Investigating the role of Sgs1 in the suppression of meiotic homeologous recombination in *Saccharomyces cerevisiae*

Thesis submitted for the degree of

Doctor of Philosophy

at the University of Leicester

by

Amit Dipak Amin

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September 2009

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PhD Thesis

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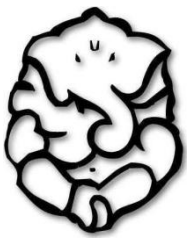
2009

for: **My Grandmother (Jyoti Patel)**
My Mum (Nita Amin)
My Dad (Dipak Amin)
My Uncle (Prakash 'Kash' Patel)
My Aunt (Rekha Ashar)



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Abstract

Homeologous recombination is the genetic exchange that occurs between diverged DNA sequences. This type of recombination can be detrimental to the cell, as it could lead to deletions, duplications and even chromosome loss. Therefore, it must be suppressed in order to maintain the integrity of the genome. The mismatch repair (MMR) complex, along with the 3'-to-5' helicase Sgs1, has been implicated in this early role in meiosis. We propose a model in which the MMR complex scans the genome searching for single end invasion (SEI) events occurring between diverged sequences. On finding such events, the MMR complex binds to them and impedes their progression. The MMR complex then recruits the Sgs1 helicase which unwinds the heteroduplex DNA, allowing the invading strand to continue its homology search. To test this hypothesis, we investigated whether the interaction between Sgs1 and one member of this MMR complex, Mlh1, affected the ability of Sgs1 to suppress homeologous recombination in the baker's yeast *Saccharomyces cerevisiae*. In addition, we investigated which domains of Sgs1 were required for this suppression. The data presented here show that the interaction between Sgs1 and Mlh1 may be important in the suppression of homeologous recombination at the SEI stage. In addition to this, we present data suggesting that the interaction between Sgs1 and the type IA topoisomerase Top3 may also be important in the resolution of recombination intermediates formed between diverged sequences. We suggest that there may still be additional factors that are utilised by the cell in order to maintain the barrier to inter-species recombination.

Sgs1 has also been suggested to function at a later stage of meiosis, in the decatenation of Holliday junctions. This process was proposed to be carried out in concert with Top3. We present data that conflict with this hypothesis. We propose that interactions between Sgs1 and the type II topoisomerase Top2 are required in the decatenation of pre-existing replication errors prior to the onset of meiosis. The data implicate Sgs1 in the pre-meiotic replication checkpoint to aid in the repair of errors caused during DNA replication prior to meiosis. We also hypothesise an additional role of Sgs1 in the activation of this pre-meiotic replication checkpoint for the process of sporulation. This investigation therefore emphasises the importance of Sgs1 in the early stages of meiosis.

Acknowledgements

The first person I must thank is my Boss, Professor Rhona Borts. I remember meeting you during the first year of my undergraduate course at the University of Leicester about 9 years ago ... which was also your first year at Leicester. I am very proud to say that we have been friends ever since (Dr. B!) ... although I dread to think what your first impression of me was, as despite getting the work done, we made sure we had a good time whilst doing it! I'm guessing that your impression of me wasn't all that bad, as you allowed me to work as a member of your lab during my Masters course ... I'll never forget this time, as it was the first time I was introduced to Sgs1! I guess I didn't let the problems associated with dissecting Sgs1 get to me, as I decided to work on it for three more years during this PhD. It was during this time that our friendship grew. I truly appreciate and respect you for all your help and never-ending support throughout my career at this university. I thank you for your ability to be frank yet motivating at the same time. More than anything, I am grateful for your understanding and for giving me the opportunity to work with you and to further my scientific career. Thank you, Boss!

The next person I must thank is Alex Chaix, who was my supervisor during my Masters course and has served as a mentor to me over the past few years. Your encouragement, help and support has led me to become a more competent scientist and has helped me further my understanding of yeast genetics. Your work has laid the foundation for my project and together we have uncovered some very exciting things.

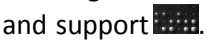
I'd also like to thank members of the lab both past and present.

