006). Coimmunoprecipitation experiments have shown that Zip3p interacts with both Zip1 and Zip2 proteins. The Zip4p also colocalises with Zip2 and Zip3 proteins and is therefore believed to be a component of the synaptonemal initiation complex. Furthermore, Zip3p is partially required for the proper localisation of Zip2p and Zip4p as shown by chromosomes spreads and immunostaining while Zip2p and Zip4p localisation are dependent of each other (Agarwal and Roeder, 2000; Tsubouchi *et al.*, 2006). These results suggest that Zip2 and Zip4 proteins function in the same complex and that Zip3p is required for the loading of this complex on chromosomes. In *zip4*Δ mutants, Red1p

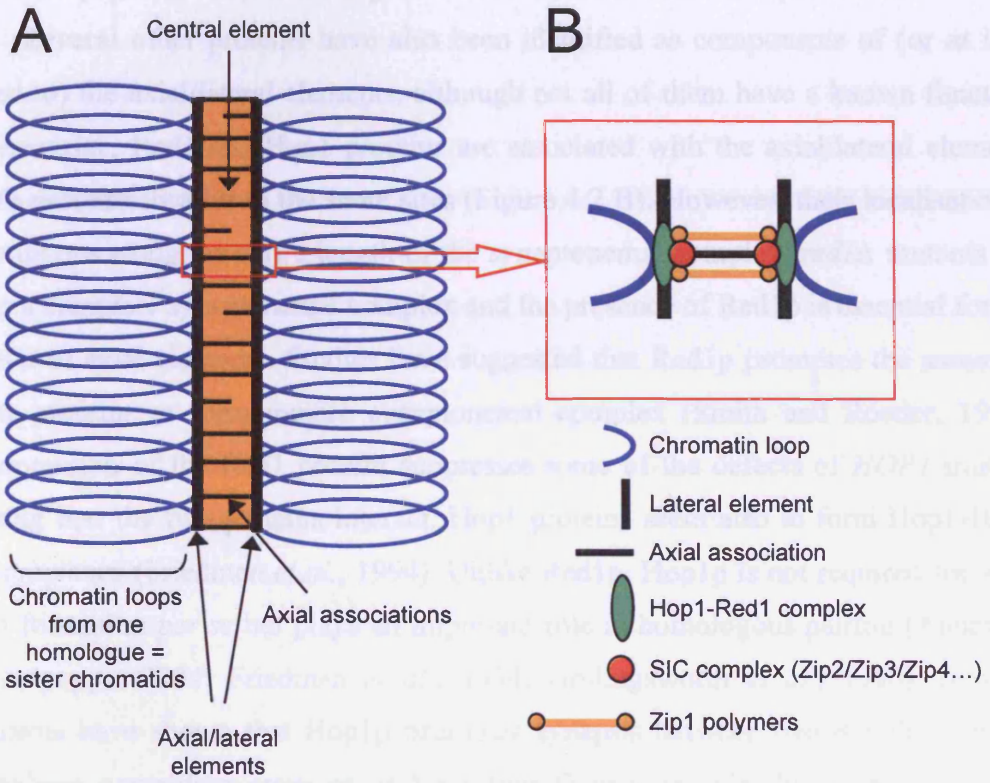


Figure 4.2: A: Schematic representation of the mature synaptonemal complex between two homologous chromosomes. The axial elements from sister chromatids develop in lateral elements, then lateral elements pair and homologous chromosomes are joined via the central element.
B: details of the axial association interaction. Polymerisation of the central element (Zip1 polymers) starts at the synaptonemal initiation complex (SIC) sites in presence of Hop1-Red1 proteins complex and SIC complex.

and Zip1p initially localise as in wild-type cells but the meiotic progression is delayed (Tsubouchi *et al.*, 2006). In *zip2Δ* (Chua and Roeder, 1998), as in *zip4Δ* (Tsubouchi *et al.*, 2006), Zip1p fails to spread along the central element of the synaptonemal.

Several other proteins have also been identified as components of (or at least localised to) the axial/lateral elements, although not all of them have a known function. In *S. cerevisiae*, Red1 and Hop1 proteins are associated with the axial/lateral elements and both proteins localise to the same sites (Figure 4.2 B). However, their localisation is not continuous along the entire length of the synaptonemal complex. *red1Δ* mutants fail to make a complete synaptonemal complex and the presence of Red1p is essential for the formation of axial elements. Studies have suggested that Red1p promotes the assembly of other proteins to form mature synaptonemal complex (Smith and Roeder, 1997). Over-expression of the Red1 protein suppresses some of the defects of *HOP1* mutant, suggesting that the two proteins interact. Hop1 proteins seem also to form Hop1-Hop1 multi-complexes (Friedman *et al.*, 1994). Unlike Red1p, Hop1p is not required for axial element formation *per se* but plays an important role in homologous pairing (Anuradha and Muniyappa, 2004; Friedman *et al.*, 1994; Hollingsworth *et al.*, 1990). *In vitro* experiments have shown that Hop1p promotes synapsis between two double stranded DNA helices containing array of at least four G residues via the formation of G-quadruplets (Anuradha and Muniyappa, 2004). Red1p dissociates from chromosomes as the synaptonemal complex disassembles in the diplotene stage, while Hop1p departs from chromosomes as axial elements become incorporated into mature synaptonemal complex (Smith and Roeder, 1997). This departure of Hop1p from the mature synaptonemal complex is consistent with an earlier function in homologous pairing. Mek1p, a meiotic specific serine/threonine DNA-damage kinase Rad53p paralog has also been shown to genetically act in the same *RED1-HOP1* pathway (Rockmill and Roeder, 1991). In Mek1 mutants, the synaptonemal complex stretches appear shorter than in wild type. Furthermore, in cells deficient for Mek1p, interhomologue recombination is reduced while intrachromosomal crossovers are not affected (Rockmill and Roeder, 1991). Therefore, apart from their function in the assembly of the synaptonemal complex, *MEK1*, *RED1* and *HOP1* are also involved in driving recombination intermediates toward homologous chromosomes in meiosis instead of sister chromatids (see § 5.1.2 for details).

The Rec8 and Smc3 proteins, part of the cohesin complex (see § 4.1.4 for details), are also required for proper synaptonemal complex formation. The deletion mutants, *rec8*Δ and *smc3*Δ fail to elongate Zip1 polymers but make normal axial elements. The localisation of both Rec8 and Smc3 proteins is more continuous along the axial/lateral element of the synaptonemal complex compared to that of Red1p (Klein *et al.*, 1999) suggesting that those proteins might be involved in the loading and extension of Zip1p polymers.

The idea that the synaptonemal complex is a prerequisite that allows homologous chromosomes to recombine has changed in recent years. In the absence of a mature synaptonemal complex, recombination still takes place although at a lower rate. For example, return to growth assays are used to monitor the commitment to homologous recombination in *zip1*Δ mutants. This technique allows for the detection of crossovers in mutants that do not sporulate. Meiosis is induced on sporulation medium but interrupted by plating on synthetic complete medium before chromosomes segregate (*i.e.* return to growth medium). Thus, crossovers induced at prophase of meiosis I can be detected in mitotic diploid cells by dissecting dyads or by random spores analysis. In *zip1*Δ, crossovers are reduced nearly 2-fold compared to wild-type cells (Sym *et al.*, 1993). In *zip2*Δ and *zip3*Δ mutants, reduced frequencies of crossovers are also detected using the same technique (Agarwal and Roeder, 2000; Chua and Roeder, 1998). Since recombination is not abolished in mutants defective for synaptonemal complex formation, the idea that the synaptonemal complex was required for homologous recombination was discredited. Furthermore, nucleofilament proteins Rad51p and Dmc1p are required to establish axial associations. In *rad51*Δ or *dmc1*Δ diploids, synapsis is delayed while homologue pairing is reduced (Rockmill *et al.*, 1995). *In vivo* meiotic-specific association between allelic loci, using *Cre/Lox* specific recombination experiments, have shown that the juxtaposition of homologous chromosomes seems to be dependent on meiotic recombination and does not require synapsis *per se* (Peoples *et al.*, 2002). Synapsis may play a role in facilitating interaction between homologues or at least stabilizing those interactions. In yeast, cytological and genetic experiments have shown that the initiation of recombination events is unaffected by the absence of synapsis. Instead, the formation of mature synaptonemal complex in *S. cerevisiae* initiates at sites of meiotic recombination events (Review in Roeder, 1997 and Bishop and Zickler, 2004).

In *S. cerevisiae, in situ* fluorescence experiments have shown that Sgs1p co-localizes with Zip3p at sites of synapsis initiation during the pachytene stage of meiosis I (*i.e.* at SIC sites). In *sgs1*Δ cells, chromosomes undergo full synapsis but the number of Zip3p foci, as monitored by immunostaining, are increased (Rockmill *et al.*, 2003). This increase in Zip3p foci probably reflects an increase in synaptonemal initiation sites. As mentioned earlier, chromosomes are unable to fully synapse in the absence of Zip1 protein. Surprisingly, in the double mutant *zip1*Δ *sgs1*Δ, the homologous chromosomes seem to be fully synapsed although Zip1p is absent. Rockmill *et al.* (2003) have referred to this association as pseudo-synapsis. Epitope tagged Sgs1 protein and immunostaining experiments have shown that Sgs1p localises to synaptonemal initiation complex sites but at a later stage than the Zip2 and Zip3 proteins (Rockmill *et al.*, 2003). Since Zip2 and Zip3 proteins localise at synaptonemal initiation sites, which might become sites for homologous recombination, the localisation of Sgs1p with the former proteins might reflect an early function of this helicase in homologous recombination.

4.1.2. Relationship between Double Strand Breaks and crossing over:

In contrast to mitosis, where DNA lesions are repaired using sister chromatids recombination (Symington, 2002), meiotic recombination primarily takes place between homologous chromosomes. As will be discussed in Chapter 5, such a difference in the choice of recombination partner is crucial for the outcome of the meiosis I division.

Recombination events initiate at double strand breaks (DSBs) sites in budding yeast. Such events are catalysed by the highly conserved topoisomerase like protein, Spo11 (Keeney, 2001). The double Holliday junction (dHJ) repair model (Szostak *et al.*, 1983) and the synthesis-dependent strand-annealing model (SDSA) are now accepted as the major repair mechanisms for meiotic DSBs (described in Chapter 1 and Figure 1.11). A given DSB can be repaired via a double Holliday junction route, creating a meiotic recombination event where strands are exchanged between the two interacting molecules with respect to proximal DNA markers (*i.e.* crossovers, Figure 1.11 D-F). The second possibility is via a non-crossover route without exchange of flanking markers (Figure 1.11 G-I). The later events play very little or no part in the correct orientation of homologous chromosomes during Meiosis I. It is the crossovers (seen as chiasmata), as discussed previously, that are essential for the outcome of meiosis I since they physically connect homologues to one another. Crossover events are therefore non-randomly

distributed between chromosomes such that each chromosome gets at least one crossover. This phenomenon is known as the "obligate chiasmata" (Jones, 1984; Jones and Franklin, 2006; Martini *et al.*, 2006; Stahl *et al.*, 2004). The distribution of crossovers along the chromosome is also regulated by a mechanism called crossover interference whereby a crossover occurring in one genetic interval reduces the likelihood of another crossover occurring in a proximal interval (Bishop and Zickler, 2004; Khazanehdari and Borts, 2000; Martini *et al.*, 2006; Muller, 1916; Novak *et al.*, 2001; Stahl *et al.*, 2004; Sym and Roeder, 1994; Tsubouchi *et al.*, 2006). The strength of this interference mechanism is inversely proportional to the distance from the crossover. The number of initiating events, as manifested by DSBs are greater in budding yeast than the number of crossovers. The majority of DSBs are therefore processed via a non-crossover pathway. The decision to create a crossover rather than a non-crossover seems to occur early during meiosis (Leptoptene/zygotene stage) (Allers and Lichten, 2001b; Borner *et al.*, 2004; Martini *et al.*, 2006) rather than late, as it was previously suggested by the dHJ repair model (Gilbertson and Stahl, 1996; Szostak *et al.*, 1983). Interestingly, in an *S. cerevisiae* strain where the numbers of DSBs are reduced (via hypomorphic mutations in Spo11p), the frequency of crossovers is not proportionally decreased. This phenotype, referred to as crossover homeostasis is at the expense of the number of non-crossovers (Martini *et al.*, 2006).

Several genes, orthologues to the mismatch repair genes *MutS* and *MutL* of *E. coli* (Chapter 1), have been implicated in meiotic recombination. *MSH4*, *MSH5*, *MLH1* and *MLH3* (review in Hoffmann and Borts, 2004; Surtees *et al.*, 2004) are involved in the stabilisation of single end invasions and joint molecules prior to the establishment of double Holliday junctions. They are therefore believed to play a role in the crossover pathway. In *S. cerevisiae msh4*Δ and *msh5*Δ show a decrease in crossovers while gene conversions are not affected (Hollingsworth *et al.*, 1995; Khazanehdari and Borts, 2000; Stone and Petes, 2006). *mlh1*Δ and *mlh3*Δ also affect the rate of recombination but to a lesser degree (Abdullah *et al.*, 2004; Hunter and Borts, 1997; Wang *et al.*, 1999). It is therefore believed that the heterodimer *MSH4/MSH5* acts prior to the *MLH1/MLH3* complex during meiotic prophase I (for review see Hoffmann and Borts, 2004). Wang *et al.* (2002) showed that Sgs1p co-immunoprecipitated with Mlh3p. They hypothesised that Sgs1p and Top3p are part of a supermolecular complex with Mlh1p/Mlh3 playing a

role during meiotic recombination. Different studies on *sgs1Δ* mutant have shown a slight increased in meiotic crossover in *S. cerevisiae* SK1 and BR strains (Jessop *et al.*, 2006; Rockmill *et al.*, 2003; Rockmill *et al.*, 2006) but no change in the proportion of non-crossover events was observed. Jessop *et al.* (2006) also showed that in *msh4Δ* strains (SK1 or BR background), mutants for either *sgs1Δ-C795* or *sgs1Δ* could restore the crossover defect of a *MSH4* deletion to a nearly wild-type level. In similar experiments, Oh *et al.* (2007) have shown that *sgs1Δ-C795* could also rescue the deficit in crossover in *msh5Δ* and *mlh3Δ* mutants to almost wild-type level. These results indicate that the helicase activity of the Sgs1 protein might antagonise the effect of pro-crossover proteins.

4.1.3. Crossover interference is linked to axial association sites:

The idea that crossovers are not evenly distributed along chromosomes length is not new (Muller, 1916; Whitehouse, 1942) but little is known about the mechanism that could account for such distribution. Recent studies on different *ZIP* and mismatch repair mutants, along with the formation of the synaptonemal complex, have shed some light on the mechanisms accountable for crossover interference. As mentioned earlier, mutants for *ZIP1*, *ZIP2*, *ZIP3*, *ZIP4* and *MSH4* genes, have a decrease in crossovers, are delayed in meiotic progression and have a defect in synaptonemal complex formation. Such mutants also exhibit defects in interference thereby allowing crossovers to take place close to each other (Novak *et al.*, 2001; Sym and Roeder, 1994; Tsubouchi *et al.*, 2006). The synaptonemal complex might therefore play a role in the establishment of crossover interference.

Two different models have been proposed to account for crossover interference. One model uses a mechanical approach where the occurrence of a crossover releases the mechanical stress intrinsic to the DNA loops in sister chromatids and therefore diminishes the probability of another crossover occurring nearby (Kleckner *et al.*, 2004). The other model, based on a more mathematical approach, hypothesises that interfering crossovers are spaced by a fixed number of non-crossovers (Stahl *et al.*, 2004). In light of the crossover homeostasis model explained above the first hypothesis seems more likely since it account for both crossover interference and homeostasis (Martini *et al.*, 2006). The idea that crossover interference relies on mature synaptonemal complex, although appealing, might not be entirely correct. Fung *et al.* (2004) have shown that

Zip2 foci (part of the SIC complexes) display cytological interference on surface spread chromosome stained with anti-Zip2p antibodies. Although *zip1*Δ and *msh4*Δ mutants don't display genetic interference (Novak *et al.*, 2001; Sym and Roeder, 1994), they do not alter distribution of the SIC complexes (i.e. they do not reduce interference between SICs) (Fung *et al.*, 2004). Interestingly, mutation in *SGS1* showed an increase in the number of Zip3 foci (Rockmill *et al.*, 2003) consistent with an increase in crossover frequency in the strain studied. This increase in Zip3 foci might be attributable to an increase in axial associations stabilised in absence of Sgs1p.

4.1.4. Hang-on to your sister:

During the first meiotic division, sister chromatids have to remain attached to one another and will only segregate during the second meiotic division. Thus, the sister chromatids must remain linked beyond meiosis I until the anaphase of meiosis II (Figure 4.3). Such cohesion of sister chromatids is regulated via a multi-subunit protein complex, cohesin. Meiotic cohesin complexes contain at least four proteins: Smc1p, Smc3p, Rec8p (the meiotic specific homologue of Scc1p) and Scc3p. Deletions of either *SMC3* or *REC8* genes increase precocious separation of sister chromatids at meiosis I, suggesting that both proteins are involved in sister chromatid cohesion (Klein *et al.*, 1999). The cohesion between sister chromatids starts to take place during pre-meiotic S-phase (Figure 4.1 B) where both DNA molecules are still close to one another (Uhlmann and Nasmyth, 1998). Rec8p localises along the entire length of the chromosomes arms until the first meiotic division. It is then removed from the chromosomes arms during anaphase I (Figure 4.3 A) but persists at the centromeres (Klein *et al.*, 1999). The cleavage of Rec8p is ensured by a specialized endopeptidase Esp1p or separin (Figure 4.3 A and Table 4.2), which is also responsible for the cleavage of cohesin complex in mitosis. The cleavage of Rec8p from the chromosome arms at anaphase I is essential for chiasmata resolution and the proper segregation of homologous chromosomes (Buonomo *et al.*, 2000). To ensure proper alignment of sister chromatids during the meiosis II division, and their accurate segregation, sufficient cohesin must remain at the centromeres (Figure 4.3 B). The dissociation of cohesin complexes from the chromosomes during meiosis is therefore done in a stepwise manner. Spo13p, Cdc20p, Cdc5p, the polyubiquinated regulated protein separin, Esp1p and securin Pds1p, regulate the dissociation of cohesin (Table 4.2). The cleavage of Rec8p is done by the separin

Esp1p in *S. cerevisiae* (Figure 4.3 and Table 4.2). Esp1p is itself regulated by securing Pds1p, which prevents Esp1p from cleaving Rec8p (Table 4.2). The inactivation of Pds1p (and therefore the activation of Esp1p) is regulated by Cdc20p (Salah and Nasmyth, 2000). During anaphase I, the cohesin that remains around the centromere region is protected from cleavage by the shugoshin protein, Sgo1p (Table 4.2). The amount of cohesin remaining is sufficient to ensure sister chromatid cohesion (Figure 4.3).

Table 4.2: Sister chromatids cohesion pathway and regulation.

Proteins	Functions	Regulated by	Degradation
Rec8p	Sister chromatids cohesion	Esp1p, Cdc5p, Spo13p	Anaphase I & II
Esp1p	Cleaves Rec8p	Pds1p	Constitutive
Pds1p	Esp1p inhibitor	Ubiquitination Cdc20-dependent	Anaphase I & II
Sgo1	Protection of Rec8p	Cdc5p and Spo13p	Anaphase II
Spo13p	Prevent degradation of Sgo1 + Protection of Rec8p	Unknown	Anaphase I

During Meiosis I, the sister kinetochores attach to microtubules issued from the same pole of the cell (same centrosome) in a syntelic attachment (kinetochores of both chromatids are attached toward the same pole). The homologous chromosomes can therefore segregate from each other to opposite poles of the cell while the sister chromatids remain attached together. At prophase II the sister chromatids still interact with each other due to the cohesin complex. The microtubule machinery reattach to the kinetochores, in an amphitelic manner (kinetochores of both chromatids are attached to microtubules in opposite direction). During the prophase II-metaphase II transition, the chromosomes, no longer in pairs, move to the equator of the meiotic spindle due to the resulting forces between the cohesin complexes (still present at the centromeres) and the microtubules. The cohesion between sister chromatids persists until anaphase II where the sister chromatids are pulled apart from one another. The cohesion complex is then disrupted in early anaphase II and the sister chromatids are pulled apart to each pole of the cell (Figure 4.3).

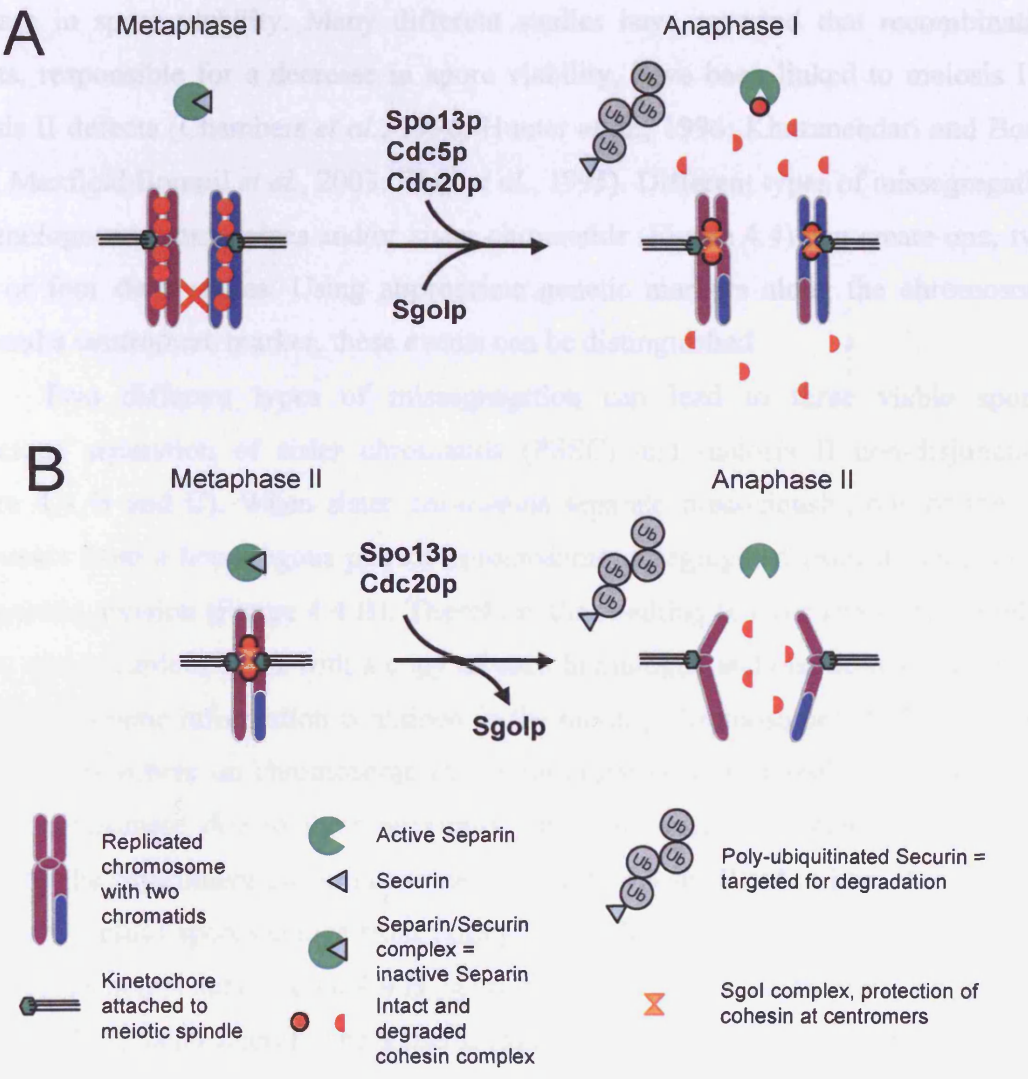


Figure 4.3: Stepwise degradation of Rec8p (cohesin) during meiosis. A: During metaphase I, Separin is render inactive via interaction with Securin. At anaphase I, Securin is targeted for degradation by polyubiquitination via Cdc20p. The cohesin is first degraded along chromosome arms during anaphase I by active Separin while it remains protected around centromers by Sgolp and Cdc5p. The sister chromatids remain attached together. B: At anaphase II the remaining cohesin is removed from centromeric region at anaphase II via degradation of Sgolp and cleavage of Rec8p by active Separin. The sister chromatids are then pull apart by the meiotic spindle.

4.1.5. Missegregation in meiosis:

Failure to properly complete either of the two meiotic divisions leads to a decrease in spore viability. Many different studies have reported that recombination defects, responsible for a decrease in spore viability, have been linked to meiosis I or meiosis II defects (Chambers *et al.*, 1996; Hunter *et al.*, 1996; Khazanehdari and Borts, 2000; Maxfield Boumil *et al.*, 2003; Watt *et al.*, 1995). Different types of missegregation of homologous chromosomes and/or sister chromatids (Figure 4.4) can create one, two, three or four dead spores. Using appropriate genetic markers along the chromosome arms and a centromere marker, these events can be distinguished.

Two different types of missegregation can lead to three viable spores: precocious separation of sister chromatids (PSSC) and meiosis II non-disjunctions (Figure 4.4 B and C). When sister chromatids separate precociously, one of the two chromatids from a homologous pair of chromosome is segregated from its sister at the first meiotic division (Figure 4.4 B). Therefore, the resulting tetrad contains two euploid spores, one aneuploid spore with a copy of each homologue and one dead spore (due to the lack of genetic information contained in the missing chromosome). *TRP1* is used to mark the centromere on chromosome IV, as no crossovers are possible between *TRP1* and the centromere due to their proximity. In PSSC events the genetic information carried by the centromere on chromosome IV (Figure 4.4 small red or blue chromosome) in the two euploid spores comes from both parents (*TRP1* and *trp1::bsu*). These spores are known as non-sisters (Figure 4.4 B, spore #1 contains a red centromere and spore #4 contains a blue centromere). The genetic information contained in the disomic spore comes from both parents (Figure 4.4 B, spore #2 contains one copy of each homologue, one red and one blue). The second event which can lead to three viable spore tetrads is a non-disjunction of sister chromatids during the equational meiotic division (*i.e.* meiosis II non-disjunction). The resulting tetrad contains one dead spore, two euploid spores and one disomic spore (Figure 4.4 C). In this case, the genetic information contained in the disomic spore comes from only one parent (Figure 4.4 C, spore #3 contains two blue chromosomes) while the two euploid spores are sisters (Figure 4.4 C, spore #1 and #2 both contain a red centromere). Thus, the events leading to three viable spores are distinguishable by means of the segregation pattern of the centromeric marker (*e.g.* *TRP1* on chromosome IV).

Events leading to two viable spore tetrads can arise due to a lack of crossover events between homologues. In the absence of chiasmata (Chambers *et al.*, 1996; Khazanehdari and Borts, 2000) random segregation at meiosis I of one particular pair of homologous chromosomes will result in two dead spores and two aneuploid spores, which are disomic (Figure 4.4 D). The genetic information, contained in the two disomes, comes from both parents (Figure 4.4 D, the two disomic spores #1 and #2 carry a red and a blue chromatid from each parent). Due to the reductional nature of meiosis I, those disomic spores are sister spores according to the segregation of the centromeric marker (Figure 4.4 D, spores #1 and #2 each contains a red centromere).