There's no better person to start with than Christian 'C.B.' Bär, who I am honoured to call one of my closest friends. Bruder, I'd like to say a massive 'TA, LAD' to you for all the great times we've had ... and there have been plenty! The letters L.E.B. will always make me smile ☺ ... and, thanks to you, I know where to go if I ever need to dry my hands! I cannot wait to hit the poker tables with you in Vegas !

I'd also like to thank Mathuravani Aaditiyaa (a.k.a. Cricket-Ben) for her encouragement, support and friendship ... and for our several-hour long chats about life! But, most of all, I want to thank you for being a true fan of Indian cricket and cricket in general, as some of my highlights during this PhD have been watching, analysing and talking about all things cricket with you ... something I know we'll continue to do for a very, very, very long time. Massive thanks go to Daniel Jablonowski (a.k.a. Hollywood Dan; a.k.a. Uncle-Social) for being such a great friend. Your helpfulness and kindness knows no limits ... plus, you come up with some of the greatest one-liners ever! I'd also like to thank my very good friend, and master of gossip, Shanow 'Shint' Uthman for being one of the funniest people I have ever met. Your story-telling ability will never be matched and will be sorely missed.

I cannot thank Dr Raffael Schaffrath enough for allowing me to work in his lab and for treating me like one of his lab members. Your acceptance, support and friendship are greatly appreciated ... Vielen Dank! I'd also like to thank Dr Flaviano Giorgini for his kindness, help and support ... but most of all, for being an Indianapolis Colts fan. I am so grateful to have someone to talk to about Cover-2 defenses, Manning's passer ratings, Bob Sanders hard-hitting abilities and, most importantly, the Colts 29-17 victory over the Bears in SuperBowl XLI!!! I must also thank my thesis committee members Dr Peter Meacock and Dr Celia May for their kind words, helpful suggestions and support throughout my degree. I'd also like to thank Celia for allowing me to carry out my undergraduate project with her. Similarly, I'd like to thank Dr Chris Talbot for not only being my personal tutor during my undergraduate degree, but for also giving me my first taste of the research world. My first experience in scientific research came in your lab during the summer of my second year of the

BSc. I am very grateful to both you and Joan Sutherland for this experience as it gave me the confidence to seriously consider a career in science.

Big thanks go to Gary Pinch for not only informing us about all things historical and factual, but for also being a great friend. I'd also like to thank Patricia 'Trish' Sawyer, Robert Mason and Victoria 'Vicky' Wheatley for being my 'fountains of knowledge'. Whenever I had any doubts, you guys were always there to help and I am eternally grateful for this. I'd also like to thank Trish for putting up all the photos and videos of her beautiful baby Emily on FaceBook (the same goes for Flav and the photos he puts up of the Z.O.G. and Emma) ... when lab-life gets us down, these pics always make us smile ☺. Huge thanks also go to the 'Dissecting Queens' Rebecca Keelagher and Maryam Rehan for their kindness, help and support  . The same goes for Jannine Clapp, Kayoko Tanaka, Max Casu, Ralitsa Kantcheva and Susie Campesan, as well as previous members of the lab Leonor Miller Fleming, Nicola 'Nicky' Butler, Eva Hoffmann, Gianni Liti, Marcus Marvin and Kees Straatman.

Big thanks go to all the gals in the office, especially Ila Patel and Sarah Laband, as well as everyone in the Media Kitchen – Keith Baker, Liz Le-Butt, Richard Sansom and Rachel Sidwell. Same goes for our resident computer expert Ian Townson who helps us with all things PC. It's been an absolute joy to work with you guys. You not only make our lives easier, but when things aren't going as planned, we can always rely on your smiling faces, friendly attitudes and understanding nature to cheer us up. You guys are absolute legends and words cannot express how truly grateful we all are for all that you do for us.

I cannot forget to thank all of the students that I have had the pleasure of teaching during my time as a PhD student. Some of you I met for the first time when you came to the lab, whereas others I had known previously. Regardless, I am proud to say that I consider each of one you as dear friends. It all started with Tom 'Indeed' Brabbs (a.k.a. Cat Butt) who I supervised during the summer of my first year ... your fascination with cats still creeps us all out! Then came the Brain-With-Feet (female) Elisa Wong, who we all predict will be running the world sooner rather than later! Along with Elisa came Team SPAA member #54 Sofia Pavlou who is my sister-from-another mister and an absolute legend! I really miss having you around Sof P ... hope Team SPAA member #189 is doing fine! I also had the pleasure of supervising Stefanie 'M.T.' Nicolaou, who eventually learned how to pronounce 'precipitation'. Along with M.T. came my Nani-Ben Maithili Shroff (a.k.a. Thep) ... I miss watching Heroes with you every Tuesday afternoon! Finally, there was Qudsia 'Quidzio' Malik who is one of the most hard working and kind-hearted people I have ever had the pleasure of knowing. Each one of you has made my time in the lab more enjoyable, and I can honestly say that teaching you guys was the highlight of my PhD.

During my time as an undergrad, I met many great people who I also have to thank for making my time at the University of Leicester even more pleasurable. The BSc Kliq included Yok 'The Man' Tan (my WrestleMania buddy), Abdel 'Abu' Halim (one of the funniest people ever – both intentionally and unintentionally!), Alan Baker (one of the nicest guys to ever wield a sword), Ryan Tan (Brain-With-Feet (male)), Andrew 'Chad' Chadburn (brother of Abu) and Dr. Farah 'Yoda' Pathan  . During my time in the Masters course, I met two people who became guys I consider to be brothers. Huge thanks to Ariful 'Arif' Haque (one of the most hard working yet laid back people I have ever met) and Pawan Noel (the man with a heart-of-gold and someone I am honoured to have as my brother) for being pillars of support in my life.

As this degree comes to an end, so does my near-decade long time at the University of Leicester. In addition to the people mentioned above, there have been many other great people who have positively influenced my time at this uni and become great friends. These legends include Jan-Christoph Detering (isn't it about that time of the year to drop us a mail to say 'Tag'?), Tony

O'Tierney (the Thesaurus-With-Feet), my brother 'The GodFather' Karzan Karim along with his wife Naz (my sister-in-law) and amazing daughter Karin (my little niece), Omar Than (no euphemisms necessary!), Mohammed 'Mo' Mussa (the only doctor who wears a baseball cap in the hospital), Safeer Mughal (a fellow Leicester original), Christiana Loizou (who is never without a glass in one hand), Bubacarr 'Buba' Gibril Kaira (one of the coolest dudes I know), Rizwan 'Riz' Khan (one of the most chilled out dudes I know), Tariq Viqaas Aziz (one of the craziest dudes I know), Steve Butler (another person who appreciates things like Zone Blitzes and scrambling QBs ), Ran Ren (a fellow gamer), Kamaldeep Chana (a rascal who has great taste in TV shows), Carlo Breda (Hello Carlo, I want to play a game ), Valeria Zonato (Game Over .... EXCUSE ME?! ), Adnan Gopinadhan (a man who truly is rock'n'roll ... and never hesitates in producing a fine set of metal horns!) and Evangelia 'Small Eva' Tasouri (my future sister-in-law ... no pressure intended, C.B.). I also have to thank my brother and proctologist-to-the-stars Dr. Afzal Korim for always making us laugh with his quick wit and for being a great friend. Another person who I consider to be a brother is Kunnaal Bojwani (a.k.a. DJ Kunni a.k.a. Back Pain a.k.a. White Dog ...). We miss your stories / tall-tales / bull-sh*t on a daily basis and look forward to when we'll all get together again ... and we also look forward to seeing your autobiography in the fiction section of most good bookstores! Then, there is one of my closest friends, Eyemen Kheir. We first met when you were doing your undergraduate project in the lab and we've since had many, many, many great times together ... as well as all the TV shows we've swapped with each other! Thank you for being my brother, bro!

Finally, I'd like to thank everyone else who is a part of the Department of Genetics ... for putting up with me for as long as you have! I figure that after 10 years you guys will end up putting me on the inventory list ... so I'm getting out at nine-and-a-half!

I would be lying if I said I didn't feel a little sad that my near-decade long run at the University of Leicester is coming to an end ... however, I greatly anticipate the challenges that lie ahead. Thanks once again to ALL those who have made my time at this uni more pleasurable. It's been a wild ride.

As a great man once said: 'I'm f'n done, son ... hooray for me'!