In mammals and flies, crossovers close to centromeres have been associated with precocious separation of sister chromatids (PSSCs) (Koehler *et al.*, 1996; Lamb *et al.*, 1996). In meiosis, different *sgs1* mutants have been associated with chromosome missegregations. First, Watt *et al.* (1995) used a diploid strain marked genetically to detect meiosis I missegregation. In this strain, the *TRP1* gene, near the centromere of one of the parental chromosome IV has been disrupted by *URA3* (*trp1::URA3*) while the other haploid parent contains an endogenous *TRP1* gene (Louis and Haber, 1989). Missegregation of chromosome IV during meiosis I is the only means by which spore colonies could become Ura^+ and Trp^+. Random spore analysis has shown that *sgs1* mutant causes a 37-fold increase in chromosome missegregation during meiosis I (Watt *et al.*, 1995). The experimental design did not allow the differentiation of precocious separation of sister chromatids and meiosis I non-disjunctions. More recently, other random spore analyses have given more insight into the defects leading to an increase of meiosis I defect in *sgs1* strains. Rockmill *et al.* (2006) have used a drug resistance cassette (hygromycin resistance provided by the *HYG* gene) and a prototrophic marker (*URA3*), each inserted at one centromere of a homologous pair of chromosome III, to select for meiosis I events. These centromeric markers, combined with another drug resistance gene (*NAT*) and auxotrophic genes on chromosome III, have shown that the meiosis I defect in *sgs1Δ-C795* is due to precocious separation of sister chromatids. More specifically, this defect is associated with an increase frequency of crossovers near the centromere (Rockmill *et al.*, 2006). In their crossover analysis of selected disomes (hygromycin resistant and Ura^+ spores), Rockmill *et al.* (2006) have also shown that the contribution made by crossovers to the right of the centromere toward PSSC events was

"stronger" than those on the left of the centromere. Therefore, these authors concluded that in *SGS1* deficient cells, increased crossovers near centromeres could lead to an increase in the frequency of PSSC by destabilising the centromere cohesin complexes.

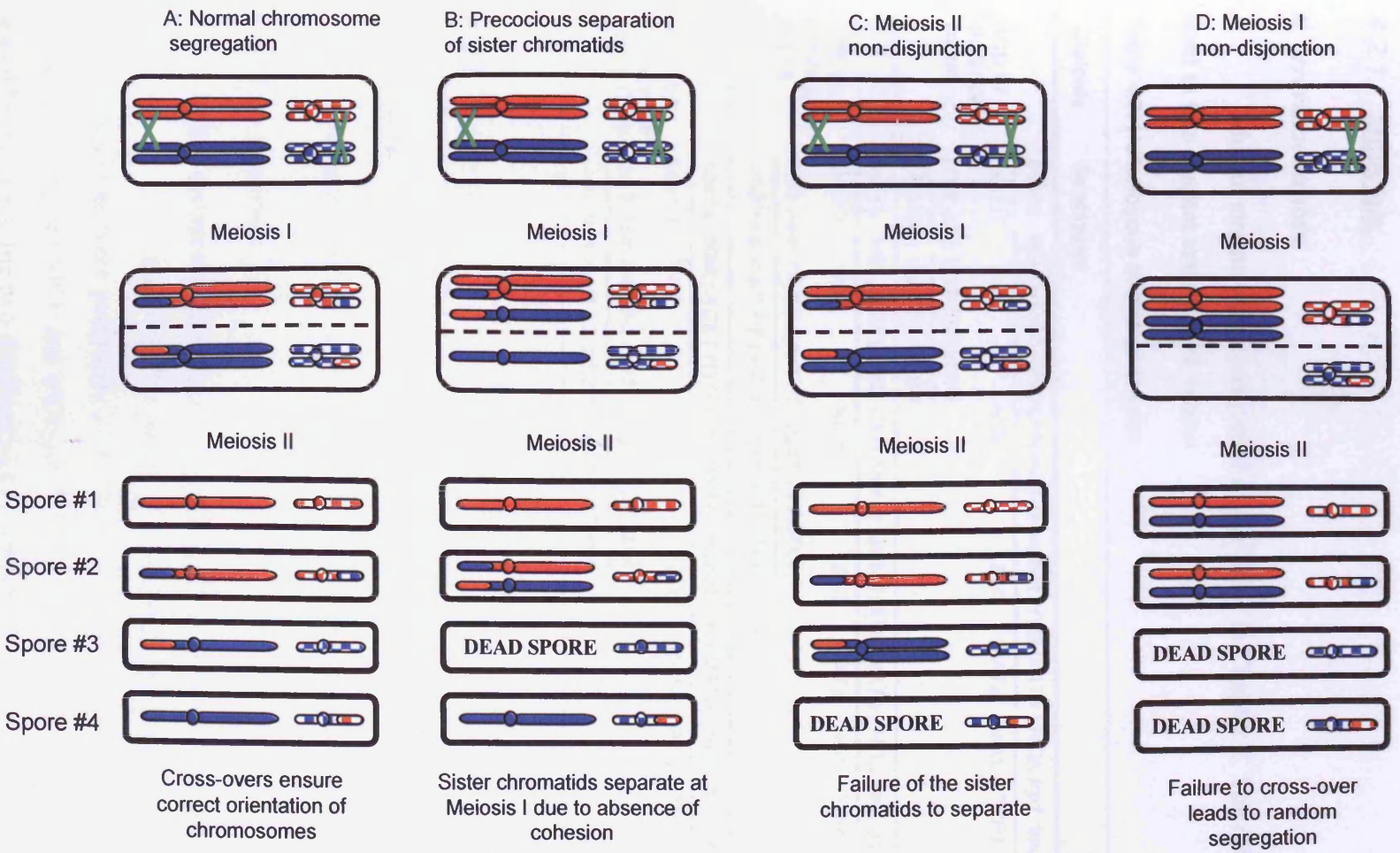


Figure 4.4: Schematic representation of normal and abnormal segregation during meiosis. Disomic spores and the genetic information carried by the centromer marker *TRP1*, on chromosome IV, can differentiate between the different events (See § 5.1.5 for details). In PSSC events, the genetic information contained in the disome is coming from both parents while in meiosis II non-disjunction, the disome only contains genetic information from one parent. In meiosis I non-disjunction tetrads, the remaining two spores are sister spores.

4.2. Experimental procedures:

4.2.1. Materials:

S. cerevisiae strains:

Mutant strains were constructed as specified in Chapter 2. The different diploids used in this chapter are detailed below:

Table 4.3: Homologous strains genotypes.

Diploids	Genotypes
ACD 97 *SGS1* WT control	*ade1Δ* *HML::ADE1* *HYG-CYH:his4-r1* *leu2-r1:HYG* *MATa* *HOΔ* *trp1::bsu36* *lys2-d* *ade1-1* *HML* *HIS4* *LEU2* *MATα* *HOΔ* *TRP1* *LYS2* *ura3::nco* *met13-2 cyh2-1* *ura3::nco* *met13-2 cyh2-1*
ACD 95 homozygote *sgs1Δ*	*ade1Δ* *HML::ADE1* *HYG-CYH:his4-r1* *leu2-r1:HYG* *MATa* *HOΔ* *trp1::bsu36* *lys2-d* *ade1-1* *HML* *HIS4* *LEU2* *MATα* *HOΔ* *TRP1* *LYS2* *ura3::nco* *met13-2 cyh2-1* *sgs1::KANMX4* *ura3::nco* *met13-2 cyh2-1* *sgs1::KANMX4*
ACD 107 homozygote *sgs1Δ-C795*	*ade1Δ* *HML::ADE1* *HYG-CYH:his4-r1* *leu2-r1:HYG* *MATa* *HOΔ* *trp1::bsu36* *lys2-d* *ade1-1* *HML* *HIS4* *LEU2* *MATα* *HOΔ* *TRP1* *LYS2* *ura3::nco* *met13-2 cyh2-1* *sgs1::NATMX4* *ura3::nco* *met13-2 cyh2-1* *sgs1::NATMX4*

4.2.2. Methods:

Tetrads analysis:

Tetrads dissection and tetrads analysis were performed as explained in Chapter 2.

Crossover frequency:

Map distance were calculated according to the formula of Perkins:

$$f(co) = \frac{1}{2}(TT + 6NPD) / (PD + NPD + TT)$$ (Perkins, 1949).

Analyses were performed on the different distributions of parental ditype (PD), non-parental ditype (NPD) and tetratype (TT) (see § 4.3.1 for details) to accept or refute the null hypothesis, the two distributions are different.

Interference:

For interference analysis, the expected frequencies of NPDs were calculated according to the Papazian's formula using the frequencies of Tetratypes.

$$f(NPD_{expected}) = \tfrac{1}{2}\,[1 - f(TT) - (1 - {}^{3}/_{2}\, f(TT))^{2/3}]$$

$$\text{And } NPD_{expected} = f(NPD_{expected}) \times (PD + NPD_{observed} + TT)$$

The ratio $r = (NPD_{expected} / NPD_{observed})$ was used to assess the degree of interference in a given interval. if $r < 1$ there is positive interference, if $r \sim 1$ there is no interference and if $r > 1$ there is negative interference in such interval (Papazian, 1952).

Gene conversions:

The number of gene conversion for a given marker was assessed by counting the number of tetrads with non-Mendelian segregation (3:1 or 1:3) compared to the 2:2 normally expected. Percentage of gene conversion were calculated for the markers *HIS4,* mutated via base pair replacement, forming an *EcoR1* site and thus creating the *his4-r1* allele, *LEU2* (*leu2-r1* allele) on chromosome III and *LYS2* (*lys2-c* allele) on chromosome VII.

Statistical analysis:

We used the G-test or the contingency Chi-square tests, when appropriate, to accept or reject the null hypothesis "the two distributions are different". Tests were conducted in 95% confidence limits (unless stated otherwise). If $p < 0.05$, the two distribution are different. If $p > 0.05$, the two distributions are not different.

4.3. Results:

4.3.1. *Increased crossover frequencies are not detected in SGS1 mutants:*

Several groups have reported a small increase of meiotic crossovers in the four viable spore tetrads class in *S. cerevisiae* strains mutant for *SGS1* (Jessop *et al.*, 2006; Oh *et al.*, 2007; Rockmill *et al.*, 2003; Rockmill *et al.*, 2006). These studies were performed in the *S. cerevisiae* SK1 and BR strain backgrounds.

We assessed the frequency of crossovers on chromosome III in *S. cerevisiae* Y55 strains. Frequencies were calculated in three different intervals: *HML-HIS4* (Table 4.4), *HIS4-LEU2* (Table 4.5) and *LEU2-MAT* (Table 4.6). The diploids tested were homozygous for *SGS1*, *sgs1*Δ or *sgs1*Δ*-C795*.

Table 4.4: Tetrad distributions and map distances in the *HML-HIS4* interval for tetrads with four viable spores.

Genotype	Tetrad distribution			Four viable spore tetrads	Map distances (cM)	G-test*, p values
	PD	NPD	TT			
SGS1	196	20	363	579	41.7	n.a
*sgs1*Δ	85	9	109	203	40.1	0.08
*sgs1*Δ*-C795*	84	8	109	201	39.1	0.107

Note: PD: Parental Ditypes; NPD: Non-Parental Ditypes; TT: TetraTypes. * G-test compared the distribution of PD, NPD and TT between the wild type and mutant strains.
p values < 0.0166 were considered significant (Three comparisons).

Table 4.5: Tetrad distributions and map distances in the *HIS4-LEU2* interval for tetrads with four viable spores.

Genotype	Tetrad distribution			Four viable spore tetrads	Map distances (cM)	G-test*, p values
	PD	NPD	TT			
SGS1	404	2	165	571	15.5	n.a
*sgs1*Δ[a]	154	5	43	202	18.1	0.006
*sgs1*Δ*-C795*	152	3	56	211	17.5	0.255

Note: PD: Parental Ditype; NPD: Non-Parental Ditype; TT: TetraType. * G-test compared the distribution of PD, NPD and TT between the wild type and mutant strains.
p values < 0.0166 were considered significant (Three comparisons).
[a] *sgs1*Δ and *sgs1*Δ*-C795* distributions are also different from one another (p=0.011).

Table 4.6: Tetrad distributions and map distances in the *LEU2-MAT* interval for four viable spore tetrad class.

Genotype	Tetrad distribution			Four viable spore tetrads	Map distances (cM)	G-test*, p values
	PD	NPD	TT			
SGS1	340	15	250	604	28.1	n.a
sgs1Δ	124	7	100	231	30.7	0.766
sgs1Δ-C795	122	12	110	244	37.3	0.09

Note: PD: Parental Ditype; NPD: Non-Parental Ditype; TT: TetraType. *G-test compared the distribution of PD, NPD and TT between the *SGS1 WT* and mutant strains.
p values < 0.0166 were considered significant (Three comparisons).

The crossover frequencies, between the wild-type strain and the *sgs1Δ-C795* mutant, in all three genetic intervals, are not statistically different from each other (p values > 0.0166). Furthermore, the frequency of crossovers in *sgs1Δ* in the *HML-HIS4* and *LEU2-MAT* intervals is also not different from wild type. But, the distribution of PDs, NPDs and TTs in the *sgs1Δ* mutants is statistically different from wild type (p values = 0.006) in the *HIS4-LEU2* interval. Therefore, in this genetic interval, the frequency of crossovers in *sgs1Δ* is increased compared to wild type.

The G-test compares the distribution of PDs, NPDs and TTs in each genetic interval, between crosses. This statistical test was preferred to the standard error test provided by the Stahl Lab Online Tools (http://www.molbio.uoregon.edu/~fstahl/), and referenced in recent meiotic studies (Jessop *et al.*, 2006; Rockmill *et al.*, 2006), because it assesses distribution rather than the calculated centiMorgan (cM) distance. The Perkins formula was used to calculate the genetic distance based on the number of PD, NPD and TT (Perkins, 1949). Different distribution of PD, NPD and TT and therefore different crossover events (such as the number of crossover and the kind of events [single/double]), may lead to the same crossover frequency expressed in centiMorgans (cM) for a given interval. An example of how different distribution of PD, NPD and TT can lead to the same frequency is given in Table 4.7.

Table 4.7: Different distributions can lead to the same genetic distance.

Strain	Intervals	PD	NPD	TT	Genetic distance (cM)	G-test values	Stahl lab online tool
wild type	*HML-HIS4*	196	20	363	41.7	p = 0.0023	p = 0.058
hypothetical	*HML-HIS4*	172	14	429	41.7		
wild type	*HIS4-LEU2*	404	2	165	15.5	p = 0.029	p = 0.052
hypothetical	*HIS4-LEU2*	266	6	70	15.5		

PD: Parental Ditypes; NPD: Non-Parental Ditypes; TT: TetraTypes.
$p < 0.05$ were considered significant

In Table 4.7, although the genetic distances, expressed in centiMorgans, are the same, the distributions of PD, NPD and TT are statistically different from each other as shown by the G-test. Using the analysis of the difference in term of map distance, as provided Stahl Lab Online Tool, would miss important changes in the distribution of crossovers.

Map distances in *sgs1Δ-C795* are 1.1 times higher than wild type in the *HIS4-LEU2* interval, which is not statistically different (p value 0.255). Rockmill *et al.* (2003) reported a 1.3 fold increase of crossovers in the *LEU2-MAT* interval for *sgs1Δ-C795*. In the present study, the calculated fold increase for this mutant is of the same magnitude (1.32 fold), although the distribution and frequency of tetrad classes is not statistically different from the SGS1 WT distribution using a G-test (p value = 0.09). However, the absence of statistical difference in our *sgs1Δ-C795* could be explained by the smaller size of our dataset.

In the Y55 *S. cerevisiae* background, *SGS1* mutants such as *sgs1Δ* and *sgs1Δ-C795* do not seem to increase the frequency of crossovers. The map distances between *sgs1Δ* and *sgs1Δ-C795* were not different from one another except for the strain and interval where higher proportions of NPDs were recovered (i.e. *sgs1Δ* in *HIS4-LEU2* interval).

4.3.2. SGS1 mutants decrease crossover interference:

For a given interval, NPDs come from the resolution of a double crossover between four chromatids. Since statistical differences between *sgs1Δ* and wild type but also *sgs1Δ* and *sgs1Δ-C795*, could be detected in the *HIS4-LEU2* interval, where higher

proportions of NPDs were recovered (Table 4.5), *sgs1* strains were assessed for crossover interference. Decreases in interference were assessed by the ratio (r) between the expected numbers of NPDs in absence of interference and the observed numbers of NPDs in a given interval. The expected numbers of NPDs were calculated using the Papazian's formula (Experimental procedures § 4.2.2 for details). If (r) < 1, there is positive interference, *i.e.* the likelihood of crossovers occurring closely from one another is reduced. If (r) = 0, there is no interference and if (r) > 1, there is negative interference, *i.e.* the likelihood of crossovers occurring close to one another is increased. Interference values were calculated for three different intervals, *HML-HIS4* (Table 4.8), *HIS4-LEU2* (Table 4.9) and *LEU2-MAT* (Table 4.10).

Table 4.8: NPD ratio as a measure of interference in the *HML-HIS4* interval.

Strains	Number of tetrads	NPD_{obs}	non-NPD_{obs}	NPD_{exp}	non-NPD_{exp}	NPD ratio (r)	Chi-square, p value
SGS1	579	20	559	63.84	515.16	0.31	$5.99x10^{-9}$
sgs1Δ	203	9	194	12.93	190.07	0.7	0.258
sgs1Δ-C795	201	8	193	13.19	187.81	0.61	0.139

NPD_{obs}: NPD observed in the population; non-NPD_{obs}: Number of tetrads minus the number of NPD observed; NPD_{exp}: NPD expected as calculated by the Papazian's formula; Non-NPD_{exp}: Number of tetrads minus the number of NPD expected; NPD ratio (r): NPD_{exp}/NPD_{obs}; Chi-square tests are performed for each strains for the NPD/non-NPD observed and the NPD/non-NPD expected. p values < 0.05 were considered significant.

Table 4.9: NPD ratio as a measure of interference in the *HIS4-LEU2* interval.

Strains	Number of tetrads	NPD_{obs}	non-NPD_{obs}	NPD_{exp}	non-NPD_{exp}	NPD ratio (r)	Chi-square, p value
SGS1	571	2	569	7.52	563.48	0.27	0.042
sgs1Δ	202	5	197	1.35	200.65	3.7	0.0016
sgs1Δ-C795	211	3	208	2.29	208.71	1.3	0.081

(See Table 4.8) p values < 0.05 were considered significant.

Table 4.10: NPD ratio as a measure of interference in the *LEU2-MAT* interval.

Strains	Number of tetrads	NPD_{obs}	non-NPD_{obs}	NPD_{exp}	non-NPD_{exp}	NPD ratio (r)	Chi-square, p value
SGS1	605	15	590	18.75	586.25	0.80	0.379
sgs1Δ	231	7	224	8.07	222.93	0.87	0.701
sgs1Δ-C795	244	12	232	18.19	234.52	1.27	0.403

(See Table 4.8) p values < 0.05 were considered significant.

Although these data sets are small with respect to interference analysis of previous studies (Chua and Roeder, 1998; Novak *et al.*, 2001; Sym and Roeder, 1994; Tsubouchi and Roeder, 2006), decreases in the frequency of double crossovers in the *SGS1* strain were observed in *HML-HIS4* and *HIS4-LEU2* intervals as expressed by the NPD ratio ($r = 0.31$, $p = 5.99 \times 10^{-9}$ and $r = 0.27$, $p = 0.042$, respectively, Tables 4.8 and 4.9). The distribution of the observed NPD events is statistically different from that of the NPD expected in the *SGS1* strain. Furthermore, the number of NPD events observed is reduced compared to the expected number of NPDs. Therefore, the *SGS1* strain shows interference in the *HML-HIS4* and *HIS4-LEU2* intervals. Thus, the likelihood of a second crossover is reduced when a strand exchange has already occurred in this interval. In the *LEU2-MAT* interval, interference was not observed for the *SGS1* strain ($r = 0.80$, $p = 0.379$, Table 4.10). The absence of detectable interference could be explained by the size of the interval, the presence of the centromere and/or the small number of tetrads analysed.

In the *sgs1Δ-C795* mutant strain, the number of NPDs observed is not statistically different from the number of NPD expected in the *HML-HIS4*, *HIS4-LEU2* and *LEU2-MAT* intervals (p values > 0.05, Tables 4.8, 4.9 and 4.10). Although, the same is true for the *sgs1Δ* strain in the *HML-HIS4* and *LEU2-MAT* intervals (p values > 0.05, Tables 4.8, and 4.10), in the *HIS4-LEU2* interval, the observed number of NPDs is greater than expected (p value = 0.0016, Table 4.9). This distribution difference and an NPD ration greater than one ($r = 3.7$; $p = 0.0016$, Table 4.9) are representative of negative interference in the *HIS4-LEU2* interval for *sgs1Δ* cells. In *sgs1Δ*, the likelihood of a double crossover occurring in this interval is increased compared to wild type. The NPD ratio in the *HML-HIS4* interval for both *sgs1Δ* and *sgs1Δ-C795*, although less than 1, is higher than the NPD ratio in *SGS1* WT cells (0.7 and 0.61, respectively compared to

0.31 in wild type) suggesting that interference is decreased in this interval but not abolished. In the *HIS4-LEU2* interval and *LEU2-MAT* interval, the NPD ratio for *sgs1Δ-C795* is higher than 1 (r = 1.3, p = 0.081; Table 4.9 and r = 1.27, p = 0.403; Table 4.10) and the number of NPDs observed is not statistically different from the expected number of NPDs. This suggests that interference is abolished in the *HIS4-LEU2* and *LEU2-MAT* intervals for the *sgs1Δ-C795* mutant.

4.3.3. Increase of gene conversion in SGS1 mutant strains:

As seen in Chapter 1 § 1.6.2, gene conversion can arise from correction of mismatched DNA during the repair of a double strand break (Paques and Haber, 1999). An increase in gene conversion might reflect an increase in stable strand invasion regardless of the subsequent outcomes.

The percentages of gene conversion were calculated at *HIS4* (Table 4.11), *LEU2* (Table 4.12) and *LYS2* (Table 4.13) in wild type, *sgs1Δ* and *sgs1Δ-C795*.

Table 4.11: Gene conversion at the *HIS4* locus among the four viable spore tetrads class.

Strains	3:1[a]	1:3[b]	2:2[c]	Number of tetrads	Gene conversion (%)[d]	G-test, p values[e]
SGS1	14	20	580	614	34/614 (5.5%)	n.a
sgs1Δ	17	13	204	234	30/234 (12.8%)	0.001
sgs1Δ-C795	15	18	215	248	33/215 (13.3%)	0.001

[a]: 3:1, number of tetrads where three out of four spores were prototrophic for histidine.
[b]: 1:3, number of tetrads where one out of four spores was prototrophic for histidine.
[c]: 2:2, number of tetrads where Mendelian segregation was observed.
[d]: Gene conversion were calculated as total number of events (3:1 events + 1:3 events).
[e]: G-tests were performed on the distribution of 3:1, 1:3 and 2:2 segregation patterns for the different mutant and compared to wild type. p values < 0.025 were considered significant (2 comparisons).

Table 4.12: Gene conversion at the *LEU2* locus among the four viable spore tetrads class.

Strains	3:1[a]	1:3[b]	2:2[c]	Number of tetrads	Gene conversion (%)[d]	G-test, p values[e]
SGS1	3	6	605	614	9/614 (1.4%)	n.a
sgs1Δ	2	1	231	234	3/234 (1.2%)	0.586
sgs1Δ-C795	1	3	244	248	4/248 (1.6%)	0.942

(a, b, c, d, and e: see Table 4.11) p values < 0.025 were considered significant (2 comparaisons).

Table 4.13: Gene conversion at the *LYS2* locus among the four viable spore tetrads class.

Strains	3:1[a]	1:3[b]	2:2[c]	Number of tetrads	Gene conversion (%)[d]	G-test, p values[e]
SGS1	0	0	614	614	0/614 (0%)	n.a
sgs1Δ	1	2	231	234	3/234 (1.28%)	0.0054
sgs1Δ-C795	1	3	244	248	4/244 (0.81%)	0.0254

(a, b, c, d, and e: see Table 4.11) p values < 0.025 were considered significant (2 comparaisons).

No increase in gene conversion was observed at the *LEU2* locus for any of the mutants. At the *LYS2* locus, only the *sgs1Δ* mutant displayed an elevated frequency of gene conversion compared to *SGS1* WT cells (p value < 0.025). The *sgs1Δ* and *sgs1Δ-C795* mutants both display an increase in gene conversion frequencies compared to wild type at the *HIS4* locus (2.3 and 2.4-fold, respectively; p values < 0.025). The distribution of 3:1, 1:3 and 2:2 events in the four viable spore tetrad classes were statistically different from that of the wild type using a G-test. Thus, *SGS1* mutants seem to increase gene conversions at the *HIS4* locus.

The strains used in this assay carried a hygromycin resistant gene (HYG^R) 3′ proximal of *HIS4* and another HYG^R gene 5′ of *LEU2*. It is therefore possible to assess the number of gene conversions associated with crossovers for this interval (Table 4.14). If a crossover occurs between the two HYG^R genes, the resulting spores will be 3:1 hygromycin resistant (see Chapter 5 for details).

Table 4.14: Distribution of gene conversion at the *HIS4* locus associated or not with crossovers.

Strains	GC with CO[a] (%)	GC without CO[b] (%)	G test, p values[c]
SGS1	22 (64.7%)	12 (35.3%)	n.a
sgs1Δ	14 (46.7%)	16 (53.3%)	0.153
sgs1Δ-C795	18 (54.5%)	15 (45.5%)	0.408

[a]: gene conversion associated with a crossover between the two *HYG* genes.
[b]: gene conversion not associated with crossover;
[c]: G tests compared the distribution of gene conversion in both mutants to that of the *SGS1* strain.

The distributions of gene conversion events associated with crossovers and not associated with crossovers in *sgs1Δ* and *sgs1Δ-C795* mutants are not different from the distribution in the *SGS1* strain. Therefore, the increase of gene conversions in *sgs1* strains (Table 4.11) is not preferentially due to an increase specific to either double Holliday junction repair model or the synthesis-dependent strand-annealing pathway. The absence of bias toward the crossover or non-crossover pathway might suggest that Sgs1p plays a very early role during homologous recombination (See Discussion, Chapter 5 and Chapter 7 for details). Although, this absence of statistical difference between the different distributions could be due to the relatively small sample size.

4.3.4. *The three viable spore tetrad class is enriched in crossovers:*

SGS1 mutant strains have been shown to increase meiosis I missegregation *i.e.* meiosis I non-disjunction (two viable spore tetrads) or precocious separation of sister chromatids (three viable spore tetrads) (Watt *et al.*, 1995). Rockmill *et al.* (2006) reported that in *sgs1Δ-C795* mutant, the increase of three viable spore tetrads could be due to precocious separation of sister chromatids due to an increase in crossovers near centromeres (§ 4.1.5). To look for PSSC in the *sgs1Δ* strain, we analysed 90 tetrads containing three-viable spores by CHEF (see Chapter 2 for experimental procedure) to detect disomes (Khazanehdari and Borts, 2000). In these 90 tetrads analysed, no disomes were detected in the *sgs1Δ* strain. However, the number of tetrads might have been too small to detected disomes using this technique. However, in the three viable spore tetrad class, precocious separation of chromatid from chromosome III leads to a disomic spore, which is a "non-mater" in absence of crossover between *MAT* and the centromere (aneuploid spore colonies possess two chromosomes III, one from each parent, Figure 4.4 B, spore #2). No non-mating spore colonies were detected in the 39 three viable wild-type tetrads analysed, nor the 323 *sgs1Δ* or the 206 *sgs1Δ-C795* three viable spore tetrads recovered after dissection.