Abbreviations

4-NQO	4-Nitoquinoline 1-Oxide
5-FOA	5-Fluororotic Acid
ALT	Alternative Lengthening of Telomeres
A. thaliana	*Arabidopsis thaliana*
ADP	Adenosine Diphosphate
AR	Acidic Region
ATP	Adenosine Triphosphate
BASC	BRCA1-Associated Genome Surveillance Complex
BIR	Break Induced Repair
BLAP18	BLM-Associated Polypeptide, 18 kDa
BLAP75	BLM-Associated Polypeptide, 75 kDa
bp	Base Pair
BrdU	Bromodeoxyuridine
BS	Bloom's Syndrome
CHEF Gels	Clamped Homogenous Electric Field Gels
cM	centiMorgans
'cut' phenotype	Cell Untimely Torn phenotype
dHJ	double Holliday Junction
DNA	Deoxyribonucleic Acid
dNTPs	Deoxyribonucleoside Triphosphates
DSB	Double-Strand Break
DSBR	Double Strand Break Repair
E. coli	*Escherichia coli*
EDTA	Ethylenediaminetetraacetic Acid
ELISA	Enzyme Linked Immunosorbent Assay
EMSA	Electrophoretic Mobility Shift Assay
ENs	Early Nodules
ERCs	Extrachromosomal rDNA Circles
ES cells	Embryonic Stem cells
G418	Geneticin
GCRs	Gross Chromosomal Rearrangements
HCl	Hydrochloric Acid
H. pylori	*Helicobacter pylori*

HMGB1	High Mobility Group Box 1
HNPCC	Hereditary Non-Polyposis Colon Cancer
HRDC domain	Helicase-and-RNaseD-C-terminal domain
HU	Hydroxyurea
IDLs	Insertion / Deletion Loops
kb	Kilo Base
LB	Luria Broth media
LMP Agarose	Low Melting Point Agarose
LNs	Late Nodules
MEPS	Minimal Efficient Processing Segment
MMR	Mismatch Repair
MMS	Methyl Methane Sulfonate
MLH	MutL Homolog
MRX complex	Mre11-Rad50-Xrs2 complex
MSH	MutS Homolog
NaCl	Sodium Chloride
NaOH	Sodium Hydroxide
NLS	Nuclear Localisation Signal
NMR	Nuclear Magnetic Resonance
NPD	Non-Parental Ditypes
PCNA	Proliferating Cell Nuclear Antigen
PCR	Polymerase Chain Reaction
PD	Parental Ditypes
PEG	Polyethylene Glycol
PIP box	PCNA Interacting Protein box
PML	Promyelocytic Leukemia Nuclear Bodies
PMS	Post Meiotic Segregation
psi	Pounds Per Square Inch
PSSC	Precocious Separation of Sister Chromatids
RAPADILINO	Radial hypoplasia, Patella hypoplasia and cleft of Arched palate, Diarrhoea and dislocated joints, Little size and limb malformation, Nose slender and normal intelligence syndrome
rDNA	Ribosomal Deoxyribonucleic Acid
RFC	Replication Factor C

Rmi1	RecQ Mediated Genome Instability 1
Rmi2	RecQ Mediated Genome Instability 2
RPA	Replication Protein A
RQC domain	RecQ Conserved domain
RTS	Rothmund Thompson Syndrome
S. carlsbergensis	*Saccharomyces carlsbergensis*
S. cerevisiae	*Saccharomyces cerevisiae*
S. paradoxus	*Saccharomyces paradoxus*
S. pneumonia	*Streptococcus pneumoniae*
S. pombe	*Schizosaccharomyces pombe*
S. typhi	*Salmonella typhi*
S. typhimurium	*Salmonella typhimurium*
SC	Synaptonemal Complex
SCB elements	Swi4-Swi6 cell cycle box elements
SDS	Sodium Dodecyl Sulphate
SDSA	Synthesis Dependent Strand Annealing
SEI	Single End Invasion
SIC	Synapsis Initiation Complex
Sgs1	Slow Growth Suppressor 1
sgs1-K706A	Helicase-defective mutation of Sgs1
sgs1-mlh1-id	Mutation in the Mlh1 Interacting Domain of Sgs1
sgs1-top3-id	Mutation in the Top3 Interacting Domain of Sgs1
SSA	Single Strand Annealing
SSB	Single Stranded Binding protein
SSC	Sodium Chloride and Sodium Citrate
ssDNA	Single Stranded DNA
dsDNA	Double Stranded DNA
TBE	Tris-Borate EDTA
TE	Tris-HCl EDTA
TT	Tetratypes
USCE	Unequal Sister Chromatid Exchange
UV	Ultra-Violet light
WS	Werner's Syndrome
YEPD	Yeast Extract Peptone Dextrose