Based on the suggestion by Rockmill *et al.* (2006), we decided to test again this hypothesis using a genetic approach. If the increase of three viable spore tetrads is due to crossovers proximal to centromeres, the dead spores in the three viable spore tetrad class should have an increase in crossovers in the *LEU2-MAT* interval compared to the four viable spore tetrad class. The potential increase of crossovers in this interval will only account for events occurring between pairs of chromosome III. These events occurring

on chromosome III might therefore be "diluted" by crossovers occurring on the other fifteen chromosome pairs and causing PSSC. To test this hypothesis, the distribution of crossovers between the four and three viable spore tetrads classes for different intervals, including *LEU2-MAT* which contains the centromere (Table 4.15 to 4.17) were compared.

Table 4.15: Distribution of Parental Ditypes (PD), Non-Parental Ditypes (NPD) and TetraTypes (TT) for the four and three viable spore tetrad class in the *HML-HIS4* interval.

Strain	Class	PD	NPD	TT	Total	Genetic distance (cM)	G-test, p values
sgs1Δ	4 viable spores	85	9	109	203	40.15	0.327
sgs1Δ	3 viable spores	109	21	161	291	49.31	
sgs1Δ-C795	4 viable spores	84	8	109	201	39.05	0.353
sgs1Δ-C795	3 viable spores	83	14	99	196	46.68	

p values < 0.05 were considered significant.

Table 4.16: Distribution of Parental Ditypes (PD), Non-Parental Ditypes (NPD) and TetraTypes (TT) for the four and three viable spore tetrads class in the *HIS4-LEU2* interval.

Strain	Class	PD	NPD	TT	Total	Genetic distance (cM)	G-test, p values
sgs1Δ	4 viable spores	154	5	43	202	18.07	0.026
sgs1Δ	3 viable spores	189	5	92	286	21.33	
sgs1Δ-C795	4 viable spores	152	3	56	211	17.54	0.15
sgs1Δ-C795	3 viable spores	126	2	70	198	20.71	

p values < 0.05 were considered significant.

Table 4.17: Distribution of Parental Ditypes (PD), Non-Parental Ditypes (NPD) and TetraTypes (TT) for the four and three viable spore tetrads class in the *LEU2-MAT* interval.

Strain	Class	PD	NPD	TT	Total	Genetic distance (cM)	G-test, p values
sgs1Δ	4 viable spores	124	7	100	231	30.74	0.353
sgs1Δ	3 viable spores	147	16	125	288	38.37	
sgs1Δ-C795	4 viable spores	122	12	110	244	37.3	0.819
sgs1Δ-C795	3 viable spores	107	9	86	202	34.65	

p values < 0.05 were considered significant.

Interestingly, the distributions of PD, NPD and TT, which reflect the number of crossover occurring in a given interval, were not different between the four and three viable spore tetrad classes in the *sgs1Δ-C795* mutant. The distribution of events was solely statistically significant for the *sgs1Δ* mutant in the *HIS4-LEU2* interval where higher numbers of NPD were recovered. We were therefore unable to detect an increase of crossover events associated with the three viable spore tetrads.

Since crossovers proximal to centromeres could potentially lead to an increase of PSSC and therefore cell death (Rockmill *et al.*, 2006), dead spores in the three spore viable class should be increased for crossovers. To test this hypothesis, the number of dead spores associated with crossovers in the *LEU2-MAT* interval was calculated among the number of recombinant three viable spores. If death is associated with recombination events, the distribution of recombinant dead spores *vs.* non-recombinant dead spores should be different from random. Therefore, the numbers of dead spores associated or not associated with crossovers were compared with that of a random distribution for death events, thereafter designated as "random death" (Table 4.18). The comparisons between the number of dead spores associated (or not associated) with crossovers and that of a random death was done using a contingency Chi-square test.

Table 4.18: Number of dead spores associated with a crossover in the recombinant three viable spore tetrad class in the *LEU2-MAT* interval:

Strains	PD	NPD	TT	Total	Recomb. Tetrads	Recomb. Tetrads: dead spore w/ c.o.	Recomb. Tetrads: dead spore w/o c.o.
sgs1Δ	147	16	125	288	141	74	67 (p=0.118)
sgs1Δ-C795	107	9	86	202	95	59	36 (p=0.018)

Recomb.: Recombinant tetrads; w/: with; w/o: without; c.o: crossovers.
p values < 0.05 were considered significant.

As hypothesised, the dead spores in the recombinant three viable spores tetrad class seem to be increased for crossover events in *sgs1Δ-C795*. In the *sgs1Δ* strain, an increase in crossovers in the dead spore could not be detected. The *sgs1Δ* defect might be "lost" due to recombination events occurring between other pairs of chromosomes, or the mitotic defect of *sgs1Δ* strain, as seen in chapter 3, might prevent the detection of such defect in this interval.

Due to the presence of the centromere marker, *TRP1*, on chromosome IV, it was possible to assess, in the three viable spore tetrads class, among the tetratype class, the number of recombinant dead spores where crossovers had occurred in the *LEU2-CEN3* or in the *CEN3-MAT* intervals (Table 4.19).

Table 4.19: Number of dead spores associated with a crossover in the recombinant three viable spore tetratype class in the *LEU2-MAT* interval:

	LEU2-CEN3	*CEN3-MAT*	
Strains	TT where dead spore is recombinant	TT where dead spore is recombinant	Chi-square, p values*
sgs1Δ	23	35	0.115
sgs1Δ-C795	25	26	0.888

Note: TT: Tetratypes; *p value < 0.05 were considered significant while comparing the distribution of dead spores in the *LEU2-CEN3* and *CEN3-MAT* intervals to that of a random death distribution (contingency Chi-square test).

No difference in terms of where the crossovers occurred relative to the centromere could be detected in the dead spores (p values > 0.05). Rockmill *et al.* (2006) reported that the region to the right of *CENIII* had a 'stronger' effect on PSSC than the region on the left of the centromere. Since no difference could be detected in our strain background, the frequencies of crossover events, in intervals where no centromeres were present, were assessed by the same method (Table 4.20 and 4.21).

Table 4.20: Number of dead spores associated with a crossover in the recombinant three viable spore tetrad class in the *HML-HIS4* interval:

Strains	PD	NPD	TT	Total	Recomb. Tetrads	Recomb. Tetrads: dead spore w/ c.o.	Recomb. Tetrads: dead spore w/o c.o.
sgs1Δ	109	21	161	291	182	110	72 (p=0.004)
sgs1Δ-C795	83	14	99	196	113	68	45 (p=0.03)

p values < 0.05 were considered significant.

Table 4.21: Number of dead spores associated with a crossover in the recombinant three viable spore tetrad class in the *HIS4-LEU2* interval:

Strains	PD	NPD	TT	Total	Recomb. Tetrads	Recomb. Tetrads: dead spore w/ c.o.	Recomb. Tetrads: dead spore w/o c.o.
sgs1Δ	189	5	92	286	97	53	44 (p=0.36)
sgs1Δ-C795	126	2	70	198	72	43	29 (p=0.09)

p values < 0.05 were considered significant.

In the *HML-HIS4* interval, which does not contain a centromere, we were able to detect an increase in crossover events in the dead spores for both *SGS1* mutant strains (*sgs1*Δ and *sgs1*Δ-*C795*, p values = 0.004 and 0.03, respectively – Table 4.20). This *sgs1* defect, *i.e.* increased crossover frequency in the dead spore, might be linked to a recombination defect in cells lacking the Sgs1 protein rather than the sole contribution of crossovers near centromeres. Furthermore, this specific *sgs1* defect is linked to the absence of the C-terminal part of the Sgs1 protein.

4.3.5. *The two viable spores tetrad class is enriched in non-sister spores in SGS1 mutant strains:*

To test the hypothesis that Sgs1p might play an active role during homologous recombination we assessed the segregation of the centromere marker *TRP1* in the two viable spore tetrad classes.

As seen previously, *SGS1* mutant strains increase meiosis I errors (Watt *et al.*, 1995). As previously reported (Jessop *et al.*, 2006; Rockmill *et al.*, 2006; Watt *et al.*, 1995), and shown in Chapter 3 § 3.2.3, *S. cerevisiae* strains mutated for *SGS1* have an increase in three and two viable spore tetrads. We have seen in the introduction of this chapter that crossovers are essential for the faithful segregation of homologous chromosomes during meiosis I. A lack of crossovers will increase the frequency of meiosis I non-disjunction and therefore the proportion of two viable spores tetrads (Chambers *et al.*, 1996; Khazanehdari and Borts, 2000). The mating phenotype and the segregation of a centromere marker can be used to determine the nature of the event leading to the two viable spore tetrads (§ 4.1.5). Meiosis I non-disjunction of chromosome III will yield two non-mating spores. The two viable spores are disomic, containing one copy of each parental chromosome III. The disomic spores are also sister spores as shown by the segregation of the centromere marker, *TRP1* on chromosome IV (see § 4.1.5 for details).

Among the populations of two viable spore tetrads, the proportion of sister spores and non-sister spores were analysed for the various mutant strains (Table 4.22).

Table 4.22: Distribution of sister and non-sister spores in the two viable spore tetrad classes.

Strains	Two viable spores tetrads	Meiosis I non-disjunction[b]	Sister spores (%)[c]	Non-sister spores (%)[c]	Chi-square, p values[d]
msh4Δ[a]	36	28/193	32 (88.8%)	4 (11.2%)	3.06×10^{-6}
sgs1Δ	301	0/1215	110 (36.5%)	191 (63.5%)	3×10^{-6}
sgs1Δ-C795	145	0/668	68 (46.9%)	77 (53.1%)	0.006

a: data from Khazanehdari *et al.* (2000).
b: the proportion of meiosis I non-disjunction were calculated as the number of chromosome III non-disjunction events (non-maters) divided by the total number of tetrads dissected except for *msh4Δ* data where non-disjunctions were detected genetically and by CHEF.
c: percentage of sister or non-sister spores for a given strain.
d: Chi-square tests were used to compare the distribution of sister and non-sister spores to that of a random distribution. p values < 0.05 were considered significant.

In *msh4Δ*, the increase in sister spores in the two viable spore tetrad class is indicative of a meiosis I defect as shown by the proportion of meiosis I non-disjunction detected both genetically, by means of heterozygosity to drug resistance cassettes, and by CHEF (Khazanehdari and Borts, 2000). In the *sgs1Δ* and *sgs1Δ-C795* strains no meiosis I non-disjunction of chromosome III could be detected. More interestingly, none of the *SGS1* mutants were enriched for sister spores in the two viable spore tetrad classes. This absence of enrichment supports the idea that the increase in two viable spore tetrads in *SGS1* mutants is not due to meiosis I non-disjunction. Furthermore, in *sgs1Δ* and *sgs1Δ-C795*, the distributions of sister/non-sister spores in the two viable spore tetrad classes were also different from a random distribution as shown by Chi-square tests (p values $<< 0.05$). In both *SGS1* mutants, the two viable spore tetrad classes were enriched in non-sister spores (Table 4.22). The influence of Sgs1p on the segregation of sister spores *vs.* non-sister spores might be dependent on the C-terminal part of the protein since no difference could be made between the full deletion of the gene (*sgs1Δ*) and the partial deletion (*sgs1Δ-C795*).

4.4. Discussion:

4.4.1. *Early activity of Sgs1p during homologous recombination:*

In the four viable tetrads, no increase of crossovers was detected compared to the wild type, except in the interval where a higher proportion of NPDs were recovered (*HIS4-LEU2*). This increase of NPDs in *sgs1*Δ could account for the statistical difference between *sgs1*Δ and the *SGS1* WT strain. Interestingly, in this same interval, negative interference was detected in *sgs1*Δ cells, while *sgs1*Δ-*C795* did not show any interference. These phenotypes *i.e.* increased NPDs and absence of interference were also observed by Oh *et al.* (2007). Furthermore, these authors have identified by 2D-gels, in wild type and *sgs1*Δ-*C795* cells, higher molecular weight recombinant molecules. Oh *et al.* (2007) proposed that the second end of a DSB could also invade either the sister or the homologue chromatid. These second end-invasion events lead to tertiary and quaternary molecules where three chromatids (tertiary molecules) or four chromatids (quaternary molecules) are linked by two double Holliday junctions (Figure 4.6 A and B). These events, referred to as multi-joint molecules, have also been observed physically by electron microscopy. In absence of the Sgs1 proteins, these multi-joint molecules are increased by 3-fold (Oh *et al.*, 2007) compared to wild type. The increase of NPD events in *sgs1*Δ could be the genetic manifestation of the resolution of those quaternary molecules since NPDs arise from the resolution of double crossovers (Figure 4.7 A). In addition, Oh *et al.* (2007) have suggested that the higher proportion of multi-joint molecules in *sgs1* mutant could be due to an early activity of the Sgs1 protein during meiotic recombination. In *SGS1* WT cells, Sgs1p might remove some of the single-end invasion events involving the second end of the DSB.

Evidence in support of an early function of Sgs1p comes from the gene conversion data. As explained earlier, gene conversions arise by correction of DNA mismatches in heteroduplex DNA during strand invasion (Figure 1.14). In the Y55 *S. cerevisiae* strain background, gene conversion rates were increased in *SGS1* mutants in the *HIS4* locus on chromsome III (~2.3 fold increase compared to wild type) and *LYS2* locus on chromosome VII. The increase gene conversion rates might therefore arise from increased strand invasion events. Unfortunately, due to the absence of auxotrophic markers and/or drug resistance genes proximal to *LYS2*, the distinction between gene

conversions associated or not associated with crossovers could not be made at this locus. However, at the *HIS4* locus, such distinction is possible. Compared to *SGS1* WT cells, the distribution of gene conversions associated with crossovers is not different in *SGS1* mutants. The absence of a difference in term of distribution between wild type and *SGS1* mutants suggest that although the interactions between chromatids are increased in *SGS1* mutants (as monitored by the increase of gene conversion), those increased interactions take place early in prophase I, before the decision of making a crossover or a non-crossover is taken. Furthermore, Sgs1p itself does not seems to be involved in the decision process has no difference in term of distribution between crossovers and non-crossovers could be detected in absence of Sgs1p.

In summary, the Sgs1 protein might play an early role in meiotic prophase I by removing invading strands. This activity seems to be dependent on the C-terminal part of the Sgs1p and could involve the helicase activity of the protein. Such early activity of *SGS1* will be further discussed in the next chapter.

4.4.2. Absence of PSSC in SGS1 mutants:

We were not able to find any evidence of meiosis I defect in *SGS1* mutant strains in contrast to previous reports (Rockmill *et al.*, 2006; Watt *et al.*, 1995). While Watt *et al.* (1995) used the Y55 strain for their study, Rockmill *et al.* (2006) carried their experiment in the BR strain background. The absence of PSSC in the strain used in this work cannot therefore be attributed to a strain difference. The major difference between the data collected in the previous studies and the data presented here, resides in the experimental design. While, Watt and Rockmill selected disomes from random spore experiments, we analysed dissected tetrads for potential disomic spores. As discussed in the introduction of this chapter, *sgs1*Δ cells have an increase of chromosome non-disjunction associated with crossovers or gene conversions during mitosis (Ajima *et al.*, 2002; Watt *et al.*, 1995). Thus, *sgs1* diploid cells could potentially enter meiosis with an extra chromosome. If proven to be the case, and in absence of a fully functional spindle checkpoint, those (2n+1) diploids could lead to disomic spores after meiosis. Large-scale deletion strain experiments have identified 363 genes involved in sporulation (Deutschbauer *et al.*, 2002). While most of these deleted genes confer a sporulation-deficiency phenotype, nearly a third of them enhance sporulation when deleted (102 genes). These later genes, called negative regulator of sporulation comprise regulators of

transcription, cell cycle control genes and genes involved in pseudohyphal differentiation. More recently, microarray experiments have analysed the effect of aneuploidy, and more specifically the gain of a chromosome, on *S. cerevisiae* cell physiology (Torres *et al.*, 2007). First, these authors have shown that in yeast, most of the genes present on the disomic chromosomes are expressed. As such, 93% of genes carried on the chromosome present in two copies were over-expressed by at least 1.3-fold, while 83% of them were over-expressed by a factor of 1.5 or more. Secondly, this genetic over-expression was reflected by in an increased translation of some of the proteins such as Cdc28p. It is then reasonable to assume that an aneuploid diploid with an extra chromosome carrying genes regulating sporulation negatively, might have a decrease sporulation efficiency compared to an euploid. This sporulation defect might be due to the expressed two copies of these genes. Since Ajima *et al.* (2000) and Watt *et al.* (1995) have shown that, in mitosis, *sgs1*Δ diploids are increased in crossover-associated non-disjuntions, the increase in PSSC events seen by Rockmill *et al.* (2006) might be linked to a mitosis defect rather than a purely meiotic one. Since Rockmill *et al.* selected disomes from random spores, they might have inadvertently select for spores coming from asci containing one, two or three spores. Since we only dissected asci containing four spores, we were unable to detect disomes, which might have arise as described above.

4.4.3. Possible late role in meiotic recombination for the Sgs1 protein:

We have shown in Chapter 3 of this thesis that both *sgs1*Δ and *sgs1*Δ-*C795* have an increased in three and two viable spore tetrads compared to wild type. This specific meiotic defect of *SGS1* mutants, which might be due to the absence of the helicase domain in the C-terminal part of the protein (see § 3.3.3 and § 3.4 for details), has also been reported by others (Gangloff *et al.*, 1999; Miyajima *et al.*, 2000b; Rockmill *et al.*, 2003). In the present work, we report two unique phenotypes for *sgs1*Δ and *sgs1*Δ-*C795*. Both recombination-associated death, in three viable spore tetrads, and two viable spore tetrads enriched in non-sister spores, are increased in *sgs1*Δ and *sgs1*Δ-*C795* compared to wild type. The *sgs1*Δ-*C795* has little mitotic-contributed death in meiosis (Chapter 3 and Rockmill et al. (2003)). Since, the meiotic defects described above can still be detected in the *sgs1*Δ-*C795* mutant, the contribution of a mitotic S-phase defect is less likely; although, it could not be discounted.

The increased interaction between chromatids in *sgs1Δ-C795* seen by Oh *et al.* (2007) might give an insight into the understanding of the meiotic defect in *SGS1* mutants. In the three viable spore tetrads class, both *sgs1Δ* and *sgs1Δ-C795* are increased for recombination events in the dead spores. This *sgs1* defect might be a result of crossover events involving more than two chromatids. Although, tetratypes are due to single crossover events between non-sister chromatids, they can also arise from closely spaced double crossovers between three chromatids. If such closely spaced double crossovers failed to be repaired in *SGS1* mutant strains, they could increased the proportion of recombinant dead spores (Figure 4.7 B).

Since two viable spore tetrads are increased in non-sister spores in *SGS1* mutants, we propose that death might be associated with the failure to resolve crossovers between homologues. Oh *et al.* (2007) have proposed that two successive end invasions, by the same DSB-end, can occur on two different templates (Figure 4.6 B – quaternary molecule). Then, if their model is correct, two successive end invasions of the same template (*i.e.* the same homologue) by the same DSB end might be possible. If such events occur, they could lead to two closely spaced double Holliday junctions between two chromatids (Figure 4.6 C). These secondary molecules (as opposed to tertiary and quaternary described by Oh *et al.*) would not be discernible from normal double Holliday junctions on 2D-gels. Failure to resolve these secondary molecules might lead to an increase in non-sister spores in the two viable spore tetrad class (Figure 4.7 C).

Since Sgs1p and Top3p have been shown to dissolve Holliday junction-like molecules in mitosis (see Chapter 1 § 1.4 for details), their function also might be required to dissolve closely spaced double Holliday junctions during meiosis. If proven, this function will confer a late role in meiosis to the Sgs1 protein.

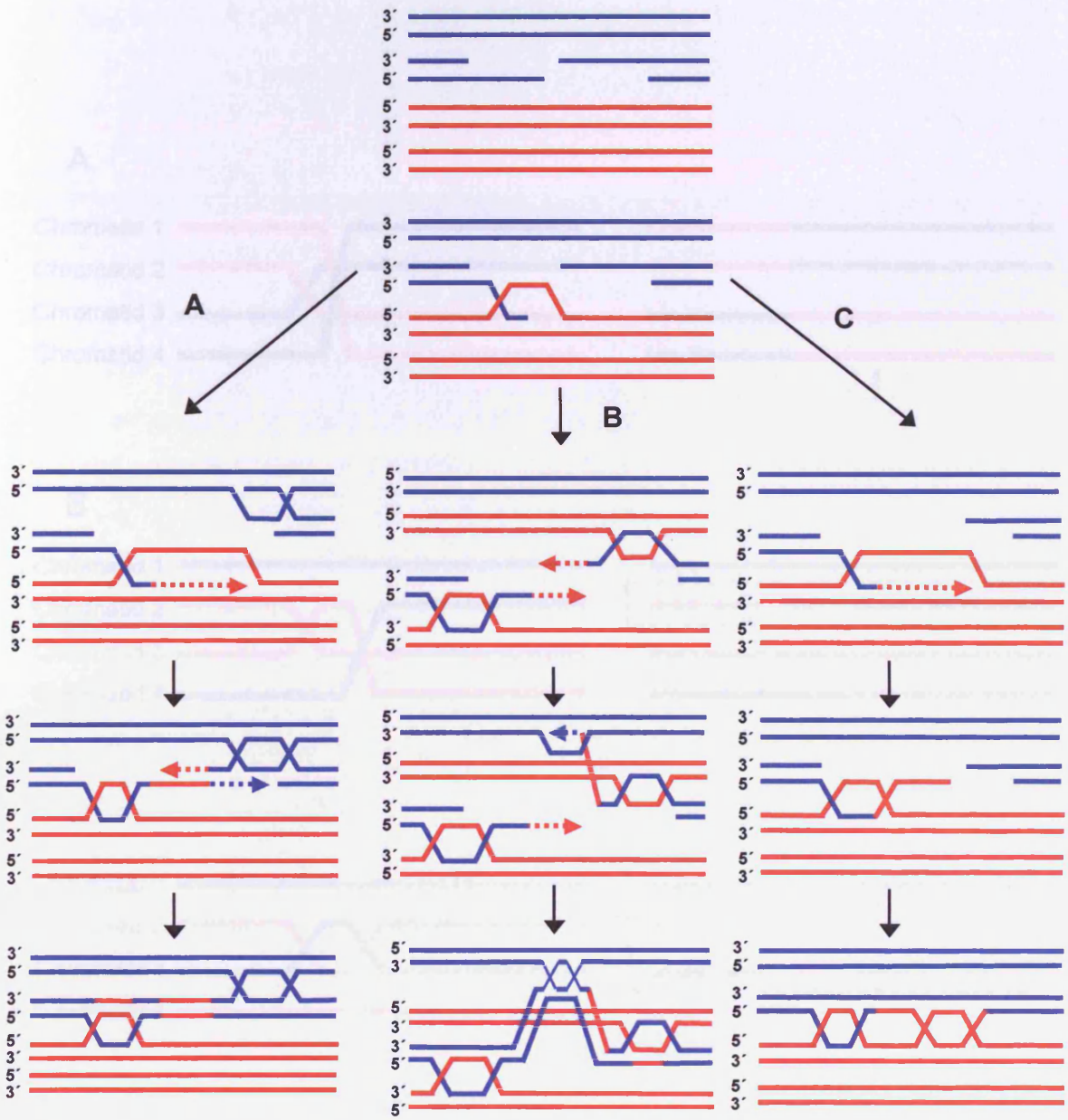


Figure 4.6: Schematic representation of molecular events leading to closely spaced double crossovers in *SGS1* mutants. Parental chromosomes and double stranded DNA are shown in blue and red.
A: After one end of the resected DSB has invaded one homologue, the other end of the break invades a sister chromatid. Such events lead to two dHJs involving three chromatids.
B: One end of the 3´-overhang of the DSB invades one homologue while the other end invades the other homologue. After the dHJs have collapsed, one 3´-overhang tail invades a sister chromatids creating three dHJs connecting the four chromatids. Such events, although complex seem to be resolved properly increasing the proportion of NPDs in the four viable spore tetrad class.
C: We hypotheses that instead of invading the sister chromatids (or one homologue), the collapsed dHJ could re-invade the same chromatid before being captured by the second end of the DSB. If occurring these events will lead to two dHJs involving two chromatids.
DSB: Double Strand Break; dHJ: double Holliday Junction.
Figure adapted from Oh *et al.* (2007).

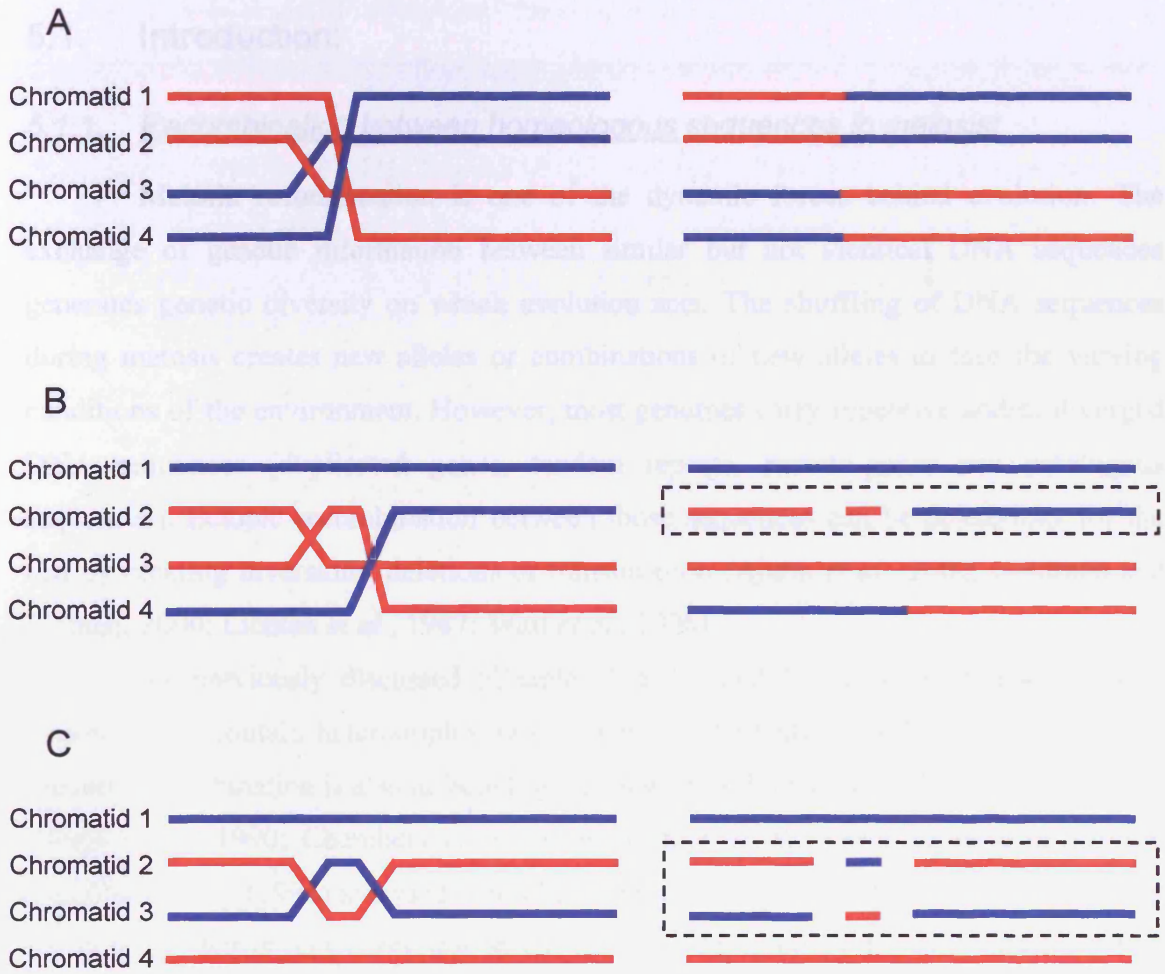


Figure 4.7: Schematic representation of events leading to an increase of NPDs, increased recombinant dead spores in the three viable spore tetrad class and increased non-sister spores in the two viable spore tetrad class. Blue and red lines represent parental chromatids. Dashed boxes mark dead spores. **A**: Double crossovers involving four chromatids create non-parental ditypes in the four viable spore tetrads class. **B**: Closely spaced double crossovers involving three chromatids might not be resolved properly. These unresolved structures lead to cell death creating an increase of recombinant dead spore in the three viable spore tetrads class. **C**: Closely spaced double crossovers involving two chromatids might not be resolved properly and lead to cell death creating an increase of non-sister spores in the two viable spore tetrads class.

Chapter 5: Sgs1p prevents inappropriate recombination events in meiosis:

5.1. Introduction:

5.1.1. *Recombination between homeologous sequences in meiosis:*

Meiotic recombination is one of the dynamic forces behind evolution. The exchange of genetic information between similar but not identical DNA sequences generates genetic diversity on which evolution acts. The shuffling of DNA sequences during meiosis creates new alleles or combinations of new alleles to face the varying conditions of the environment. However, most genomes carry repetitive and/or diverged DNA sequences (duplicated genes, tandem repeats, pseudo-genes and paralogous sequences). Ectopic recombination between those sequences can be deleterious for the cell by creating inversions, deletions or translocation (Ajima *et al.*, 2002; Goldman and Lichten, 2000; Lichten *et al.*, 1987; Watt *et al.*, 1996).

As previously discussed (Chapter 1 § 1.5 and § 1.6.3), single-end invasion intermediates contain heteroduplex DNA (Allers and Lichten, 2001a). Like in mitosis, meiotic recombination is also affected by the degree of divergence in heteroduplex DNA (Borts *et al.*, 1990; Chambers *et al.*, 1996; Hunter *et al.*, 1996; Selva *et al.*, 1995). Chambers *et al.*, (1996) analysed recombination in a strain where the entire chromosome III of a *Saccharomyces cerevisiae* strain has been replaced by the chromosome III of another close related species, *Saccharomyces paradoxus*. This strain is referred to as a "partial hybrid" as it carries the *S. paradoxus* chromosome III in an otherwise *S. cerevisiae* background. When crossed to a strain with a normal *S. cerevisiae* chromosome III, the resulting diploid becomes homeologous for chromosome III. The percentage of identity between the two chromosomes varies from 80 to 92% depending on the intervals. Experiments with this strain have demonstrated an increase in chromosome III missegregation. These events are mostly meiosis I non-disjunction due to failure of the homeologous chromosomes to recombine with each other (Chambers *et al.*, 1996). Other experiments involving a homeologous pair of chromosome V (one *S. cerevisiae* and one *S. carlsbergensis*) in an otherwise *S. cerevisiae* background have come to similar conclusions. The two homeologous chromosomes in this case share a

71% sequence identity along their entire length (Maxfield Boumil *et al.*, 2003). Furthermore, both studies have found that the reduced frequency in crossing over events is not due to a decrease in double-strand breaks (Chambers, 1999; Maxfield Boumil *et al.*, 2003). In addition, the decreased crossover frequency in these homeologous strains is dependent on the mismatch repair genes *MSH2*, *MSH3*, *MSH6* and, to a lesser extent, *PMS1* (Chambers *et al.*, 1996; Chambers, 1999; Chen and Jinks-Robertson, 1999; Hunter *et al.*, 1996; Maxfield Boumil *et al.*, 2003). Furthermore, a correlation between the degree of divergence and the rate of recombination has been made in *MMR* mutants in *S. cerevisiae* (*msh2*Δ and *pms1*Δ) and *Arabidopsis thaliana* (*AtMSH2*$^{-/-}$)(Chen and Jinks-Robertson, 1999; Li *et al.*, 2006b). In both organisms, the effect of different degrees of homeology, ranking from 0% to 15% divergence between a chromosome pair, have been analysed. The ratio of crossover frequency in *msh2*Δ to the crossover frequency in wild type increases with the sequence divergence until it reaches a plateau. Above 9% of divergence, the cumulative effect of the sequence divergence is no longer increased. This observation suggests that MMR proteins, such as Msh2p, can detect a certain level of sequence divergence. However, above this level, the sequence similarity between homeologue might not be sufficient to allow single-ends invasion to take place and therefore, the mismatch repair proteins to act (Chambers *et al.*, 1996; Chen and Jinks-Robertson, 1999; Li *et al.*, 2006b). The mismatch repair system seems to provide a safeguard against deleterious rearrangements by not allowing recombination to take place between diverged DNA sequences. The regulation of recombination between homeologous sequences is therefore of importance to provide the balance between the fidelity of recombination and the plasticity needed for the creation of diversity.

As seen in the introduction of this thesis (Chapter 1 § 1.6.3), RecQ helicases such as BLM in human and Sgs1p in *S. cerevisiae*, co-immuoprecipitate with the mismatch repair proteins Mlh1p and Msh6p in mitosis. Therefore, we hypothesis that the helicase activity of the Sgs1 protein might be responsible for the unwinding of heteroduplex DNA during homeologous recombination.

5.1.2. Partner choice during meiosis I:

Contrary to mitosis, where recombination uses sister chromatids to repair DNA lesions (Kadyk and Hartwell, 1992; Symington, 2002), meiotic recombination primarily takes place between homologues. This is to ensure, as discussed earlier, the correct orientation of homologous pairs. In meiosis, there is a real bias toward homologue recombination compared to sister chromatid recombination. The rate of recombination between non-sister chromatids is between 3 to 10-fold greater than between sister chromatids (Petes and Pukkila, 1995).

It was first hypothesised that the partner choice during meiosis was driven by the meiosis specific *RecA* homologue, *DMC1* (Bishop *et al.*, 1992). Although appealing, the idea of an active recombination bias toward the homologous chromatids does not seem to be the whole story. Both strand exchange proteins, Rad51p and Dmc1p, play a role during meiotic recombination. In diploid cells deleted for *DMC1* no interhomolog-strand invasion takes place and DSBs are left unrepaired and hyperresected (Bishop *et al.*, 1992; Hunter and Kleckner, 2001). The impaired inter-homologue recombination phenotype of *dmc1*Δ can be compensated by over-expression of *RAD51* (Tsubouchi and Roeder, 2003). In *rad51*Δ mutants, the repair of double-strand breaks is also affected, but more importantly, the joint-molecules formed have an 8-fold decrease of inter-homologue recombination events compared to inter-sister recombination (Schwacha and Kleckner, 1997). Thus, *DMC1* and *RAD51* might act together during homologous recombination in yeast. Other evidence suggests that the bias toward the homologues might be due to a barrier to sister chromatid recombination (Niu *et al.*, 2005). Various studies have shown that proteins involved in axial associations are also involved in partner choice. The Red1/Hop1/Mek1 protein complex plays an essential role in blocking inter-sister chromatid recombination rather than promoting recombination toward the homolog (Niu *et al.*, 2005; Niu *et al.*, 2007; Sheridan and Bishop, 2006; Wan *et al.*, 2004). Experiments with a drug inducible *mek1* phosphorylation-deficient mutant (*mek1-as1*) have shown that the kinase activity of Mek1p is essential to block recombination between sister chromatids. In a *dmc1*Δ *mek1-as1* double mutant double-strand breaks are repaired using the sister chromatid. The kinase activity of Mek1 requires Red1/Hop1 complex and is also dependent on Mek1 dimerisation (Niu *et al.*, 2005; Wan *et al.*, 2004). Recently, another meiosis specific protein has been identified,

Hed1p. In *hed1Δ dmc1Δ* double mutants, the phenotypes generally associated with *dmc1Δ*, such as unrepaired DSBs, sporulation and viability defects, are improved (Tsubouchi and Roeder, 2006). In the BR and SK1 strain backgrounds, deletion of *HED1* in *dmc1Δ* diploid rescues sporulation to a wild type level (BR strain) while viability is also greatly improved. Interestingly, these phenotypes cannot be rescue when *RAD51* is deleted in the *hed1Δ dmc1Δ* strain. Thus, Rad51p is essential for the sporulation and the viability of *hed1Δ dmc1Δ* mutants. These authors have also shown that the *HED1* deletion mimics the effect of Rad51p overexpression in *dmc1Δ* cells. Not surprisingly, Hed1p and Rad51p interact in a yeast two-hybrid assay (Tsubouchi and Roeder, 2006). Furthermore, ectopic expression of the Hed1 protein in vegetative cells impairs mitotic DSB repair. Tsubouchi *et al.* (2006) have therefore proposed that Hed1p down regulates the activity of Rad51p during meiotic recombination. Sheridan and Bishop (2006) hypothesize that Hed1p could be phosphorylated by Mek1p and therefore re-enforces the barrier toward sister chromatid recombination by inactivating the strand invasion activity of Rad51p toward the sister chromatids. At this point, the bias toward the homologues seems to involve blocking recombination between sister chromatids rather than actively promoting recombination between homologous chromosomes.

As mentioned earlier (Chapter 1 § 1.3), a key phenotype in Bloom's patients, in mitosis, is the high level of sister chromatid exchange in cultured *bs* cells (Chaganti *et al.*, 1974). Transfection of a wild-type *BLM* cDNA gene in cultured *bs* cells restores the level of sister chromatid exchanges to normal (Ellis *et al.*, 1999). Interestingly, *sgs1* mutants have been reported to increase the level of sister chromatid exchange as monitored by unequal crossovers between sister chromatids in mitosis (Onoda *et al.*, 2000). More specifically, the *sgs1Δ* unequal sister chromatid exchange phenotype could not be complemented with a plasmid carrying an allele of *SGS1* mutated in the helicase domain (*sgs1-hd* – mutation K706A) (Onoda *et al.*, 2000). Thus, in mitosis, the helicase domain of Sgs1p is essential for the suppression of unequal crossover events between sister chromatids. Study of the double mutants *sgs1Δ rad52Δ* and *sgs1Δ msh2Δ* shows that the increase of unequal sister chromatid exchange in *sgs1Δ* cells is dependent on recombination (*RAD52* pathway) but also upon Msh2p. Interestingly, analysis of an *MSH2* point mutant defective for mismatch repair correction could also rescue the sister chromatid exchange phenotype of *sgs1Δ* cells, implying that the mismatch repair function of Msh2p is not required for this function (Onoda *et al.*, 2004).

Based on the above data, we hypothesise that Sgs1p and MMR proteins might play a role in suppressing inappropriate events such as homeologous recombination and unequal sister chromatid recombination during meiosis.

5.2. Experimental procedures:

5.2.1. Plasmid construction

A pRS306 plasmid, carrying a *SacI-SacII* fragment carrying the *CYH2* ORF (cycloheximide sensitive gene) was digested by the restriction enzymes *SacI-ClaI* (to clone out the *CYH2* ORF) and *AatII-NcoI* (to digest the remaining plasmid). The *SacI-ClaI* fragment was then ligated at 16°C for 12 hours, using T4 DNA ligase, into the pAG32 vector (*HYGMX4*, Chapter 2) pre-digested with *SacI* and *ClaI*. The products of ligation were used to transform *E. coli* DH5α cells (Invitrogen). After transformation, *E. coli* cells were plated on Luria-Broth medium supplemented with ampicillin. Plasmids from ampicillin resistant clones were extracted using the methods described in chapter 2. Confirmation of the insertion of the *CYH2* gene in pAG32 was performed using restriction digestion with *HindIII* and *XhoI* enzymes. One positive clone was re-extracted using a Qiagen mini-prep® extraction kit. This plasmid was named pRED548 and was used to PCR-amplify the *HYG-CYH2* genes for transformation in *S. cerevisiae*.

5.2.2. Strain construction:

Insertion of the HYG-CYH2 and HYG drug resistance genes:

The *HYG-CYH2* and *HYG* cassettes were inserted on chromosome III via PCR-replacement method and crossing. First, the *HYG-CYH2* genes were PCR amplified from the plasmid pRED548 and inserted via a one step replacement method (Chapter 2) between the *RRP7* and *HIS4* genes in a *MAT a* strain. The *HYG* cassette was PCR amplified from pAG32 and inserted via the same method between *LEU2* and *NFS1* in a *MAT α* strain. The different primers used for amplification are described in Table 2.3 (Chapter 2). The transformed *MAT a* and *MAT α* strains were then crossed to each other. The resulting diploids were dissected after four days on sporulation medium. Crossovers between the two *HYG* genes were identified as tetrads with three hygromycin resistant spores (3:1 HYG^R: HYG^S, Figure 5.1). Confirmation of the co-segregation of both drug resistance cassettes was done via junction PCR (Chapter 2 § 2.2.13). Strains carrying the *HYG-CYH2/HYG* cassettes were used to monitor intra and inter-chromatids recombination events.

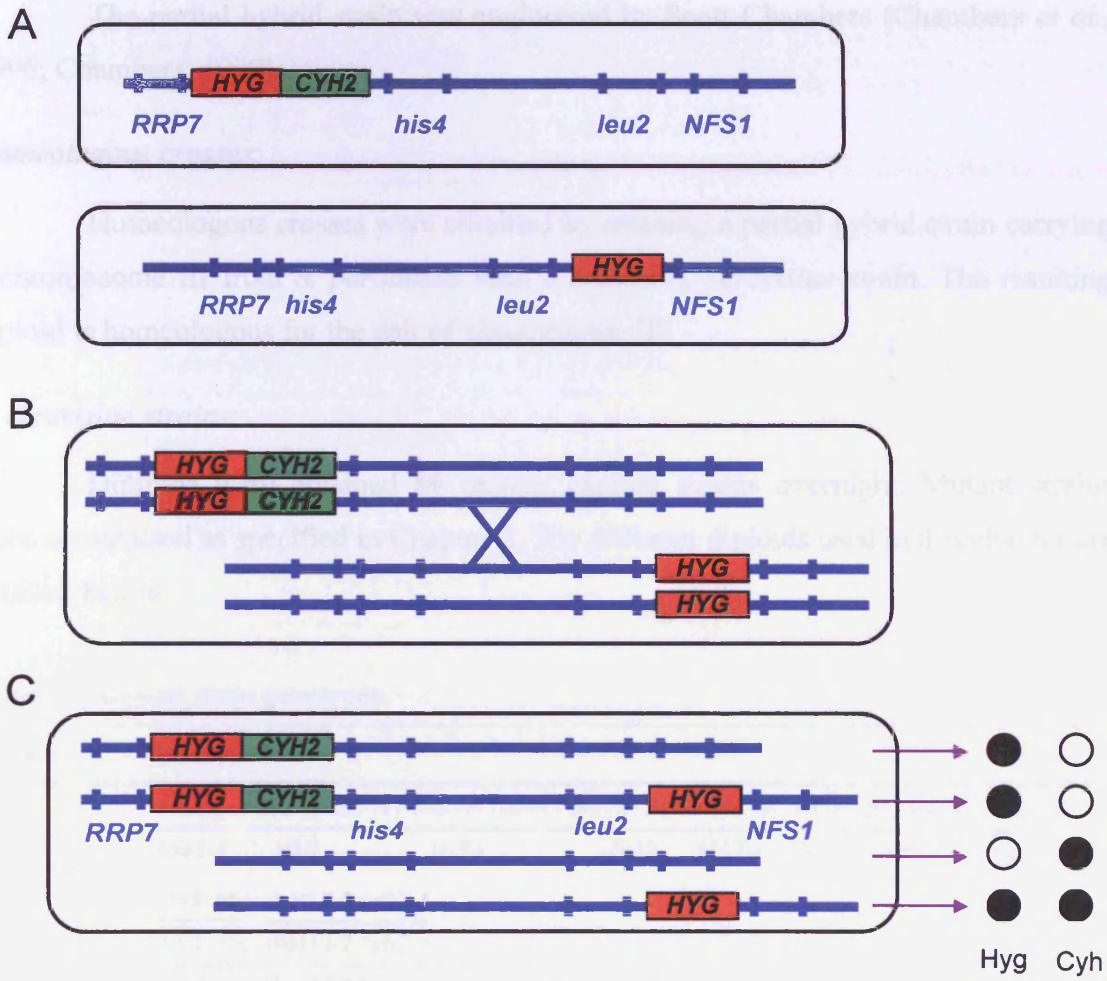


Figure 5.1: Construction of the *HYG-CYH2/HYG* strain.

A: The *HYG-CYH2* and *HYG* cassettes were each inserted on chromosome III of two different strains via one-step PCR.

B: After mating the two strains and tetrad dissection, four viable spore tetrads were screened for crossovers between *HIS4* and *LEU2*.

C: Schematic representation of a meiosis event where a crossover occurred between *HIS4* and *LEU2*. The resulting four viable spore tetrad colony has a 3:1 Hyg^R:Hyg^S segregation pattern when plated on hygromycin supplemented medium (Hyg). On cycloheximide supplemented medium (Cyh), the same meiosis has a 2:2 Cyh^R:Cyh^S segregation pattern. Thus, in this tetrads one spore colony is Hyg^S and Cyh^R, inferring that it does not contain either cassettes. Another spore colony is Hyg^R and Cyh^R, inferring that it does not contain the *HYG-CYH2* cassette but has retained the *HYG* cassette. Spore colonies which were HYG^R and $CYH2^S$ were further analysed by junction-PCR to determine the ones containing both the *HYG-CYH2* and *HYG* cassettes.

Partial hybrid strains:

The partial hybrid strain was engineered by Scott Chambers (Chambers *et al.*, 1996; Chambers, 1999).

Homeologous crosses:

Homeologous crosses were obtained by crossing a partial hybrid strain carrying a chromosome III from *S. paradoxus* with a normal *S. cerevisiae* strain. The resulting diploid is homeologous for the pair of chromosome III.

S. cerevisiae strains:

Diploids were obtained by mating haploid strains overnight. Mutant strains were constructed as specified in Chapter 2. The different diploids used in this chapter are detailed below:

Table 5.1: Diploid strain genotypes.

Strains	Genotypes
ACD 97 *SGS1* WT control	*ade1Δ HML::ADE1 HYG-CYH:his4-r1 leu2-r1:HYG MATa HOΔ trp1::bsu36 lys2-d* *ade1-1 HML HIS4 LEU2 MATα HOΔ TRP1 LYS2* *ura3::nco met13-2 cyh2-1* *ura3::nco met13-2 cyh2-1*
ACD 94 *SGS1* WT homeologous control	*ade1Δ HML::ADE1 HYG-CYH:his4-r1 leu2-r1:HYG MATa HOΔ trp1::bsu36 lys2-d* *ade1-1 HML HIS4 LEU2 MATα HOΔ TRP1 LYS2* *ura3::nco met13-2 cyh2-1 KAR1* *ura3::nco met13-2 cyh2-1 kar1Δ*
ACD 95 homozygote *sgs1Δ*	*ade1Δ HML::ADE1 HYG-CYH:his4-r1 leu2-r1:HYG MATa HOΔ trp1::bsu36 lys2-d* *ade1-1 HML HIS4 LEU2 MATα HOΔ TRP1 LYS2* *ura3::nco met13-2 cyh2-1 sgs1::KANMX4* *ura3::nco met13-2 cyh2-1 sgs1::KANMX4*
ACD 96 Homeologous homozygote *sgs1Δ*	*ade1Δ HML::ADE1 HYG-CYH:his4-r1 leu2-r1:HYG MATa HOΔ trp1::bsu36 lys2-d* *ade1-1 HML HIS4 LEU2 MATα HOΔ TRP1 LYS2* *ura3::nco met13-2 cyh2-1 KAR1 sgs1::KANMX4* *ura3::nco met13-2 cyh2-1 kar1Δ sgs1::KANMX4*

Table 5.1 (continue): Diploid strain genotypes.

Strains	Genotypes
ACD 107 homozygote *sgs1Δ-C795*	*ade1Δ HML::ADE1 HYG-CYH:his4-r1 leu2-r1:HYG MATa HOΔ trp1::bsu36 lys2-d* *ade1-1 HML HIS4 LEU2 MATα HOΔ TRP1 LYS2* *ura3::nco met13-2 cyh2-1 sgs1::NATMX4* *ura3::nco met13-2 cyh2-1 sgs1::NATMX4*
ACD 108 homeologous heterozygote *sgs1Δ-C795*	*ade1Δ HML::ADE1 HYG-CYH:his4-r1 leu2-r1:HYG MATa HOΔ trp1::bsu36 lys2-d* *ade1-1 HML HIS4 LEU2 MATα HOΔ TRP1 LYS2* *ura3::nco met13-2 cyh2-1 KAR1 sgs1::NATMX4* *ura3::nco met13-2 cyh2-1 kar1Δ sgs1::KANMX4*
ACD 127 homozygote *msh6Δ*	*ade1Δ HML::ADE1 HYG-CYH:his4-r1 leu2-r1:HYG MATa HOΔ trp1::bsu36 lys2-d* *ade1-1 HML HIS4 LEU2 MATα HOΔ TRP1 LYS2* *ura3::nco met13-2 cyh2-1 msh6::KANMX4* *ura3::nco met13-2 cyh2-1 msh6::KANMX4*
ACD 133 homeologous homozygote *msh6Δ*	*ade1Δ HML::ADE1 HYG-CYH:his4-r1 leu2-r1:HYG MATa HOΔ trp1::bsu36 lys2-d* *ade1-1 HML HIS4 LEU2 MATα HOΔ TRP1 LYS2* *ura3::nco met13-2 cyh2-1 KAR1 msh6::KANMX4* *ura3::nco met13-2 cyh2-1 kar1Δ msh6::KANMX4*
ACD 138 homozygote *sgs1Δmsh6Δ*	*ade1Δ HML::ADE1 HYG-CYH:his4-r1 leu2-r1:HYG MATa HOΔ trp1::bsu36 lys2-d* *ade1-1 HML HIS4 LEU2 MATα HOΔ TRP1 LYS2* *ura3::nco met13-2 cyh2-1 sgs1::KANMX4 msh6::KANMX4* *ura3::nco met13-2 cyh2-1 sgs1::KANMX4 msh6::KANMX4*
ACD 139 homeologous homozygote *sgs1Δmsh6Δ*	*ade1Δ HML::ADE1 HYG-CYH:his4-r1 leu2-r1:HYG MATa HOΔ trp1::bsu36 lys2-d* *ade1-1 HML HIS4 LEU2 MATα HOΔ TRP1 LYS2* *ura3::nco met13-2 cyh2-1 KAR1 sgs1::KANMX4 msh6::KANMX4* *ura3::nco met13-2 cyh2-1 kar1Δ sgs1::KANMX4 msh6::KANMX4*

5.2.3. PCR from CHEF plugs:

After CHEF gel analyses were performed, the remainder of the plugs were used for DNA extraction and PCRs. An Eppendorf tube containing a third of an agarose plug was chilled on ice for 30 minutes with 40µl of 1X ß-agarase buffer (NEB). After this incubation time, the solution was discarded and replaced with fresh buffer. The incubation was continued for another 30 minutes. Upon completion, the buffer was discarded and the plug was incubated at 65°C for 10 minutes. After the agarose had melted, 1.5µl of ß-agarase was added and the solution was incubated at 42°C for 1h 30 min. The tube was then place on ice to ensure that no solid agarose remained. 200µl of 1X TE was added to the solution and normal phenol-chloroform extraction were performed to remove the enzyme. DNA was then ethanol precipitated and resuspended in 50µl of 1X TE. DNA was used for PCR reactions and sequencing (Chapter 2).

5.2.4. DNA probe:

Southern blot analyses were performed as explained in chapter 2. The plasmid pRS306 carrying the *CYH2* gene and a *URA3* gene was fluorescein-labelled (random prime dUTP, GE-Healthcare) and used as a probe. Detection of the hybridized probe was performed using the Gene Image, CDP-Star detection system (GE-Healthcare) according to the manufacturer recommendations.

5.2.5. Statistical analysis:

A Chi-square test referred to as the synthetic lethal test was used to assess if the defects associated with two different mutants were additive or not. The expected pattern for individual synthetic lethal defect was calculated as follow:

Given a strain "A" associated with a viability defect. The tetrad class distribution for such strain is: a4 (number of 4 viable spore tetrads), a3 (number of 3 viable spore tetrads), a2 (number of 2 spore tetrads), a1 (number of 1 spore tetrads) and a0 (number of 0 spore tetrads).

The frequencies associated with such distribution are:

f(a4) = a4/(a4+a3+a2+a1+a0)

f(a3) = a3/(a4+a3+a2+a1+a0)

f(a2) = a2/(a4+a3+a2+a1+a0)

f(a1) = a1/(a4+a3+a2+a1+a0)

f(a0) = a0/(a4+a3+a2+a1+a0)

Given a strain "B" also associated with a viability defect. The tetrad class distribution in strain "B" is: b4 (number of 4 spore tetrads), b3 (number of 3 spore tetrads), b2 (number of 2 spore tetrads), b1 (number of 1 spore tetrads) and b0 (number of 0 spore tetrads) associated with the frequencies:

f(b4)
f(b3)
f(b2)
f(b1)
f(b0)

} as calculated previously.

It is possible to estimate the death that would be associated in the double mutant "AB" if the effects of the two mutations were independent (also called additive).

The estimated frequency of 4 viable spore tetrads in the hypothetical double mutant (AB) is given by:

f(ab4) = f(a4) x f(b4)

The estimated frequency of 3 viable spore tetrads in such strain will be the contribution of death events associated with A times the death events associated with B and ¼ of the death event associated with A and B (1 dead spore out of 4):

f(ab3) = f(a4)f(b3) + f(a3)f(a4) + f(a3)f(b3)/4

The estimated frequency of 2 viable spore tetrads in the hypothetical strain is the contribution of the death associated with strain A and strains B in the 4, 3 and 2 viable spore tetrads:

f(ab2) = f(a4)f(b2) + f(a2)f(b4) + ¾ f(a3)f(b3) + ½ [f(a3)f(b2) + f(a2)f(b3)] + $^{1}/_{6}$ f(a2)f(b2)

The same calculations are done to estimate the frequencies of 1 viable spore tetrads and 0 viable spore tetrads in the "AB" strains.

This expected distribution of spore classes was compared to the observed distribution in the double mutant using Chi-square test. The null hypothesis is: "the two distributions are different". p values < 0.05 were considered significant.

5.3. Results:

5.3.1. *SGS1 and MSH6 mutants restore crossover defects in homeologous strains:*

In the wild-type homeologous strain, the deficit of crossovers between homeologous chromosome III (Chambers *et al.* (1996); this study, tables 5.2-4) was attributed to the lack of homology in the different intervals. Since drug resistance cassettes were inserted on the *S. cerevisiae* chromosome III, crossover frequencies were re-analysed.

Table 5.2: Decrease of crossovers in WT homeologous strain in the *HML-HIS4* interval.

	Tetrad distribution			Four viable spores tetrads	Map distance (cM)	G-test, p values [a]
	PD	NPD	TT			
SGS1 homologous	196	20	363	579	41.7	n.a
SGS1 homeologous	407	0	3	410	0.4	<<0.01

PD: Parental Ditype; NPD: Non Parental Ditype; TT: Tetratype.
a p values < 0.05 were considered significant.

Table 5.3: Decrease of crossovers in WT homeologous strain in the *HIS4-LEU2* interval.

	Tetrad distribution			Four viable spores tetrads	Map distance (cM)	G-test, p values [a]
	PD	NPD	TT			
SGS1 homologous	404	2	165	571	15.5	n.a
SGS1 homeologous	409	0	1	410	0.1	<<0.01

PD: Parental Ditype; NPD: Non Parental Ditype; TT: Tetratype.
[a] p values < 0.05 were considered significant.

Table 5.4: Decrease of crossovers in WT homeologous strain in the *LEU2-MAT* interval.

	Tetrad distribution			Four viable spores tetrads	Map distance (cM)	G-test, p values [a]
	PD	NPD	TT			
SGS1 homologous	340	15	250	605	28.1	n.a
SGS1 homeologous	399	0	10	409	1.2	<<0.01

PD: Parental Ditype; NPD: Non Parental Ditype; TT: Tetratype.
[a] p values < 0.05 were considered significant.

In all three intervals (*HML-HIS4*, *HIS4-LEU2* and *LEU2-MAT*) the frequency of crossovers is reduced in the homeologous cross compared to the wild type homologous strain (Table 6-2 to 6-4).

A genetic screen for hyper and hypo-recombination using a wild-type partial hybrid strain transformed with a transposon library (Burns *et al.*, 1994) has shown that Sgs1p might play a role in this hypo-recombination phenotype (Chambers, 1999). Therefore, strains mutant for *sgs1* and *msh6* were tested for their recombination phenotypes in the partial hybrid strain.

Table 5.5: Crossovers in homeologous strains in the *HML-HIS4* interval.

	Tetrad distribution			Four viable spores tetrads	Map distance (cM)	G-test, p values [a]
	PD	NPD	TT			
SGS1 homeologous	407	0	3	410	0.4	n.a
sgs1Δ homeologous	95	0	5	100	2.5	0.028
sgs1Δ-C795 homeologous	141	0	8	149	2.7	< 0.0125
msh6Δ homeologous	200	0	9	209	2.2	0.0129
sgs1Δmsh6 homeologous	52	0	7	59	5.9	< 0.0125

PD: Parental Ditype; NPD: Non Parental Ditype; TT: Tetratype.
[a] p values < 0.0125 were considered significant compared to WT.

Table 5.6: Crossovers in homeologous strains in the *HIS4-LEU2* interval.

	Tetrad distribution			Four viable spores tetrads	Map distance (cM)	G-test, p values [a]
	PD	NPD	TT			
SGS1 homeologous	409	0	1	410	0.1	n.a
sgs1Δ homeologous	97	0	3	100	1.5	0.05
sgs1Δ-C795 homeologous	144	0	3	147	1	0.126
msh6Δ homeologous	209	0	1	210	0.2	0.89
sgs1Δmsh6Δ homeologous	57	0	2	59	1.7	0.09

PD: Parental Ditype; NPD: Non Parental Ditype; TT: Tetratype.
[a] p values < 0.0125 were considered significant compared to WT.

Table 5.7: Crossovers in homeologous strains in the *LEU2-MAT* interval.

	Tetrad distribution			Four viable spores tetrads	Map distance (cM)	G-test, p values [a]
	PD	NPD	TT			
SGS1 homeologous	399	0	10	409	1.2	n.a
sgs1Δ homeologous	90	1	9	100	7.5	< 0.0125
sgs1Δ-C795 homeologous	132	2	13	147	8.5	<< 0.0125
msh6Δ homeologous	188	0	23	211	5.5	<< 0.0125
sgs1Δmsh6Δ homeologous	52	0	7	59	5.9	< 0.0125

PD: Parental Ditype; NPD: Non Parental Ditype; TT: Tetratype.
[a] p values < 0.0125 were considered significant compared to WT.

Due to the poor sporulation and spore viability of the different mutants analysed only a small data set was collected. This, along with the confidence interval of 98.75% due to multiple tests, affected the statistical analysis of the various mutants. Although the different *sgs1* mutants, *msh6Δ* and the double mutant *sgs1Δmsh6Δ* have an increase of crossovers from 2-fold to 15-fold, this increase is not reflected by a statistical difference in all genetic intervals. In the *HML-HIS4* interval (Table 5.5), only the partial mutant *sgs1Δ-C795* (6.75-fold increase) and the double mutant *sgs1Δmsh6Δ* (14.75-fold increase) are statistically different from the *SGS1* homeologous strain (p values < 0.0125). Interestingly, none of the mutants that increased crossover frequencies are different from wild type in the *HIS4-LEU2* interval (Table 5.6). In the *LEU2-MAT* interval, all four mutants, *sgs1Δ*, *sgs1Δ-C795, msh6Δ* and *sgs1Δmsh6Δ* have a significant increase of crossovers compared to the SGS1 WT homeologous strains (Table 5.7).

5.3.2. Meiosis I defects are associated with a deficit of crossovers:

In the wild-type homeologous strains, the increase in two viable spore tetrads can be attributed to meiosis I defects (Chambers *et al.*, 1996). Due to a deficit in crossovers between the chromosomes III pair, homeologues segregate to either or both pole of the dividing cell at meiosis I (see § 4.1.5 for details). Although, past studies have shown that, as in *D. melanogaster*, the segregation of homologue lacking crossovers is not random in yeast (Dawson *et al.*, 1986; Guacci and Kaback, 1991). This specific segregation, also called distributive segregation, ensures that non-recombinant

chromosomes or non-homologous chromosomes can still segregate properly during meiosis I. If both chromosomes III segregate to the same pole, the resulting two viable spore tetrad will be aneuploid with two chromatids, one chromatid carrying a *MATα* loci and the other a *MATa* loci. They will therefore be non-maters (Figure 4.4). In the *SGS1* homeologous strain the deficit of crossovers associated with homeology can be linked to an increase of meiosis I non-disjunction. The proportion of meiosis I non-disjunctions was assessed by the number of non-maters and two viable sister spore tetrads (Table 5.8).

Table 5.8: Proportion of meiosis I non-disjunction in SGS1 WT homeologous strain.

	Meiosis I non-disjunction[a] of chromosome III	Sister spores[b]	Non-sister spores
SGS1 homeologous	78/682 (11.44%)	78/93	15/93

[a] Meiosis I non-disjunctions were assessed by the numbers of non-maters pairs/total number of tetrads dissected.
[b] Pairs of sister *vs.* non-sister spores were distinguished via the segregation of the centromere marker TRP1 on chromosome VII.

In the *SGS1* homeologous strains, the increase of two viable spores (Figure 5.3) is due to an increase of meiosis I non-disjunction as assessed by the frequency of non-maters (11.44%) and the increase of pairs of sister spores (Table 5.8). The distribution of pairs of sister spores *vs.* non-sister spores in the *SGS1* homeologous strain is different from that of a random distribution (Chi-square test, $p < 0.01$). The increase of sister spores is solely due to the meiosis I non-disjunction phenotype since all 78 sister spores were non-maters (Table 5.8). These phenotypes are consistent with those previously reported (Chambers, 1999; Hunter *et al.*, 1996; Roeder, 1990; Tsubouchi and Roeder, 2003).

5.3.3. *Deletion of SGS1 or MSH6 reduces meiosis I non-disjunctions:*

The main cause of meiosis I non-disjunction is a lack of crossovers between pairs of chromosomes (Chambers *et al.*, 1996; Hunter *et al.*, 1996; Roeder, 1995). Although the increase of crossover frequencies between the pair of homeologous chromosomes III (Tables 5.5-7) is not restored to a *SGS1 WT* homologous level (Table 5.2-4), it might be sufficient to alleviate some of the missegregation of the chromosomes III pairs during meiosis I.

Table 5.9: Reduced meiosis I non-disjunction in *sgs1* and *msh6* homeologous mutant strains.

Strains [a]	Meiosis I non-disjunction[b] of chromosome III (%)	# Tetrads [c]	Sister spores[d] (%)	Non-sister spores[e] (%)
WT	78 (11.44%)	682	78[¥] (11.43)	15 (2.19)
*sgs1*Δ	10[†] (1.61%)	620	68 (10.96)	77 (12.41)
*sgs1*Δ-*C795*	23[†] (3.89%)	591	68 (11.50)	86 (13.87)
*msh6*Δ	20[†] (6.07%)	329	32[¥] (9.72)	15 (4.55)
*sgs1*Δ*msh6*Δ	10[†] (2.82%)	354	52 (14.68)	54 (15.25)

[a] strains were homeologous for chromosome III.
[b] meiosis I non-disjunctions were assessed by the number of non-mating spores/total number of tetrads dissected. Sisters where only one spore was non-mating were included.
[c] # Tetrads: total number of tetrads dissected.
[d] Increased sister spore is due to meiosis I non-disjunction.
[e] Increased non-sister spores is to sgs1 defect (none of the non-sisters were non-maters).
[†] p value < 0.0125 were considered statistically different from WT.
[¥] p value < 0.05 were considered different from a random distribution.

As shown in § 5.3.2 and Tables 5.8 and 5.9, the *SGS1* homeologous strain is enriched in meiosis I non-disjunction. This defect is partially suppressed in *sgs1* mutants (*sgs1*Δ, *sgs1*Δ-*C795* and *sgs1*Δ*msh6*Δ) and *msh6*Δ (Table 5.9). This decrease of meiosis I non-disjunction in mutant strains is most likely linked to an increase of crossovers (Tables 5.5-7, § 5.3.3).

In the wild type, the meiosis I defect contributes to an increase in sister spores in the two viable spore tetrad class (Tables 5.8 and Figure 5.3). Furthermore, in the *msh6*Δ homeologous strain, although the meiosis I non-disjunctions are partially rescued compared to wild type (Table 5.9, p value < 0.0125), the two viable spore tetrads are enriched in pairs of sister spores (p value < 0.05). This enrichment suggests that deletion of the *MSH6* gene in meiosis is not accompanied with an increase of non-sister spores as in the *sgs1* mutants. However, due to the meiotic defect of *sgs1* mutants (*i.e.* increase in non-sister spores, § 4.3.3, Table 4.22) and the increase of sister spores due to the homeology between chromosomes III, the distribution of pairs of sister spores *vs.* pairs of non-sister spores in the *sgs1* mutants (*sgs1*Δ and *sgs1*Δ-*C795*, table 5.9) is no longer different from that of a random distribution (p values 0.45 and 0.14, respectively). The random distribution of sister and non-sister spore tetrads in *sgs1* homeologous mutants might be due to the equal contribution of the *sgs1* meiotic defect and the homeology defect in such strains.

5.3.4. *The overall pattern of spore viability indicates that sgs1Δ rescues the death associated with homeology:*

Chambers *et al.* (1996) have demonstrated that the decrease in spore viability in *SGS1 WT* homeologous diploids was due to meiosis I non-disjunction and a specific crossover defect. These authors have hypothesized that, in the *SGS1 WT* homeologous strain, the increase of three viable spore tetrads is due to "half-crossovers" (Figure 5.2). "Half crossovers" might come from crossovers where the D-loops failed to recapture the second resected end of a DSB. This hypothesis is supported by the recent physical study on double-strand break repair where single end invasion and strand re-capture have been shown to be two separable events (Hunter and Kleckner, 2001).

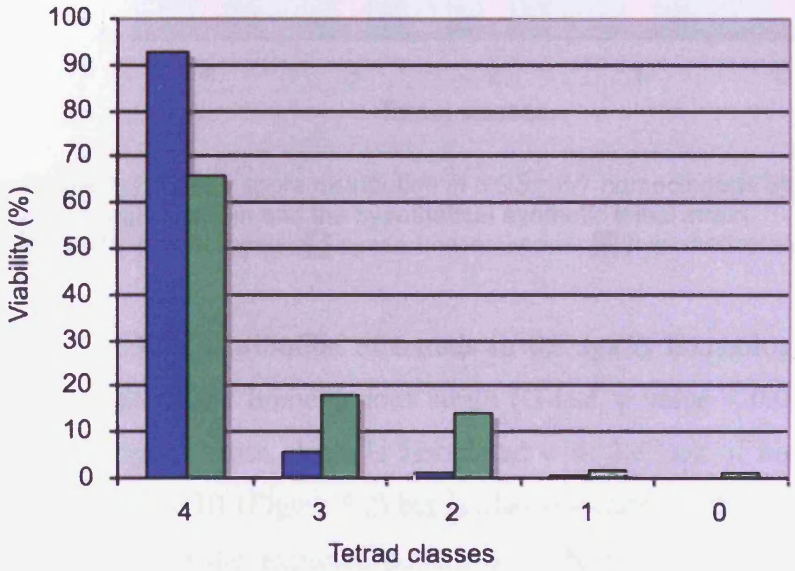


Figure 5.2: Tetrad spore distribution in *SGS1 WT* homologous strain compared to the *SGS1 WT* homeologous strain.
■ WT homologous; ■ WT homeologous.
G-test: $p = 3 \times 10^{-26}$

The viability in the *SGS1* homeologous strain is decreased compared to the *SGS1* homologous strain (p value < 0.05, G-test). This decreased viability is manifested by a decreased frequency in four viable spore tetrads and an increased frequency in three and two viable spore tetrads (Figure 5.2). We confirmed earlier in this chapter that the increase in two viable spore tetrads in *SGS1* homeologous diploids is due mainly to meiosis I non-disjunction (see § 5.3.2 for detail).

"Synthetic lethality" tests, described in the experimental procedures of this chapter (§ 5.2.3) can be used to test if the death associated with two particular mutations or defects are additive (*i.e.* independent – Figure 5.3).

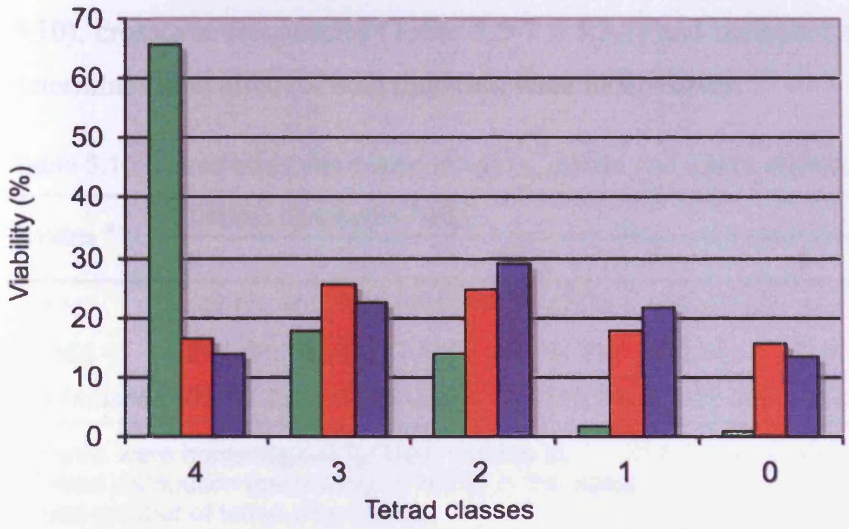


Figure 5.3: Tetrad spore distribution in *SGS1 WT* homeologous strain compared to the *sgs1*Δ homeologous strain and the hypothetical synthetic lethal strain.
SGS1 homeologous; *sgs1*Δ homeologous; hypothetical synthetic lethal strain.

The distribution of tetrads in the *sgs1*Δ homeologous strain is different from that of the *SGS1* homeologous strain (G-test, p value < 0.01, Figure 5.3). In the *sgs1*Δ homeologous cross, death is associated with the lack of homology between the pair of chromosome III (Figure 5.2) but is also associated with an *sgs1* defect (Figure 3.2). We can calculate the expected pattern of viability if the two defects are independent by imposing the pattern of homeology onto the pattern of *sgs1*Δ. Statistical comparison (Chi-squared test) between the distribution of viable spores in the *sgs1*Δ homeologous strain and this "synthetic lethal strain" shows that the distributions are statistically different (Figure 5.3, $p < 0.01$). Therefore the better viability (higher number of four and three viable spore tetrads and less two and one viable spores) associated with the *sgs1*Δ homeologous strain suggests that the *sgs1* mutation suppresses some of the defects associated with homeology.

5.3.5. *SGS1 is epistatic to MSH6 in the homeologous strains.*

As shown previously, both *SGS1* and *MSH6* mutants can alleviate some of the crossover and meiosis I non-disjunction defects in the homeologous strains. The effect of the double mutant *sgs1Δ msh6Δ* was therefore analysed in terms of viability (Table 5.10), crossover frequencies (Table 5.5-7 § 5.3.1) and meiosis I defects (Table 5.11) to determine if the effect of both mutation were independent.

Table 5.10: Tetrad class distribution in *sgs1Δ*, *msh6Δ* and *sgs1Δ msh6Δ*.

Strains [a]	Tetrads distribution [b] (%)					Total [c]	G-test [d]
	4	3	2	1	0		
sgs1Δ	102 (16.45)	159 (25.65)	153 (24.68)	110 (27.08)	96 (15.48)	620	n.a
msh6Δ	213 (64.74)	58 (17.63)	47 (14.29)	6 (1.82)	5 (1.52)	693	<<0.025
sgs1Δmsh6Δ	61 (17.23)	73 (20.62)	106 (29.94)	71 (20.06)	43 (12.15)	354	0.122

[a] Strains were homeologous for chromosome III.
[b] Tetrad distribution (percentage of tetrad in the class).
[c] Total number of tetrad dissected.
[d] G-tests compared the distribution of tetrads in *msh6Δ* and *sgs1Δmsh6Δ* to that of *sgs1Δ*; p values < 0.025 were considered significantly different from sgs1Δ.

The distribution of tetrad classes in the *sgs1Δ* homeologous strain is statistically different form the distribution of tetrad classes in the *msh6Δ* homeologous strain (G-test, p value < 0.025, Table 5.10). However, the difference in distribution between *sgs1Δ* and *sgs1Δ msh6Δ* is not of statistical significance (G-test, p value 0.122). This result suggests that the *sgs1Δ* mutation could be epistatic to the *msh6Δ* mutation.

Other lines of evidence suggest an epistasis relation between *SGS1* and *MSH6*. These are the crossover defect (Table 5.5-7; § 5.3.1) and the meiosis I non-disjunction phenotype (Table 5.11) in the *sgs1Δ msh6Δ* homeologous strain compared to the *sgs1Δ* homeologous strain. As seen previously *sgs1Δ* and *msh6Δ* mutants restore some of the crossovers due to the lack of homology between the homeologous chromosomes III. When comparing the distribution of crossovers in the intervals *HML-HIS4*, *HIS4-LEU2* and *LEU2-MAT* (Table 5.5-7), no differences were observed between *sgs1Δ* and the double mutant *sgs1Δmsh6Δ* (*HML-HIS4* interval: p value = 0.3; *HIS4-LEU2* interval: p value = 0.99 and *LEU2-MAT* interval: p value = 0.53). Although the data set is small, the absence of cumulative crossover effects between the single *sgs1* mutant and the double

mutant *sgs1Δ msh6Δ*, suggests that *SGS1* and *MSH6* might act in the same pathway. This hypothesis was confirmed by analysing the frequencies of meiosis I non-disjunctions in the double mutant (Table 5.11).

Table 5.11: Meiosis I non-disjunction in *sgs1Δ msh6Δ* homeologous strain:

Strains [a]	Meiosis I non-disjunction [b] (%)	# Tetrads [c]	Sister spores (%)	Non-sister spores (%)
sgs1Δ	10 (1.61%)	620	68 (10.96)	77 (12.41)
msh6Δ	20[†] (6.07%)	329	32[¥] (9.72)	15 (4.55)
sgs1Δmsh6Δ	10 (2.82%)	354	52 (14.68)	54 (15.25)

[a] strains were homeologous for chromosome III.
[b] meiosis I non-disjunction were calculated by means of non-mating two viable spore tetrads.
[c] # Tetrads: total number of tetrads dissected.
[†] p value = 0.0003, p value < 0.0125 were considered statistically different from *sgs1Δ*.
[¥] p value = 0.013, p value < 0.05 were considered different from a random distribution.

The distribution of meiosis I non-disjunctions in *sgs1Δ* is statistically different from the distribution in the *msh6Δ* mutant (Table 5.11, p value = 0.0003). Interestingly, the distribution in the double mutant *sgs1Δmsh6Δ* is no different from that of *sgs1Δ* (p value = 0.2) suggesting that *sgs1Δ* is epistatic to *msh6Δ*. Therefore they might act in the same pathway. As seen previously, in *SGS1* cells, meiosis I non-disjunctions increase the frequency of sister spores (Table 5.9). This is confirm in *msh6Δ* mutant where the proportion of two spore viable containing sister spores is also increased. The distribution of two viable spore tetrads in the *sgs1Δ msh6Δ* mutant is random (Chi-square test, p value > 0.05). The random distribution of pairs of sister spores and non-sister spores is probably due to the meiotic defect of the *sgs1Δ* mutant as seen in § 4.3.5 and explained in § 5.3.3.

5.3.6. Differentiation of intra- and inter-sister chromatid events:

The strain carrying the *HYG-CYH2* and *HYG* cassettes is hygromycin resistant and cycloheximide sensitive due to the insertion of the dominant *HYG* genes and the dominant *CYH2* sensitive gene, on chromosome III. However, this strain also carries the recessive *cyh2* resistant allele at its endogenous location, on chromosome VII. In haploid cells, deletion or mutation of the *CYH2* gene on chromosome III will give rise to cycloheximide resistant colonies. After mating of the *HYG-CYH2/HYG* strain with an *S.*

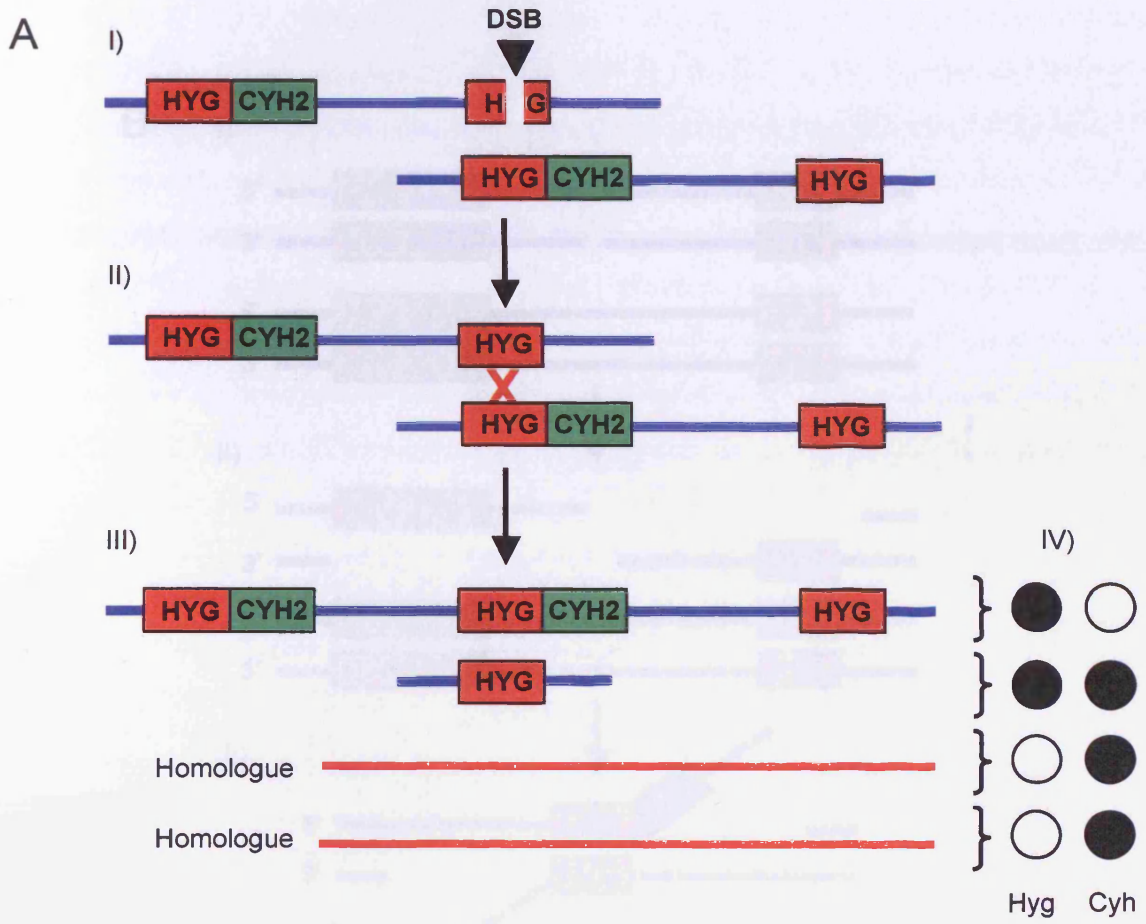
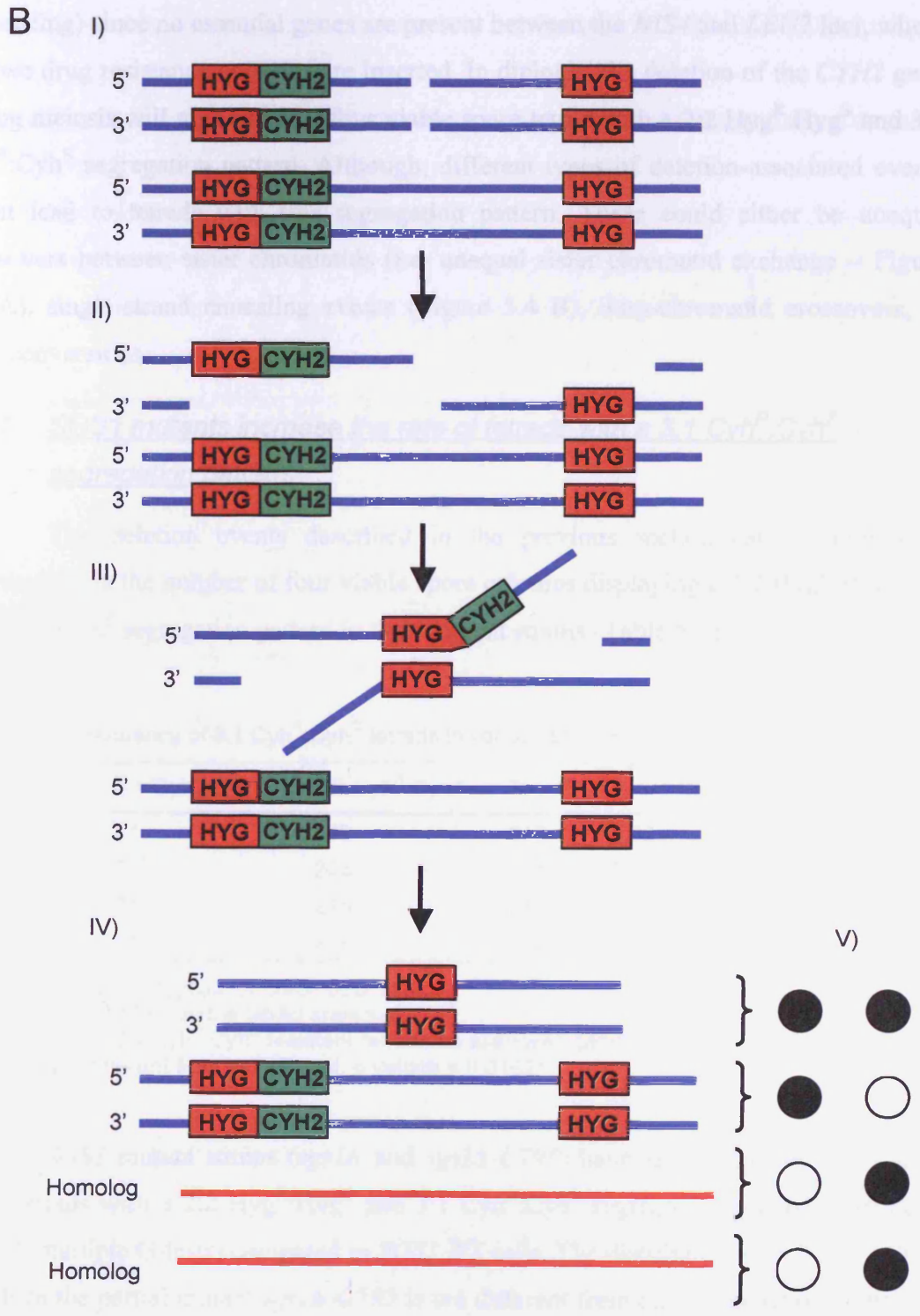


Figure 5.4: Schematic representation of inter- and intra-sister chromatid events leading to deletion of the *CYH2* gene. **A:** Deletion of the *CYH2* gene by unequal sister chromatid exchange. Only one parental pair of sister chromatids is represented. I) Double strand break occurs in one of the *HYG* genes. Sister chromatids are misaligned. II) Due to lack of homology with the homologue, the 3´-end invades the *HYG* gene on the misaligned sister chromatid and creates a double Holliday junction. III) Resolution of the double Holliday junction leads to the duplication of the *CYH2-HYG* interval on one chromatid and its deletion on the sister chromatid. IV) Schematic representation of the tetrad spore colonies with a 2:2 Hyg^R:Hyg^S segregation pattern on hygromycin supplemented medium (Hyg). The same tetrad will have a 3:1 Cyh^R:Cyh^S segregation pattern on cycloheximide (Cyh) supplemented medium plates.

B (next page): Deletion of the *CYH2* gene by single strand annealing (SSA). I) DSB occurs between the two *HYG* gene. II) Sae2, Mre11 and Exo1: long track resection leaving 3´overhangs. III) No invasion of the sister chromatid nor the homologue. Intra-chromatid homology search takes place instead. The two *HYG* genes anneal to one another. IV) The 3´ tails are cleaved leading to the deletion of the *CYH2* gene. IV) Schematic representation of tetrad spore colonies on hygromycin supplemented medium (Hyg) (2:2 Hyg^R:Hyg^S segregation pattern) and cycloheximide supplemented medium (Cyh) (3:1 Cyh^R:Cyh^S segregation pattern).


B
I)
5'
3'
HYG
CYH2
II)
III)
IV)
V)
Homolog
Homolog

cerevisiae strain carrying a normal chromosome III, one can study the segregation of the *HYG* and *CYH2* markers in the progeny. Deletions of the *CYH2* gene are detectable genetically (drug resistance) and physically (CHEF gels, Southern blotting and sequencing) since no essential genes are present between the *HIS4* and *LEU2* loci, where the two drug resistance cassettes are inserted. In diploids, the deletion of the *CYH2* gene during meiosis will give rise to a four viable spore tetrad with a 2:2 Hyg^R:Hyg^S and 3:1 Cyh^R:Cyh^S segregation pattern. Although, different types of deletion-associated events might lead to tetrads with this segregation pattern. These could either be unequal crossovers between sister chromatids (*i.e.* unequal sister chromatid exchange – Figure 5.4 A), single-strand annealing events (Figure 5.4 B), intra-chromatid crossovers, or gene conversions.

5.3.7. *SGS1 mutants increase the rate of tetrads with a 3:1 Cyh^R:Cyh^S segregation pattern:*

The deletion events described in the previous section can be monitored genetically via the number of four viable spore colonies displaying a 2:2 Hyg^R:Hyg^S and 3:1 Cyh^R:Cyh^S segregation pattern in the different strains (Table 5.12).

Table 5.12: Frequency of 3:1 Cyh^R:Cyh^S tetrads in various strains:

Strains [a]	3:1 Cyh^R:Cyh^S	2:2 Cyh^R:Cyh^S	Total [b]	% 3:1 Cyh^R:Cyh^S [c]
WT	31	582	613	5.32%
sgs1Δ	31[†]	203	234	13.24%
sgs1Δ-C795	31[†]	215	246	12.6%
msh6Δ	11	219	230	4.8%

[a] Strains were homologous for chromosome III.
[b] Total number of four viable tetrad analysed.
[c] Percentage of 3:1 Cyh^R:Cyh^S resistant tetrads as assessed genetically.
[†] Statistically different from WT (G-test, p values < 0.0169)

SGS1 mutant strains (*sgs1Δ* and *sgs1Δ-C795*) have an increase in four viable spore tetrads with a 2:2 Hyg^R:Hyg^S and 3:1 Cyh^R:Cyh^S segregation pattern (p value < 0.0169, multiple G-tests) compared to *SGS1 WT* cells. The distribution of 3:1 Cyh^R:Cyh^S tetrads in the partial mutant *sgs1Δ-C795* is not different from that of the total deletion of the *SGS1* gene (*sgs1Δ*, p value = 0.83). This suggests that the Sgs1p domain that is responsible for the increase of 3:1 Cyh^R:Cyh^S tetrads is contained in the C-terminal 795

amino acids of the protein. Furthermore, *msh6Δ* mutants are not increased for this specific segregation pattern compared to wild type (p value = 0.86) but are still different from *sgs1Δ* (p value < 0.0169). This results suggest that *MSH6* is not involved in the different mechanisms that could lead to the increase resistance to cycloheximide in those tetrads.

The rates of cycloheximide resistant spore colonies recovered after meiosis cannot be due to a deletion of the *CHY2* gene in diploid cells since these events will give rise to tetrads with a 2:2 Hyg^R:Hyg^S and 4:0 Cyh^R:Cyh^S segregation pattern. However, it could be due to events that occurred during mitotic growth after meiosis. Although these events should give rise to sectored colonies 1:1 or 1:0 Cyh^R:Cyh^S, we decided to monitor the rate of cycloheximide resistance in mitotic dividing cells. The rate of haploid cycloheximide resistant cells in dividing yeast culture is given in Table 5.13.

Table 5.13: Rate of cycloheximide resistant cells in mitosis.

Haploid strains[a]	Mitotic rate[b] of Cyh^R	T-test, p value[c]
SGS1	7.14×10^{-6}	n.a
sgs1Δ	4.13×10^{-5}	<< 0.001
sgs1Δ-C795	3.06×10^{-5}	0.008

[a] Haploid strains were carrying the *HYG-CYH2* and *HYG* cassettes.
[b] Mitotic rate of cycloheximide resistant cells was calculated as the mean of three independent experiments.
[c] T-tests were used to compare the frequency of cycloheximide resistant cells in wild type to that of *sgs1Δ* and *sgs1Δ-C795*, p value < 0.0166 were considered significant (Three comparaisons).

The mitotic rates of cycloheximide resistance, in haploids cells, is of 5-6 orders of magnitude lower than the rate of 3:1 Cyh^R:Cyh^S tetrad spore colonies recovered after meiosis. This lower rate of cycloheximide resistance in mitotic cells therefore cannot account for the rate seen in meiosis (5-13.24%, Table 5.11). Interestingly, in mitosis, both *sgs1* mutant strains have an increase rate of *cyh2* resistance compared to *SGS1* cells (*sgs1Δ*: 5.78-fold increase, p value << 0.001 and *sgs1Δ-C795*: 4.28-fold increase, p value = 0.008) consistent with an increase in genomic instability in mitotically dividing *sgs1* mutants (Onoda *et al.*, 2000). In addition, the mitotic rate of cycloheximide in *sgs1Δ* is not statistically different from the rate observed in *sgs1Δ-C795* (p value = 0.209)

As explained in § 5.3.6 and in Figure 5.4, various mechanisms such as an unequal crossover between sister chromatids, an intra-chromatid crossover, a single-strand annealing event and a gene conversion, could lead to a deletion of the *CYH2* gene. To differentiate between these different events, tetrads with 3:1 Cyh^R:Cyh^S spores were analysed by CHEF gel and Southern blotting.

5.3.8. *Deletion of SGS1 increases interactions between sister chromatid:*

Among all the 3:1 Cyh^R:Cyh^S tetrads from the various strains analysed genetically some but not all were analysed by CHEF gel and Southern blotting. Table 5.13 summarises the total number of 3:1 Cyh^R:Cyh^S tetrads analysed by CHEF gel for the various strains.

Table 5.14: Number of 3:1 Cyh^R:Cyh^S tetrads analysed by CHEF gel:

Strains [a]	# tetrads [b]	Sub-population analysed [c]	# CHEF [d]
WT	31/613	255	17
sgs1Δ	31/203	178	23
sgs1Δ-C795	31/246	177	16
msh6Δ	11/230	230	11

[a] Strains were homologous for chromosome III.
[b] Total number of 3:1 Cyh^R:Cyh^S tetrads/ Total number of 4 viable spore tetrads.
[c] Number of 4 viable spore tetrads in the sub-population of tetrads analysed.
[d] Number of real events analysed by CHEFs (events in the sub-population).

The frequency of tetrads with three cycloheximide resistant spore colonies and one cycloheximide sensitive spore colonies in the different sub-populations of tetrad analysed physically are not statistically different from the distribution of events in the main populations (G-tests, p values > 0.05) for the different strains. Therefore, the physical analysis of events leading to 3:1 Cyh^R:Cyh^S tetrads in the sub-populations will be representative of the events taking place in each strain.

All four spores from tetrads showing a 3:1 Cyh^R:Cyh^S segregation pattern (Table 5.14, #CHEF[c]) were run on CHEF gels and Southern blotted as explained in experimental procedures (§ 5.2.2). These experiments were used to differentiate between the types of events leading to the deletion of the *CYH2* gene on chromosome III. Figure 5.5 is an example of the three types of events detected.

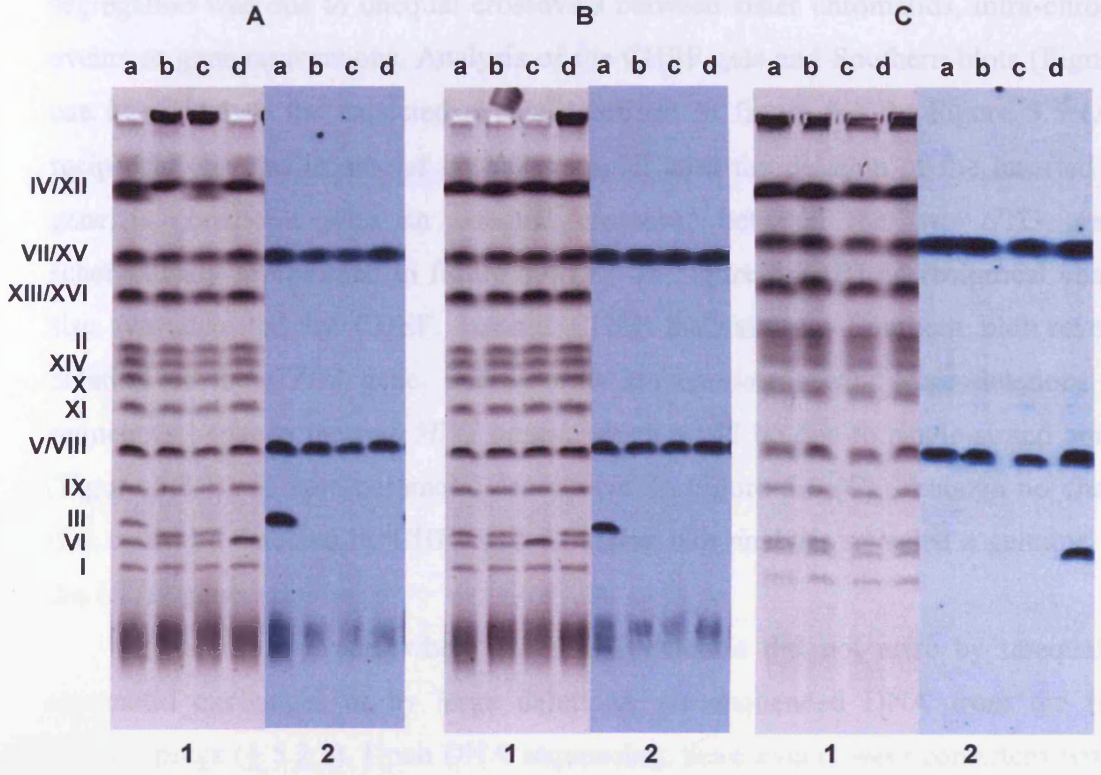


Figure 5.5: Physical analysis of tetrads containing 3:1 Cyh^R:Cyh^S spore colonies.
1: Ethidium bromide stained CHEF gels.
2. Southern blots of each CHEF gel were probed with *CYH2-URA3* labelled plasmid.
A: The larger chromosome III in spore "a" and smaller chromosome III in spore "d" indicates reciprocal events. Only spore "a" contains the *CYH2* gene as detected by the labelled probe. these are therefore reciprocal events (Figure 5.4 A)
B: The smaller chromosome III in spore "c" which had lost the *CYH2* gene represents intra-chromatid events (Figure 5.4 B)
C: No evident changes in size could be detected on the CHEF gel (1) while only one band could be detected via southern blotting (2). These events are indicative of a small deletion of the *CYH2* gene and could occur via gene conversion.
Note: The *URA3* gene is on chromosome V while the endogenous *cyh2* gene is on chromosome VII.

The size changes of chromosome III detected by CHEF gels were confirmed by Southern blot analysis. As mentioned earlier (Figure 5.5), the labelled probe used to detect deletion of the *CYH2* gene is a fluorescent-labelled plasmid carrying the *CYH2* and *URA3* genes (pRS306 – see experimental procedures § 5.2.2 for details). The labelled pRS306 plasmid provides a positive internal control detecting the endogenous *URA3* gene on chromosome V. This confirms that the hybridisation and detection of the probes worked. The detection of the *CYH2* gene on chromosome III was performed using the same labelled plasmid.

A number of tetrads with a 3:1 Cyh^R:Cyh^S segregation pattern from each strain (the number for each strain is given in Table 5.13) were analysed to detect if their segregation was due to unequal crossovers between sister chromatids, intra-chromatids events or gene conversions. Analysis of the CHEF gels and Southern blots (Figure 5.5) can be linked to the expected events described in figure 5.4. In Figure 5.5 (A), the reciprocal changes in size of chromosome III with the deletion of the inserted *CYH2* gene is consistent with an unequal crossover between the two *HYG* genes as schematically represented in figure 5.4 (A). In Figure 5.5 (B) no reciprocal change in size was detected by CHEF. Instead CHEF analysis and Southern blot revealed a deletion of the *CYH2* gene. Such events are consistent with large deletions of the sequences between the two *HYG* genes, which could be due to single-strand annealing (Figure 5.4-B) or intra-chromatid crossovers. In Figure 5.6 (C), although no change in size could be detected by CHEF gel, Southern blot analysis revealed a genuine loss of the *CYH2* gene.

For those events where the *CYH2* deletion did not arise by unequal sister chromatid exchanges or by large deletions, we sequenced DNA from the residual agarose plugs (§ 5.2.2). Upon DNA sequencing, these events were consistent with gene conversions using the homologous chromosome as a template (data not shown). Interestingly, the frequency of *CYH2* deletions arising from gene conversion is independent of the mutants used (Table 5.15, G-tests p value > 0.0125)

Table 5.15: Distribution of *CYH2* deletion arising from gene conversion event:

Strains[a]	Gene conversion[b] (% of events)	G-test[c]
WT	11/255 (4.3%)	n.a
sgs1Δ	5/178 (2.8%)	0.4
sgs1Δ-C795	4/177 (2.6%)	0.23
msh6Δ	4/230(1.7%)	0.09
sgs1Δmsh6Δ	3/118(2.5%)	0.66

[a] Strains were homologous for chromosome III.
[b] Number of gene conversions/Number of 4 viable spore tetrads.
[c] G-test compared distribution of gene conversions in mutant strains to that of the *SGS1 WT* distribution. p values < 0.0125 were considered significant (multiple tests).

The frequency of large deletions is not affected in the *sgs1* or *msh6* mutants. This is in contrast to data presented in Chapter 4 (§ 4.3.5) where gene conversions, arising from the correction of heteroduplex DNA, are increased in *sgs1Δ* strains compared to wild type. This difference might be due to the increased length of resection necessary to gene convert a deletion. Due to the homogenous distribution of large gene conversions among the different strains, the differences in distribution of inter-chromatid events and intra-chromatid events can be evaluated without including large gene conversions.

Table 5.16: Distribution of unequal sister chromatid exchanges and intra-chromatid events in *SGS1 WT* and mutant strains:

Strains[a]	USCE[b] (% of events)	Intra-chr. events[c] (% of events)	G-test[d]
WT	6/255 (2.4%)	0/255 (0%)	n.a
sgs1Δ	13/178 (7.3%)	5/178 (2.8%)	<<0.0125
sgs1Δ-C795	4/177 (2.6%)	8/177 (4.5%)	<<0.0125
msh6Δ	3/230 (1.3%)	4/230 (1.7%)	0.034
sgs1Δmsh6Δ	3/118 (2.5%)	4/118 (3.4%)	<0.0125

[a] Strains were homologous for chromosome III.
[b] Number of unequal sister chromatid exchanges (USCE)/Number of 4 viable spore tetrads.
[c] Number of intra-chromatid deletion events/Number of 4 viable spore tetrads.
[d] G-test compared distribution of events in mutant strains to that of the *SGS1 WT* distribution. p values < 0.0125 were considered significant.

The distribution of unequal crossover between sister chromatids and intra-chromatid deletion, in all *sgs1* mutant strains (*sgs1Δ*, *sgs1Δ-C795* and *sgs1Δmsh6Δ*), is statistically different from wild type (G-test, p values < 0.0125 – Table 5.16). However, their distribution is not statistically different between *sgs1Δ* and *sgs1Δ-C795* (G-test, p value = 0.055). The deletion of the *MSH6* gene does not seem to affect the distribution of intra- and inter-chromatids deletions since the distribution of events in *msh6Δ* is not different from wild type (p value = 0.034). However, it is different from *sgs1Δ* (p value = 0.005). In addition, the distribution of events in *sgs1Δmsh6Δ* is not different from *sgs1Δ* mutant (G-test, p value = 0.172) despite the small number of events analysed in the double mutant. Therefore, it seems that in absence of the Sgs1 protein, more intra- and inter-chromatid interactions occur between sister chromatids. Possible reasons for this will be discussed in the next section.

5.4. Discussion:

5.4.1. *Sgs1p and Msh6p prevent meiotic recombination between diverged sequences:*

Homeologous recombination is increased in the *LEU2-MAT* interval for *sgs1 sgs1Δ*, *sgs1Δ-C795*, *msh6Δ* and *sgs1Δ msh6Δ* (§ 5.3.1 – Table 5.7). This phenotype is similar to that previously reported in mismatch repair deficient strains (Chambers *et al.*, 1996). However, increased recombination between the homeologous pair of chromosome III could not be detected for *sgs1Δ* and *msh6Δ* in the *HML-HIS4* interval (Table 5.5), nor for either of the mutants in the *HIS4-LEU2* interval (Table 5.6). This absence of increased homeologous recombination in these two intervals could be explained by the relatively small number of tetrads dissected for each cross. However, the insertion of the *HYG-CYH2* genes, proximal to the *HIS4* locus, and the insertion of the *HYG* gene, proximal to *LEU2*, might also explained the centiMorgan differences, in the *msh6Δ* mutant, observed between this study and that of Scott Chambers (1999). While Scott Chambers reported distances of 5.65 cM (*HML-HIS4*) and 1.77 cM (*HIS4-LEU2*) (Chambers, 1999), we only observed a distance of 2.2 cM for the *HML-HIS4* interval and a distance of 0.2 cM for the *HIS4-LEU2* interval (Tables 5.5 and 5.6). These discrepancies could be explained by the higher degree of divergence between the homeologous chromosome pairs due to the insertions mentioned previously. As seen in the introduction of this chapter, the frequency of homeologous recombination in mismatch repair deficient diploids is dependent on a minimal degree of homology (§ 5.1.1). Thus, further increasing divergence by inserting drug resistance cassettes on one homeologue might have reached the point whereby single-end invasions are no longer possible proximal to the *HIS4* and *LEU2* loci.

The analysis of crossover frequencies in the double mutant *sgs1Δ msh6Δ* has highlighted the epistatic relation between these two genes. As previously hypothesised by Petit *et al.* (1991) for the *MutS* and *MutL E. coli* genes, the mismatch repair gene *MSH6* could also play a role during the annealing of heteroduplex DNA. Further evidence for such role has been demonstrated in the single-strand annealing pathway where both Msh6 and Sgs1 proteins are required for heteroduplex rejection (Sugawara *et al.*, 2004). Based on the previous evidence and that presented in this thesis, we proposed

that Sgs1p might act in a complex with the protein heterodimer Msh2/Msh6 to unwind heteroduplex DNA. The unwinding activity of the Sgs1p helicase might be triggered by the degree of divergence between annealed molecules that is recognised by Msh2p and Msh6p. However, the fact that the double mutant *sgs1*Δ *msh6*Δ was unable to restore the crossover defect in the homeologous strain to a *SGS1* homologous level suggests that (1) other helicase(s) interacting with mismatch repair proteins might also be involved in this pathway or (2) the degree of divergence between the two sequences might be sometimes too low to allow strand invasion by Rad51/Dmc1 proteins (Chen and Jinks-Robertson, 1999).

The increase of the crossover frequency in *sgs1* and *msh6*Δ mutant strains discussed above can restore some, but not all, of the chromosome III segregation defects during meiosis I (§ 5.3.3 Table 5.9 and § 5.3.4 Figure 5.4). This further supports the hypothesis that other proteins might be involved in preventing recombination between diverged sequences. In addition, we know from mitotic studies of *sgs1*Δ strains (Ajima *et al.*, 2002; Miyajima *et al.*, 2000a; Miyajima *et al.*, 2000b; Watt *et al.*, 1995) and Chapter 3 (§ 3.3.4), that the *sgs1*Δ mutant has an increased genomic instability manifested by chromosome loss/non-disjunction, deletion, rearrangement and hyperrecombination. Thus, the different frequencies of meiosis I non-disjunction recovered in *sgs1*Δ and *sgs1*Δ-*C795* (§ 5.3.3, Table 5.9) might be due to death associated with the *sgs1*Δ mutant during mitosis. Therefore, the frequency of meiosis I non-disjunction in *sgs1*Δ cells might be under estimated. Evidence for such under estimation comes from the higher number of meiosis I non-disjunction in the partial mutant *sgs1*Δ-*C795* (Table 5.9). As seen by Rockmill *et al.* (2003) and in Chapter 3 (§ 3.3.5), the C-terminal truncation of the *SGS1* gene (*sgs1*Δ-*C795*) does not have a mitotic defect as severe as *sgs1*Δ. Thus, the lower frequency of meiosis I non-disjunction recovered in the *sgs1*Δ compared to *sgs1*Δ-*C795* might not be due to a better segregation of homeologous chromosome during meiosis I in the C-terminal truncation mutant but rather to the decrease spore viability associated with the *sgs1*Δ defect during mitotic cell divisions.

5.4.2. *Sgs1p but not Msh6p prevents sister chromatid interactions:*

Analysis of the events leading to tetrads with a 3:1 Cyh^R:Cyh^S segregation pattern have shown that Sgs1p is involved in preventing intra- and inter-sister chromatid interactions from happening (Table 5.12). Physical analysis by CHEF gels and Southern blotting can distinguish between the different kinds of deletion occurring (Table 5.15 and 5.16).

Firstly, gene conversion of the entire *HYG-CYH2* cassette, using the homologue as a template, occurs regardless of *sgs1*Δ or *msh6*Δ (Table 5.15). This is in contrast with previous data (Chapter 4, § 4.3.5) where gene conversions are increased at the *HIS4* and *LYS2* loci in *SGS1* mutants compared to *SGS1 WT* cells. However these phenotypes are not necessarily different. The gene conversion events leading to a complete deletion of the *HYG-CYH2* cassette will need to involve much longer resection tracks than the gene conversion occurring at the *HIS4* or *LYS2* loci. Therefore, these two gene conversion events might come from different pathways.

Secondly, in absence of the C-terminal part of the Sgs1 protein, unequal sister chromatid exchanges and intra-chromatid events (such as single-strand annealing and intra-chromatid crossovers) are increased (Table 5.16). This meiotic phenotype, which to our knowledge has never been reported before, is consistent with the phenotypes of *RecQ* mutants in mitosis. Indeed, *bs* cells and yeast *SGS1* mutants are more prone to sister chromatid recombination (Ellis *et al.*, 1999; Onoda *et al.*, 2004). Interestingly, we have also shown that this increased interactions between sister chromatids is independent of Msh6p. Onoda *et al.* (2004) reported that in mitosis, the increase sister chromatids interactions in *sgs1*Δ required Msh2p. Although, the mismatch repair function of the Msh2 protein was dispensable for this function (Onoda *et al.*, 2004). Thus, Msh2p and Msh6p functions might differ regarding sister chromatid recombination compared to the detection of homeology.

Several studies have hypothesised that the bias toward inter-homologue recombination is a barrier toward sister chromatid recombination (Niu *et al.*, 2005; Schwacha and Kleckner, 1997; Sheridan and Bishop, 2006). The axial element proteins Red1/Hop1 and the protein kinase Mek1 might decrease recombination between sister chromatids (Niu *et al.*, 2005; Schwacha and Kleckner, 1997; Wan *et al.*, 2004). Recent data also suggested that the newly identified protein Hed1 might act in the same pathway

(Sheridan and Bishop, 2006; Tsubouchi and Roeder, 2006) (see § 5.1.2 for more details). Furthermore, physical analysis of double-strand break repair and recombination intermediates has shown that in *sgs1Δ-C795* mutants, more interactions take place between sister chromatids compared to wild-type cells (Oh *et al.*, 2007). We have shown in this study that inter-sister interactions are increased in *SGS1* mutants. Based on the above evidence, we proposed that the Sgs1 protein might be part of the barrier toward sister-chromatid recombination.

Chapter 6: Discussion – Dual role of Sgs1p in meiotic recombination:

6.1. Sgs1p unwinds heteroduplex DNA:

Based on the evidence collected during the course of this PhD, we hypothesis that the Sgs1 helicase unwinds heteroduplex DNA at an early stage of meiotic recombination. In the absence of an active Sgs1 protein, crossover interference is reduced (§ 4.3.2) and gene conversions are increased in both the crossover and the non-crossover pathways (§ 4.3.3). Furthermore, in *sgs1* cells, the crossover defect associated with homeology between homologues is partially alleviated. This function of the Sgs1 protein is though to be in association with the MMR proteins Msh2p, Pms1p and Msh6p § 5.3.3 and (Chambers *et al.*, 1996; Hunter *et al.*, 1996). The fact that Sgs1 and Top3 proteins have been found in a complex with Mlh1p and Mlh3p in meiosis (Wang and Kung, 2002), while Sgs1p, Msh2p and Msh6p are required for heteroduplex rejection in single-strand annealing (Sugawara *et al.*, 2004) further support the hypothesis that the *S. cerevisiae* RecQ helicase in association with MMR proteins act as a complex to reject early recombination intermediates.

6.2. Three models could account for the late role of Sgs1p:

First, we have shown that, although *sgs1Δ-C795* is nearly wild type regarding its mitotic functions, this C-terminal truncation of the *SGS1* gene still has a meiotic defect (§ 3.3.5). As presented in the introduction of this thesis, Sgs1p is though to play three major roles during mitotic S-phase: activation of the intra-S phase checkpoint, stabilisation of DNA polymerases at replication forks and branch migration of Holliday junction-like structures into hemicatenates (§ 1.3). The first two functions might not require the C-terminal part of the protein while the third certainly does. If these functions are required during the pre-meiotic S-phase, *sgs1Δ-C795* (and therefore *sgs1Δ*) cells could enter meiosis with entangled sister chromatids. Chromatid entanglement could account for an increase in non-sisters spores in the two viable spore tetrad class. If a diploid cell enters meiosis with entangled sister chromatids (Figure 6.1 A) and during the

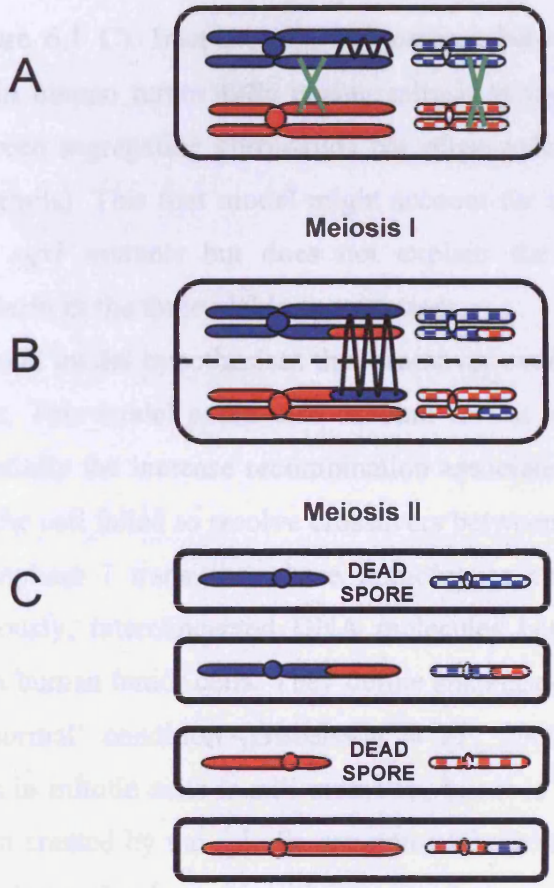


Figure 6.1: Pre-meiotic S-phase defect in *sgs1* mutants could lead to an increase of non-sister spores. **A**: Entanglement of sister chromatids occur in S-phase as *sgs1* mutants cannot process Holliday junction-like molecules into hemicatenates. **B**: A crossover between the entanglement and the centromere lead to homologue being physically connected. At anaphase of meiosis I, the segregation of homologues could result in the breaking of entangled chromatids. **C**: Because of the crossover, the dead spores in the two viable spore tetrads are non-sister spores.

prophase of meiosis I, a crossover occurs between the centromere and the entanglement, then at the metaphase of meiosis I, the homologue will still be connected via genetic material (Figure 6.1 B). During anaphase I, the segregation of homologues might break the entangled chromatids and that would result in two viable spore tetrads, which will be non-sisters (Figure 6.1 C). Interestingly, connections between sister chromatids have been identified in human tumor cells during mitosis (Gisselsson *et al.*, 2000). These DNA links between segregating chromatids are often referred to as anaphase bridges (see below for details). This first model might account for some of the increase in non-sister spores in *sgs1* mutants but does not explain the increase of recombination associated with death in the three viable spore tetrads.

The second model hypothesises that crossover events are not properly resolved in *SGS1* mutants. This model could also account for an increase in two viable spore tetrads and potentially the increase recombination associated with death in three viable spore tetrads. If the cell failed to resolve crossovers between a pair of homologues at the metaphase I /anaphase I transition, these homologues could still be connected. As mentioned previously, interconnected DNA molecules between daughter nuclei have been identified in human tumor cells. They define anaphase-bridges between chromatids in mitosis in 'normal' condition (Gisselsson *et al.*, 2000). The resolution of these anaphase-bridges in mitotic cells is still unknown, but it is likely that they might break due to the tension created by the spindle apparatus (Hoffelder *et al.*, 2004). In addition, anaphase-bridges have also been identified in *S. cerevisiae* during meiosis anaphase I (Yu and Koshland, 2005). Thus, these connections between homologues are likely to occur due to unresolved chiasmata. The force, created by the microtubules pulling homologues away from each another, might create tension between these interconnected chromatids. The release of this physical stress could occur by breakage of the meiotic anaphase-bridges. Broken chromatids could lead to loss of DNA information, as the cell might not be able to repair the damage at this late stage of the meiotic division. Therefore, if a single crossover between homologue is not resolved, the force-induced DNA break will result in two non-sister viable spore tetrads and two dead spores (Figure 6.2 A). As tetratypes can be due to double crossovers between three chromatids, one chromatid is involved in two double Holliday junctions (Figure 6.2 B). We hypothesis that the tension applied by the spindle apparatus on the chromatid involved in double crossovers might be twice as much as the tension on the other two chromatids. Thus, the

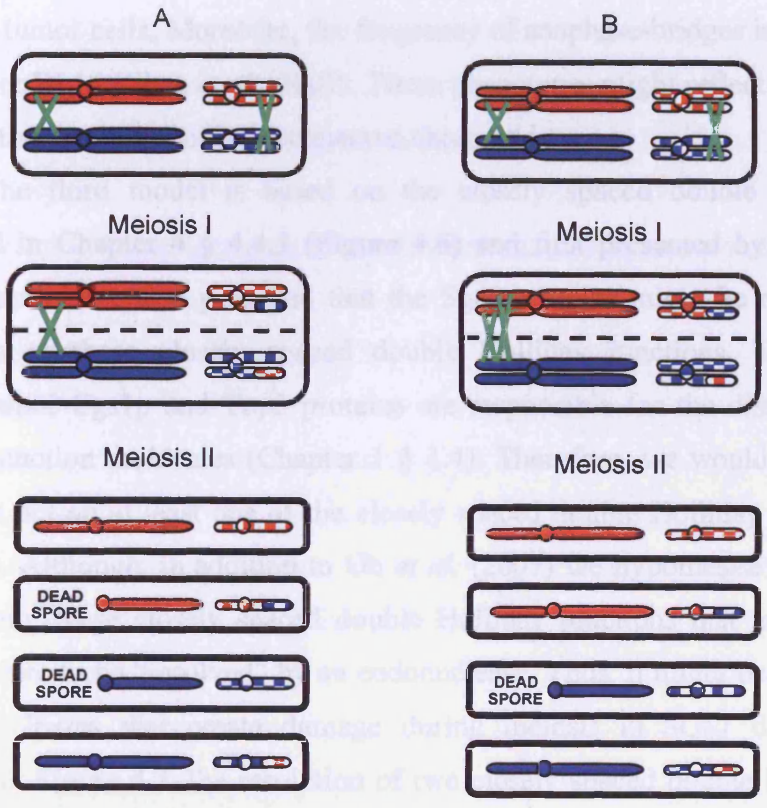


Figure 6.2: Inhability to resolve cross-overs leads to broken chromatids.
A: Crossovers between homologues are not resolved properly. The segregation of homologue at anaphase I leads to the breakage of the interacting chromatids. Genetic information is lost as the cell might not be able to repair the damage leading to two non-sister viable spore tetrads.
B: As in A but somehow, only the chromatid involved in the two crossovers break. This might result in a three viable spore tetrad with a recombinant dead spore.

break should be more likely to occur on the chromatid with the most tension. If this is the case, only one chromatid might break, leading to three viable spore tetrads where the dead spore is recombinant. Although, there is not much evidence to account for this model, the human RecQ homologue, BLM, has been found to localise at anaphase-bridges in tumor cells. Moreover, the frequency of anaphase-bridges is increased in cells deficient for BLM (Chan *et al.*, 2007). These phenotypes might reflect a role of the BLM protein in the resolution of interconnected chromatids.

The third model is based on the closely spaced double crossovers model introduced in Chapter 4 § 4.4.3 (Figure 4.6) and first presented by Oh *et al.* (2007). These authors have also proposed that the Sgs1 proteins might be responsible for the dissolution of these closely spaced double Holliday junctions. This hypothesis is plausible since Sgs1p and Top3 proteins are responsible for the dissolution of double Holliday junction molecules (Chapter 1 § 1.4). Therefore, we would like to argue that they might act on at least one of the closely spaced double Holliday junction shown in Figure 4.6. Although, in addition to Oh *et al.* (2007) we hypothesise that in absence of Sgs1 protein, these closely spaced double Holliday junctions that are not "dissolved" need therefore to be "resolved" by an endonuclease. Thus, it might be the late resolution by endonucleases that create damage during meiosis in *SGS1* deficient cells. As presented in Figure 4.7, the resolution of two closely spaced double Holliday junctions can create chromatids with two nicks and chromatids with four nicks. While cells might be able to repair two nicks in one molecule, the closely juxtaposition of four nicks might be lethal. Our model of two closely spaced double Holliday junction involving only two chromatids (Secondary molecules, Figure 4.6 C and § 4.4.3) could account for the increase of non-sister spores in the two viable spore tetrad class. Resolution of these structures might lead to two chromatids with four nicks (Figure 6.3 C). The quantification of events leading to non-sister or sister spores can be monitored on a "genome wide" scale. If recombination of two homologues of any of the 16 chromosome pairs lead to death, we will recover non-sister spores since we are using a centromere marker always segregating at meiosis I. The resolution of tertiary molecules (two closely spaced double Holliday junction between three chromatids) is more problematic. First, assuming the first end invasion takes place between the homologue, the second end invasion can occur either between sister chromatids (Figure 4.6 A and 6.3 A) or with the other homologue (Figure 4.6 B and 6.3 B). Although, large joint molecules (both tertiary

and quaternary molecules) occur in wild type, the *sgs1Δ-C795* mutation increases their levels by nearly three fold. In addition, Oh *et al.* have shown that in absence of Sgs1p, inter-sister interactions are increased by 2.5-fold. This result was confirmed by our data where unequal sister chromatid recombination is increase by 3-fold in *sgs1Δ* compared to wild type (Chapter 5 § 5.3.8). Thus, the second end invasion might preferentially invade its sister chromatid rather than the homologue (*i.e.* bias toward events represented in Figure 6.3 A). Interestingly, resolution by endonucleases of a tertiary molecule joining the homologue and a sister chromatid will result in recombinant molecule carrying four nicks. This model could therefore account for the increase of recombinant dead spores in the three viable spore tetrads. Besides its appeal, this hypothesis also raises some questions. First we, and Oh *et al.*, are only monitoring events on chromosome III. Considering that around 70 to 90 crossovers occur genome wide (Brem *et al.*, 2002; Winzeler *et al.*, 1998), if the resolution of tertiary molecules was always leading to death, one might expect to see a decrease in three viable spore tetrads and an increase in zero viable spores in *SGS1* mutant since resolution of multiple tertiary molecules on different chromosomes will have an additive effect. Thus, either these events are "rare" or they might be specific to chromosome III. Secondly, in helicase proficient cells, Sgs1p and Top3p might dissolve at least one of these closely spaced double Holliday junctions. Since inter-homologue recombination is essential for the faithful segregation of chromosome during meiosis I, only one of the double Holliday junction should be dissolved. Furthermore, Sgs1/Top3 might preferentially dissolved double Holliday junctions connecting sister chromatids since they do not play a role in segregation. How can a distinction be made between the two events? This distinction might involve Hop1p. The Hop1 protein has been found to bind to the core of Holliday junctions and changing their conformations. The conformation change of Hop1-bound Holliday junction inhibits their dissolution by the protein complex Sgs1/Top3 *in vitro* (Tripathi *et al.*, 2006). Thus, selective binding of Hop1 to inter-homologue double Holliday junction could prevent dissolution of crossover events during meiosis I by Sgs1p while sequestration of Hop1 in a protein complex will enable Sgs1 to dissolve recombination events between sister chromatids. Likewise, since Hop1p is involved in the barrier toward sister chromatid recombination (§ 5.1.2), the Hop1 protein might be recruiting the Sgs1/Top3 protein complex to dissolve recombination events occurring between sister chromatids.

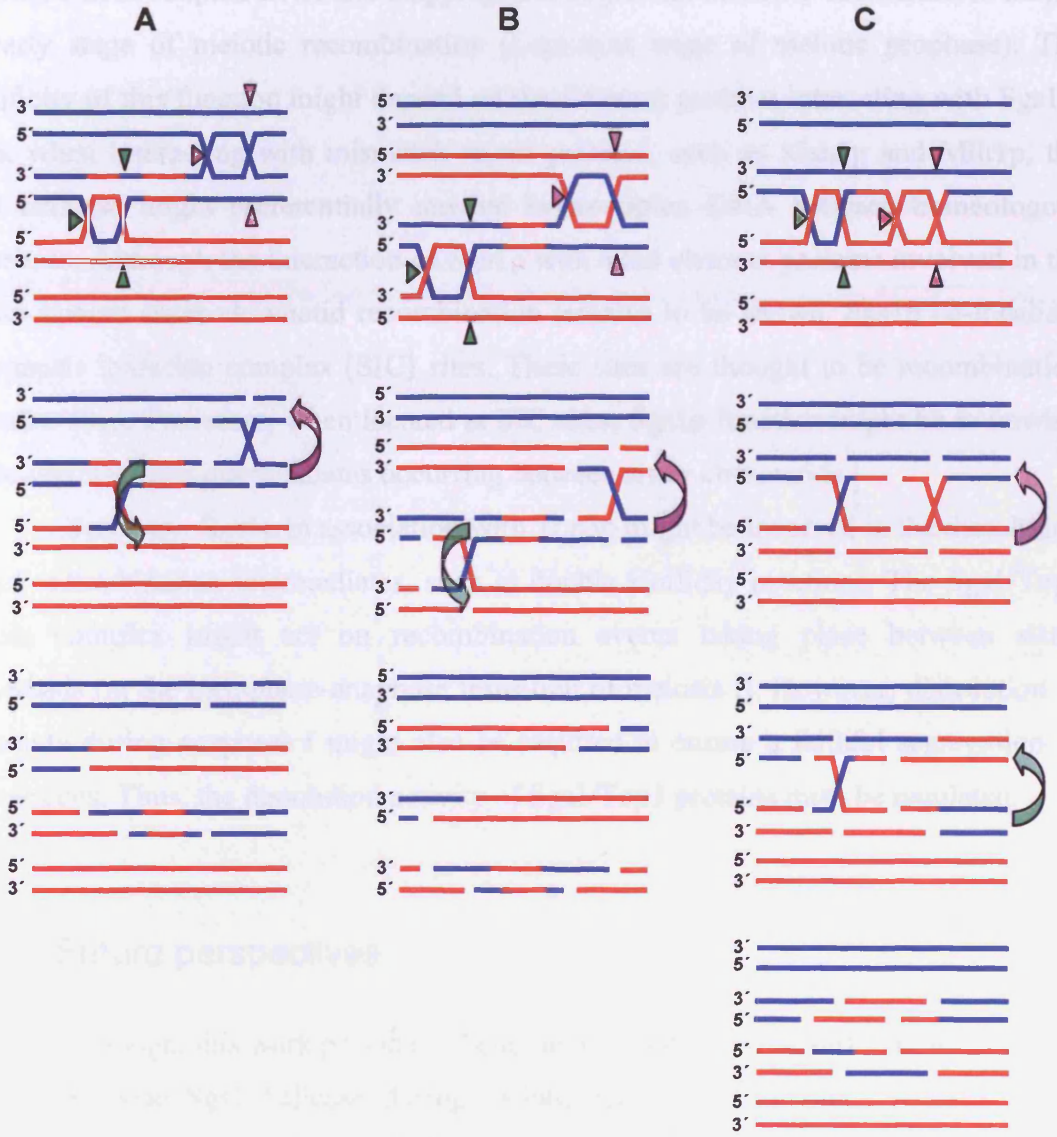


Figure 6.3: Resolution of two closely spaced double Holliday junctions. One of four equivalent possibility of resolution is represented. Each resolution will lead to recombinant molecules. One DSB-end invades a homologue while the other DSB-end can invade a sister (**A**) or the other homologue (**B**) or re-invades itself (**C**). Cutting of A creates two recombinant molecules, one carrying four nicks the other only two. Cutting of B creates two recombinant molecules each carrying only two nicks while the chromatids carrying four nicks is non recombinant. In A, the chromatid carrying four nicks is recombinant, while in B it is not. **C:** one end of the DSB invades the same chromatid twice and is recaptured by the second end of the break. In this case, the schematic representation of the resolution of the two double Holliday junctions is represented asynchronously. Cutting of two closely spaced dHJs involving two chromatids creates two recombinant molecules, each carrying four nicks.

In conclusion, the work described in this thesis has shown that the Sgs1 protein might have a dual role during homologous recombination. First, Sgs1p might be required to unwind heteroduplex DNA and inappropriate single-end invasion intermediates during an early stage of meiotic recombination (Leptotene stage of meiotic prophase). The specificity of this function might depend on the different proteins interacting with Sgs1p. Thus, when interacting with mismatch repair proteins, such as Msh6p and Mlh1p, the Sgs1 helicase might preferentially unwind heteroduplex DNA between homeologous sequences. Although the interaction of Sgs1p with axial element proteins involved in the barrier toward sister chromatid recombination remains to be shown, Sgs1p co-localises at synapsis initiation complex (SIC) sites. These sites are thought to be recombination initiation sites. Therefore, when located at SIC sites, Sgs1p function might be to unwind single-end invasion intermediates occurring between sister chromatids.

Secondly, Sgs1p in association with Top3p might be involved in the dissolution of late recombination intermediates, such as double Holliday junctions. The Sgs1/Top3 protein complex might act on recombination events taking place between sister chromatids (at the metaphase-anaphase transition of meiosis I). However, dissolution of chiasmata during anaphase I might also be required to ensure a faithful segregation of homologues. Thus, the dissolution activity of Sgs1/Top3 proteins must be regulated.

6.3. Future perspectives:

Although, this work provides a better understanding of the different functions of the *S. cerevisiae* Sgs1 helicase during meiotic recombination, much remains to be discovered. Since Msh2p is involved in the unequal sister chromatid recombination defect of *sgs1*Δ during mitosis, it will be of interest to analyse in our assay the *msh2*Δ and *msh2*Δ *sgs1*Δ during meiosis. To avoid the mutator phenotype of *MSH2* mutants and the genomic instability of *sgs1*Δ during mitotic division, both genes could be placed under the control of the *CLB2* promoter. *CLB2* is only expressed during mitosis, the transcription of the gene being repressed during meiosis. The *CLB2* promoter has been used in other studies to create conditional mutants (Lee and Amon, 2003). Furthermore, both Sgs1p and Hed1p interact with Rad51p. Since Sgs1p and Hed1p might be involved in the barrier toward sister chromatid recombination, study of both *hed1*Δ and *sgs1*Δ *hed1*Δ might be of interest to further characterise this barrier.

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Chapter 7: Appendix: Construction of a *N-Degron SGS1* mutant.

7.1. Introduction:

7.1.1. The N-end rule: a proteins degradation pathway:

In cells, the steady state level of proteins varies from a few seconds to several days depending on the regulation of their expression and their degradation. Therefore, the concentration of proteins inside a given cell is continually changing with time in order to adjust to external stimuli such as osmotic pressure and oxygen level or to adjust to internal stimuli to maintain the amino-acid pool. Different pathways are implicated in the degradation of proteins. They include the storage of proteins in organelles such as vacuoles and vesicles prior to their degradation by proteases. However, the vast majority of proteins degraded in the cell are targeted, via their polyubiquitination, to a proteolytic organelle, the proteasome. The N-end rule pathway (Figure 7.1) is the pathway responsible for the targeting of proteins to the proteasome via substrate polyubiquitination (for review see (Varshavsky, 1996)). The degradation of proteins by this pathway plays an important role in the variation of protein concentration with time. For example, a regulator protein such as Sic1p, with a short *in vivo* half-life regulated by proteolysis, has a rapid adjustment of its concentration creating a spatial regulation and/or gradient in the cell (Levy *et al.*, 1996). Furthermore, the degradation of cyclin proteins such as the protein kinase Cdc28p at specific times of the cell cycle regulates the cell growth and division (for review (Mendenhall and Hodge, 1998)). The proteasome, implicated in the degradation of polyubiquitinated proteins, generates small fragmented peptides in an ATP-dependent fashion (Figure 7.1). The N-end rule pathway of protein degradation is dependent on the presence of a destabilising residue at the N-terminal of the targeted protein and of internal lysines in its vicinity. Such signals – the N-destabilising residue and the internal lysines – are responsible for the degradation of protein via the N-end rule and are called N-Degrons (Varshavsky, 1991).

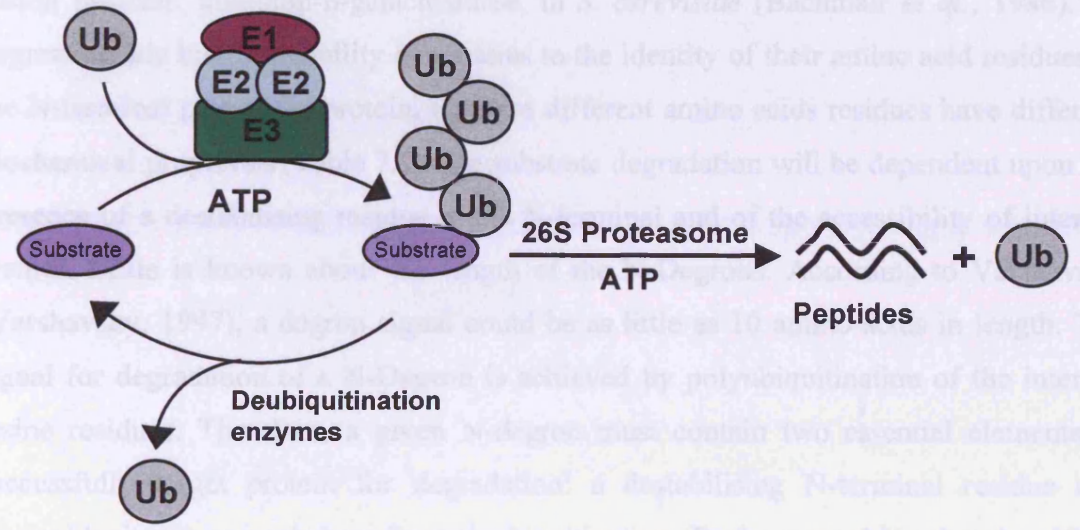

Figure 7.1: Overview of the N-end rule pathway.
E1: Ubiquitin activating enzyme.
E2: Ubiquitin conjugating enzyme.
E3: N-recogninin.
Isopeptide-linked ubiquitin chains that form on proteolitic substrate are dynamic structures, with ubiquitinating (E1, E2 and E3) and deubiquitinating enzymes rapidly modifying these adducts, Ubiquitinated substrates are degradated by a large (≈ 2000 kDa) protease, the 26S proteasome (Adapted from Hochstrasser (1996)).

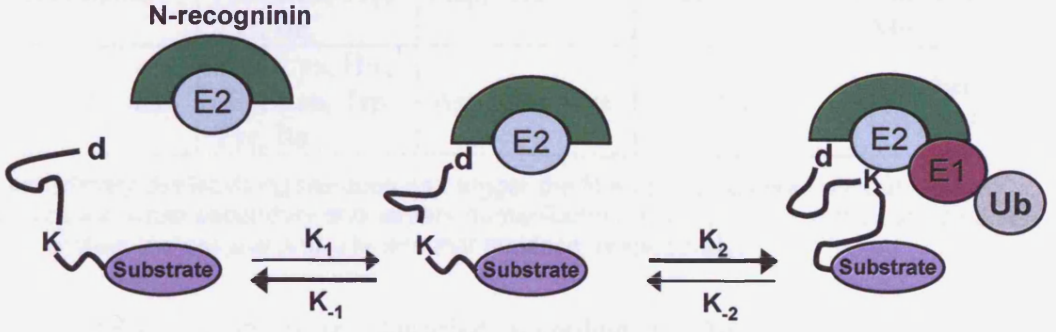

Figure 7.2: Recognition of N terminal destabilizing residue and capture of internal lysine by N-recogninin (E3)/Ubiquitinin-conjugating enzyme (E2) complex.
(Adapted from Varshavsky (1997))

7.1.2. Features of N-degron:

The first N-Degron signal was discovered in experiments following the fate of fusion proteins, ubiquitin-ß-galactosidase, in *S. cerevisiae* (Bachmair *et al.*, 1986). N-degron signals link the stability of proteins to the identity of their amino acid residues in the N-terminal part of the protein. Because different amino acids residues have different biochemical properties (Table 7.1), the substrate degradation will be dependent upon the presence of a destabilising residue at the N-terminal and of the accessibility of internal lysines. Little is known about the length of the N-Degrons. According to Varshavsky (Varshavsky, 1997), a degron signal could be as little as 10 amino acids in length. The signal for degradation of a N-Degron is achieved by polyubiquitination of the internal lysine residues. Therefore, a given N-degron must contain two essential elements to successfully target protein for degradation: a destabilising N-terminal residue and internal lysine(s) as a substrate for polyubiquitination (Bachmair and Varshavsky, 1989; Chau *et al.*, 1989; Dohmen *et al.*, 1994).

Table 7.1: Comparison of eukaryotes N-end rule residues.

	Primary destabilising residues	Secondary destabilising residues	Tertiary destabilising residues	Stabilising residues
S. cerevisiae	Arg, Lys, His, Phe, Leu, Trp, Tyr, Ile.	Asp, Glu.	Asn, Gln.	Cys, Ala, Ser, Thr, Gly, Val, Met.
Mouse L-cells	Arg, Lys, His, Phe, Leu, Trp, Tyr, Ile.	Asp, Glu, Cys.	Asn, Gln.	Ala, Ser, Thr, Gly, Val, Met.

Note: primary destabilising residues can trigger the N-end rule pathway without further modification while secondary and tertiary destabilising residues need modification by a Arg-tRNA-protein transferase and a N-terminal amidase, respectively.

Amino acids were classified according to their different destabilising or stabilising properties when featured in N-Degron. Primary destabilising residues are bound directly by N-recognin (Figure 7.2), encoded by the *UBR1* gene. Secondary destabilising amino acids (Aspartic acid and glutamic acid) acquire their destabilising property through their conversion by Arginine-tRNA-transferase (R-transferase) into arginine, one of the primary destabilising residues (Figure 7.3). Tertiary residues such as asparagine and glutamine acquire their destabilising function after their conversion into

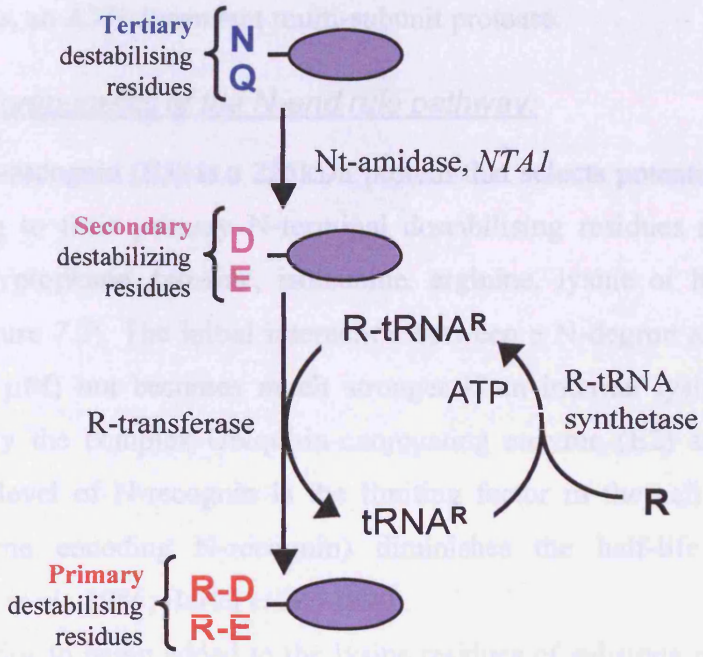

Figure 7.3: The conversion of tertiary destabilizing residues N and Q into secondary residues D and E is mediated by N-terminal amidohydrolase (Nt-amidase), encoded by *NTA1*. The conjugaison of secondary destabilising residues D and E into the primary destabilising residue R is mediated by Arg-tRNA-protein transferase (R-transferase), encoded by *ATE1*. (Adapted from Varshavsky (1997))

secondary destabilising residues by enzymatic deamination (N-terminal amidohydrolase (Nt-amidase)). Such secondary residues are themselves converted into primary destabilising residues as explain above (Baker and Varshavsky, 1995; Balzi *et al.*, 1990) (Figure 7.3). Proteins featuring a N-degron are then targeted for degradation to the 26S proteasome, an ATP-dependant multi-subunit protease.

7.1.3. Components of the N-end rule pathway:

N-recognin (E3) is a 225kDa protein that selects potential degradation proteins by binding to their primary N-terminal destabilising residues such as phenylalanine, leucine, tryptophane, tyrosine, isoleucine, arginine, lysine or histidine (Bartel *et al.*, 1990) (Figure 7.2). The initial interaction between a N-degron and N-recognin is weak (Kd $\approx$ 10 μM) but becomes much stronger if an internal lysine of the N-degron is captured by the complex Ubiquitin-conjugating enzyme (E2) and N-recognin (Figure 7.2). The level of N-recognin is the limiting factor in the cell as over-expression of *UBR1* (gene encoding N-recognin) diminishes the half-life of targeted proteins (Bachmair *et al.*, 1986; Bartel *et al.*, 1990).

Prior to being added to the lysine residues of substrate proteins, ubiquitin must be activated via adenylation by the ubiquitin-activating enzyme (E1) (Figure 7.2). The ubiquitin-conjugating enzyme (E2) catalyses the formation of isopeptide bonds between the C-terminal glycine 76 of ubiquitin, presented by the ubiquitin-activating enzyme (E1), and an ε-amino group of the acceptor lysine residue (Hershko, 1991; Jentsch, 1992). Subsequently, another ubiquitin residue is added to the internal lysine 48 of the precedent ubiquitin via its C-terminal glycine to form a chain of polyubiquitin, targeting the substrate for degradation by the 26S proteasome (Chau *et al.*, 1989).

Upon capture of the internal lysine by the E1/E2/E3 enzymatic complex, a multi-ubiquitin chain is progressively added to the targeted protein (Figure 7.1). The amount of ubiquitin on a given protein is in a dynamic state where ubiquitin residues are constantly added or removed by deubiquitination enzymes.

The 26S proteasome is a multisubunit complex made of a hollowed 20S core protease and a pair of symmetrically disposed 19S regulatory particles (Figure 7.4) where the targeted protein is degraded into small peptides. Studies have suggested that a particle of the 19S proteasome subunit complex, the 5S subunit, was responsible for the

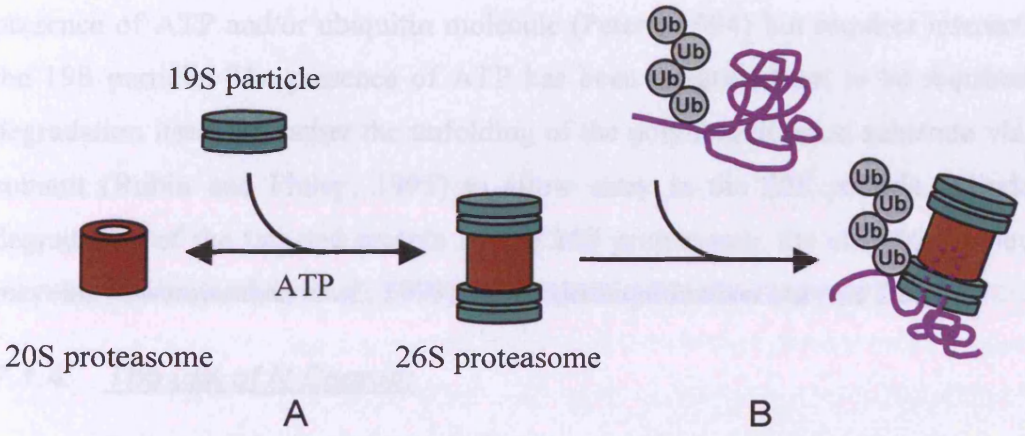

Figure 7.4: A - Assembly of the 26S proteasome. Interaction of the 20S Proteasome with 19S regulatory complex. The ~700 kDa complex 19S can bind to both ends of the C2-symmetric proteasome 20S, thereby conferring ATP- and ubiquitin-dependence on the complex. B - Hair-pin model of polyubiquitinated protein degradation. (Varshavsky (1997)).

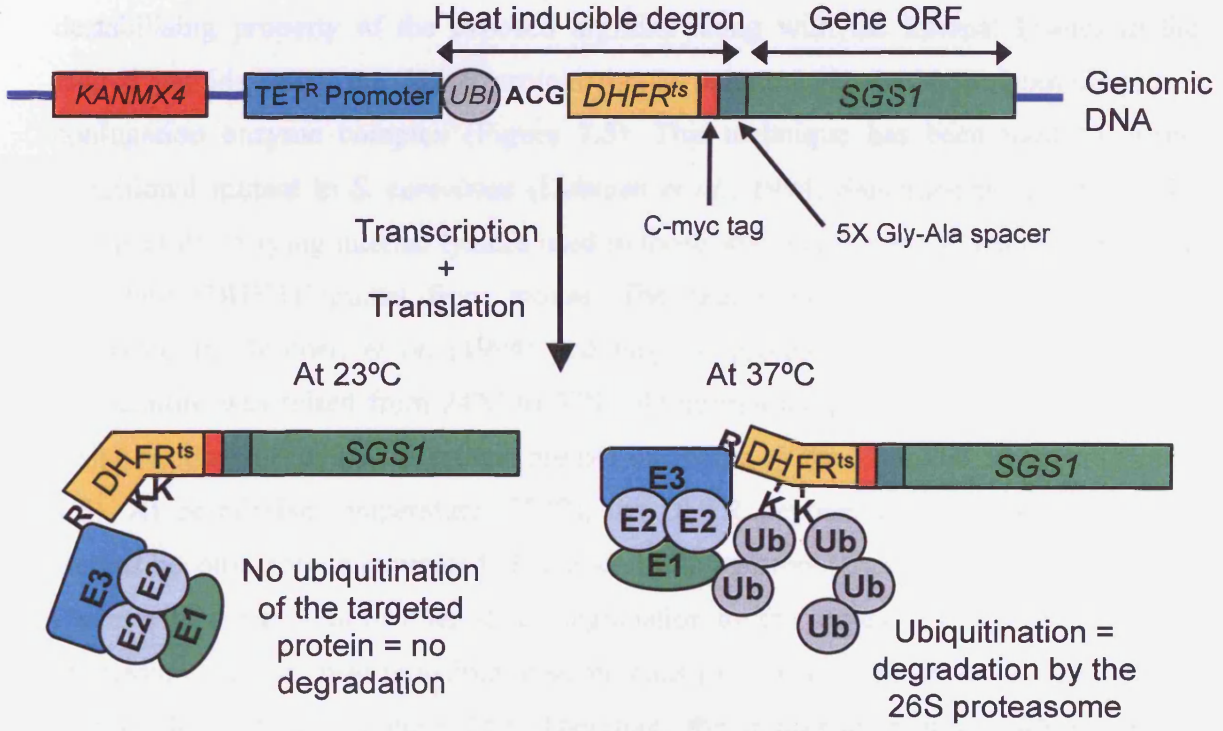

Figure 7.5: Mechanism of heat inducible degron tagged to the SGS1 open reading frame. At 23°C, the folding of the DHFRts prevents the internal lysines from being ubiquitinated. At permisive temperature, the internal lysine are polyubiquitinated and the protein is targeted for degradation by the 26S proteasome.

binding of multiubiquitinated proteins in an ATP independent manner (Deveraux *et al.*, 1994). The 20S particle is not able to degrade multiubiquitinated substrate either in presence of ATP and/or ubiquitin molecule (Peters, 1994) but requires interaction with the 19S particle. The presence of ATP has been suggested not to be required for the degradation itself but rather the unfolding of the polyubiquitinated substrate via the 19S subunit (Rubin and Finley, 1995) to allow entry in the 20S particle cylinder. After degradation of the targeted protein by the 26S proteasome, the ubiquitin molecules are recycled (Swaminathan *et al.*, 1999) by the deubiquitination enzyme Doa4p.

7.1.4. *The use of N-Degron:*

It is possible to engineer N-Degron signals to target proteins for degradation. The engineered N-Degron is made of a peptide containing internal lysine residues, which is tagged with a destabilising N-terminal arginine and an ubiquitin moiety (Figure 7.5). In yeast cells, ubiquitin C-terminal hydrolases rapidly remove the ubiquitin moiety from the fusion protein exposing the arginine residue (Bachmair *et al.*, 1986). The destabilising property of the exposed arginine along with the internal lysines in the tagged peptide target the fusion protein for degradation via the N-recognin/ubiquitin-conjugation enzyme complex (Figure 7.5). This technique has been used to create conditional mutant in *S. cerevisiae* (Dohmen *et al.*, 1994; Sanchez-Diaz *et al.*, 2004). The peptide carrying internal lysines used in those studies is a conditional dihydrofolate reductase (DHFR) mutant from mouse. The heat inducible degron was originally described in Dohmen *et al.* (1994) and targeted proteins for degradation when the temperature was raised from 24°C to 37°C. At normal temperature (24°C), the internal lysine residues of the DHFR protein are not accessible by the E1/E2/E3 complex (Figure 7.5). At permissive temperature (37°C), the DHFR peptide unfolds and the internal lysines become polyubiquitinated. The N-end rule pathway targets the DHFR protein in frame with the protein of interest for degradation by the proteasome (Figure 7.5). The expression of the heat-inducible degron construct is controlled by a tetracycline repressible promoter (Figure 7.5). Therefore, the transcription into mRNA can be repressed. Previous studies have shown that the efficient proteolysis of the degron requires increased expression of the Ubr1 protein (E3) (Bachmair *et al.*, 1986; Bartel *et al.*, 1990; Labib *et al.*, 2000).

As mentioned previously, cells lacking Sgs1p have an increased of chromosome rearrangements and deletions (Ajima *et al.*, 2002; Miyajima *et al.*, 2000a; Myung *et al.*, 2001; Watt *et al.*, 1995; Watt *et al.*, 1996) in mitosis while in meiosis *sgs1*Δ strains have a decreased sporulation and unfaithful chromosome segregation (Rockmill *et al.*, 2003; Watt *et al.*, 1995). The distinction between the mitotic and the meiotic phenotypes are often unclear and a reduction in spore viability in meiosis could be due to the mitotic defect of *sgs1*Δ strains (chapter 3 and (Miyajima *et al.*, 2000a; Rockmill *et al.*, 2003)). To overcome this problem, we decided to specifically abolish the expression of the *SGS1* gene during meiosis using the properties of the N-end rule pathway. For this purpose, we tagged the 5′ end of the *SGS1* gene with an N-degron carrying temperature sensitive degradation signals (destabilising residues and internal lysines). This "heat inducible" degron was inserted at the start of the open reading frame of the *SGS1* locus by PCR targeted replacement method (Figure 7.5).

7.2. Experimental procedures:

7.2.1. *Plasmid construction:*

Plasmid carrying the Heat inducible degron under a tetracycline repressible promoter (pKL183) and an expression *UBR1* plasmid (pSS126; 2μ *TRP1*; *UBR1*) were kindly given by Dr. Pedro San-Segundo (unpublished). The used of N-degron tag to induce conditional mutants have been described before (Dohmen *et al.*, 1994; Sanchez-Diaz *et al.*, 2004). The version used here was modified compared to the published studies as the N-degron is under a tetracycline promoter (*tet02*) and not a copper repressible promoter. The endogenous *UBR1* gene was not deleted from the tested strain but rather unconditionally over-expressed on a high copy plasmid (pSS126 derived from pRS424). The pSS126 plasmid, over-expressing the *UBR1* gene, was under an auxotrophic *TRP1* selection marker. The experimental *S. cerevisiae* strains used for this study are heterozygote *TRP1/trp1* and homozygote *ura3*. The *UBR1* gene, from pSS126, was then cloned in a *URA3* expression vector, pYES2. The *UBR1* gene from pSS126 was cloned out by restriction digests with *SalI* and *BamHI* restriction enzymes and cloned into a pYES2 vector (high expression, 2μ, *URA3*) digested with *XhoI* and *BamHI* restriction enzymes. Insertions of the *UBR1* gene were confirmed by restriction digests with *XbaI*, *SpeI*, *BamHI* and *PstI* restriction enzymes. The resulting pYES2-*UBR1* plasmid was transformed in an *ura3* strain to ensure complementation of the uracil auxotrophy.

7.2.2. *Strain construction:*

The N-degron plasmid (pKL183) was amplified using proof-reading *Pfu* polymerase and primers as described in Chapter 2. The amplification products were confirmed by electrophoresis and transformed in the *S. cerevisiae* strains ACT 53 (Table 7.1). Transformants were selected on YEPD medium supplemented with Geneticin. After DNA extraction, insertion of the degron cassette were confirmed by junction PCR and sequencing. The different strains used in this Chapter are detailed in Table 7.1.

Table 7.1: Haploid strains used:

Strains	Genotypes	Comments
ACT 53	*ade1-1; MATα; HOΔ; met13-2; ura3::nco; cyh2-1*	*SGS1*
ACT 56	*ade1-1; MATα; HOΔ; met13-2; ura3::nco; cyh2-1; sgs1::KANMX4*	*sgs1Δ*
Dg-SGS1	*ade1-1; MATα; HOΔ; met13-2; ura3::nco; cyh2-1; KANMX4; N-Degron-SGS1*	*N-Degron-SGS1*

7.2.3. MMS experiments:

The methylmethane sulfonate sensitivity of *sgs1* mutants in mitosis was used to confirm the effectiveness of degradation of the N-Degron-tagged Sgs1 protein. Prior to the experiment, serial concentrations of MMS were tested to ensure maximum efficiency of resistance/sensitivity by the wild type (ACT53) and *sgs1Δ* (ACT56) strains. The different MMS concentration tested were 0.02%, 0.05% and 0.075%. 0.075% of MMS per plate gave the best results in term of sensitivity by *sgs1Δ* cells. First, *SGS1* cells were transformed with an empty vector (pYES2) or with the *UBR1* over-expressing vector (pRED 688) to assess the effect of *UBR1* over-expression. Then, *sgs1Δ* cells (ACT 53) were also transformed with pYES2 or the *UBR1* over-expression vector (pRED 688) to confirm the MMS sensitivity of such strains. The strain carrying the *N-Degron-SGS1* (*Dg-SGS1*) construct was transformed with the *UBR1* vector (pRED688). Each different strain was pre-culture in *URA* drop out medium for 12 hours. 500 µl of each cell cultures was grown in fresh *URA* drop out medium for another 3 hours. 100 µl aliquots of each cell cultures were taken from the cell cultures and diluted into 1/10, 1/100, 1/1000 and 1/10.000 serial dilutions. Such dilutions were then plated in 5 µl dots on synthetic medium depleted of uracil and incubated at 23°C for 12 hours (growth control). Cell dilutions were also plated on URA drop out plates supplemented with 10% doxycycline and 0.075% MMS. The different plates were either incubated at 23°C or 37°C to induce degradation of the N-Degron-tagged Sgs1 protein.

7.3. Results:

In mitosis, cells lacking the Sgs1 protein are highly sensitive to DNA damaging agents such as hydroxyurea and methyl methane sulfonate (Ui *et al.*, 2001). *sgs1*Δ cells incubated on MMS-supplemented agarose plates are unable to divide effectively (Miyajima *et al.*, 2000a; Mullen *et al.*, 2000). Damage caused by MMS forces replication forks to arrest and/or collapse. In the absence of Sgs1p, the stalled replication forks are unable to restart (Cobb *et al.*, 2003). As explained in the experimental procedures (§ 7.2.3) we used this phenotype to assess the degradation of the tagged protein N-degron-Sgs1p.

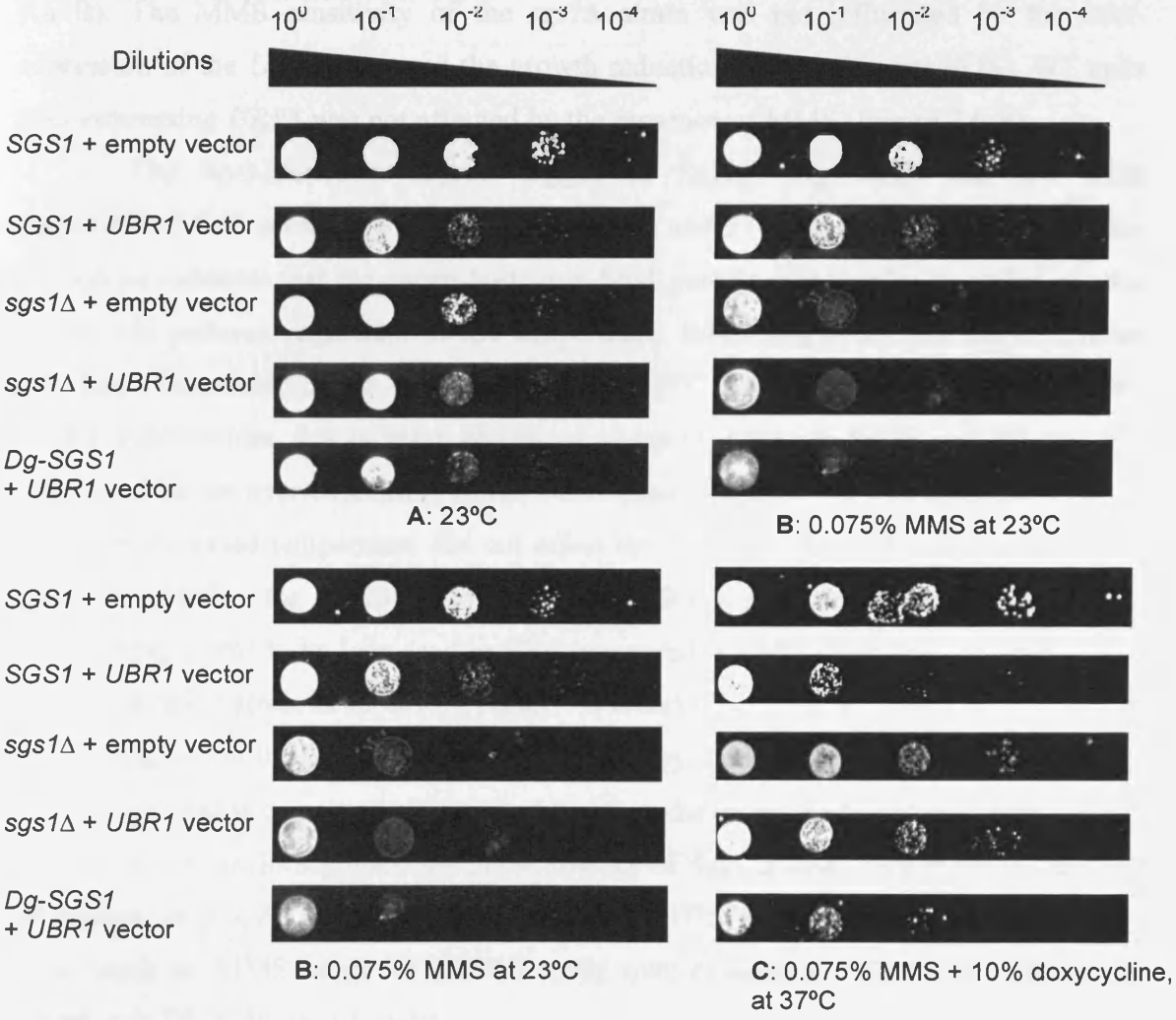

Figure 7.6: MMS sensitivity of *SGS1* mutants. 5 µl of each dilution were dotted on synthetic complete medium minus uracil ± MMS. (A): growth control at 23°C. (B): MMS sensitivity on plates supplemented with 0.075% MMS at 23°C. (C): MMS sensitivity on plates supplemented with 0.075% MMS and 10% doxycycline.

Growth on uracil drop out medium at 23°C (Figure 7.6 A) was used as a control to compare the MMS sensitivity of the different strains at 23°C and 37°C. Interestingly, the over-expression of the *UBR1* gene decreased growth in the SGS1 WT strain as shown by the small amount of growth in the 10^{-3} dilution (Figure 7.6 A). As previously reported (Watt *et al.*, 1995), *sgs1*Δ cells grow at a slower rate than SGS1 WT cells. This phenotype was also observed in our experiment where growth of *sgs1*Δ cells were reduced compared to SGS1 WT cells at 23°C. The growth in the *sgs1*Δ strain did not seem to be further reduced by over-expression of the *UBR1* gene as no further reduction was observed in the *sgs1*Δ strain over-expressing the *UBR1* plasmid (Figure 7.6 A). As seen in other studies, *SGS1* mutant strains are sensitive to MMS (Miyajima *et al.*, 2000a; Mullen *et al.*, 2000). The MMS sensitivity of *sgs1*Δ was confirmed in this study (Figure 7.6 B). The MMS sensitivity of the *sgs1*Δ strain was not influenced by the over-expression of the *UBR1* gene and the growth reduction observed in the SGS1 WT cells over-expressing *UBR1* was not affected by the presence of MMS (Figure 7.6 B).

The heat-inducible degron tagged to Sgs1p (*Dg-SGS1*) had the same constitutive MMS sensitivity as an *sgs1*Δ at 23°C and 37°C (Figure 7.6 B and C). This phenotype indicates that the entire N-degron-Sgs1 protein was rapidly degraded via the N-end rule pathway regardless of the temperature. Interestingly, at 37°C *sgs1*Δ mutant cells had a better viability on MMS plates than at 23°C (Figure 7.6 C compared to Figure 7.6 B). Furthermore, this increase resistance of *sgs1*Δ strains to MMS at 37°C was not influenced by the over-expression of the *UBR1* gene (Figure 7.6 C). In contrast to *sgs1*Δ cells, the increased temperature did not affect the resistance of the bona fide wild-type strain. In addition, the sensitivity to MMS in the SGS1 WT strain, over-expressing the *UBR1* gene, seems to be increased at 37°C compared to 23°C while the opposite is true for the *Dg-SGS1* strain as shown by Figure 7.6 B and C.

Although the tagging of the Sgs1 protein by the N-Degron cassette worked, the Dg-Sgs1 protein is constitutively targeted for degradation by the N-end rule pathway. In addition, these results suggests that in the absence of Sgs1 protein, regardless of the level of expression of *UBR1* gene, cells grow better at 37°C in presence of DNA damaging agent such as MMS while SGS1 WT cells over-expressing *UBR1* might be more sensitive to DNA damaging agent.

7.4. Discussion:

Several studies have shown that *UBR1* (E3) is the limiting factor of the N-end rule pathway (Bachmair *et al.*, 1986; Bartel *et al.*, 1990; Labib *et al.*, 2000). Therefore it is not surprising that over-expression of the *UBR1* gene in SGS1 WT cells may decrease the amount of growth in such cells by accelerating the degradation of proteins targeted by the N-end rule pathway.

A careful analysis of 30 N-terminal residues of the Sgs1 protein has highlighted the presence of several destabilising residues in this domain (Figure 7.7). Although Sgs1p has a methionine residue at the N-terminal, which is a stabilising amino acid, this amino acid is often removed from the protein during maturation by methioninine aminopeptidase. In the case of Sgs1p this cleavage is more likely to occur as the methionine residue precedes valine and threonine residues known to increase the likelihood of cleavage (85% and 30% respectively) (Sherman *et al.*, 1985). One interesting feature of the Sgs1 protein is the presence of three internal lysines (K) at position 4, 9 and 16, two of which K9 and K16 are surrounded by destabilising residues. The presence of those internal lysines in a block of destabilising residues could trigger the degradation of the Sgs1 protein via the N-end rule pathway by providing binding site for the Ubr1 protein. A study of 68 different degron signals by Susuki and Varshavsky (1999) has shown that strong degron signals carried lysine residues at position 3, 8 and 15 consistent with the topology of the E1/E2/E3 complex. If cleavage of the first methionine residue of the Sgs1 protein occurs, the internal lysine residues of the Sgs1 protein will find themselves exactly at the positions highlighted by the previous study (Suzuki and Varshavsky, 1999). Furthermore, although the Sgs1 protein does not contain a N-terminal destabilising residue essential for degradation, Varshavsky has shown that N-degron signals could be recognised in *trans* with interacting proteins carrying N-destabilising residues (Johnson *et al.*, 1990; Varshavsky, 1996). In the case of the Sgs1 protein, such *trans* activation of the degradation could occur via interaction between Sgs1 and another protein.

Study of the human RecQ homologue *RECQL4* provides further evidence for the degradation of RecQ helicases by the N-end rule pathway. Yin *et al.* (2004) have isolated the RecQl4 protein in a stable complex with human ubiquitin ligase Ubr1p and

Ubr2p. Although, RecQl4p is a long-lived protein and is not ubiquitinated *in vivo* (Yin *et al.*, 2004) its interaction with the human Ubr1p homologue of *S. cerevisiae* could suggest that the N-end rule pathway might target Sgs1p for degradation.

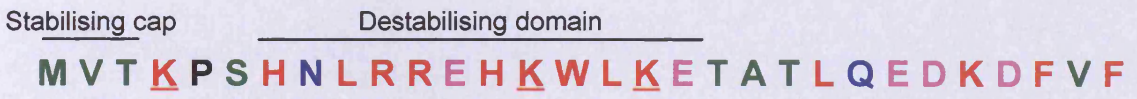

Figure 7.7: Sgs1p N-terminal Residues property (30 amino acids). The green colour highlights stabilising residues while red colour represents primary destabilising residues. Purple and blue colours highlight secondary and tertiary destabilising residues, respectively. K internal lysine hypothetic targets for ubiquitination.