Contents

Chapter 1: Introduction

The process of meiosis involves the halving of the chromosome number from a diploid to a haploid state. In contrast, mitosis involves the division of a cell into two identical daughter cells which carry the same genetic information. The process of meiosis consists of DNA replication, followed by two sequential rounds of division. The first division separates the homologous chromosomes, whereas the second division separates the sister chromatids. This ultimately leads to the production of four haploid cells. This process is important in generating genetic diversity amongst species.

1.1 DNA replication is a pre-requisite to mitotic and meiotic division

DNA replication is a pre-requisite for both mitosis and meiosis. This occurs during the S-phase of the cell cycle. For mitotic division, DNA replication is initiated at various specific points, known as origins of replication. Replication is carried out via the movement of replication forks along the DNA. The replication forks act by unwinding DNA, which exposes single-stranded DNA that is used by the enzyme DNA polymerase as a template to prime DNA synthesis. In order to control the start of S-phase, pre-replication complexes are found at the start of each origin of replication (Diffley et al., 1994). In *Saccharomyces cerevisiae*, several proteins comprise these pre-replication complexes, including the Mcm2-7 (minichromosome maintenance 2-7) family of proteins (Tye, 1999). The Mcm2-7 proteins are required for the initiation and elongation of replication forks during S-phase by functioning as helicases, unwinding the DNA ahead of the fork. They function by binding to chromatin at the end of the M-phase of the cell cycle and remain bound until the start of S-phase. The Mcm2-7 protein hexamer, aided by Cdt1 and Cdc6, is able to open up and clamp around the DNA (Cao et al., 2008, Kawasaki et al., 2006). When replication starts, they are removed from the origins of replication, which converts pre-replication complexes into post-replication complexes, which cannot initiate replication. This ensures that re-replication of the DNA does not occur (Blow and Dutta, 2005).

The process of DNA replication must be tightly regulated, as any errors that may arise could result in genomic instability. This instability is, in fact, a hallmark of many cancers (Myung and Kolodner, 2002). The cell has several mitotic checkpoints that are activated in response to DNA damage or when the replication machinery comes across problems (**Figure 1.1**). Such problems include nucleotide damage caused by exposure to UV or strand breaks caused by ionizing radiation (Rowley et al., 1999), or single stranded gaps in the DNA (Courcelle and Hanawalt, 2001, Cox et al., 2000, Cox, 2001, Oakley and Hickson, 2002). Activation of checkpoints during G1/G2 leads to cell cycle arrest, whereas activation during S-phase leads to a slowing of the rate of S phase progression (Paulovich

and Hartwell, 1995), which ensures that the DNA damage is not replicated (Lowndes and Murguia, 2000, Lydall and Weinert, 1996, Weinert, 1998, Zhou and Elledge, 2000). During S-phase, the DNA is exposed, which results in an increase in the rate of DNA damage. This means that there is an increased probability that the replication machinery could collide with DNA lesions or proteins that are bound to the DNA, which would lead to collapse of the replication fork (Kuzminov, 1999, Michel et al., 2007, Ouyang et al., 2008). When this occurs, 'sensor kinases', such as Mec1 and Rad53, are activated, which are able to control the progression of replication forks (Tercero and Diffley, 2001). This enables the stabilisation and restarting of the replication forks, as well as the prevention of any additional origins from firing and the inhibition of the onset of mitosis (Oakley and Hickson, 2002).

The entry into meiosis is regulated by a pre-meiotic checkpoint that acts after DNA replication (reviewed in Murakami and Nurse (2000)). Interestingly, some of the proteins implicated in this process are also shown to be required to regulate entry in mitosis. For example, Stuart and Wittenberg (1998) showed that *CLB5* and *CLB6* are essential for this pre-meiotic DNA replication checkpoint. In mitosis, these genes promote the G1-to-S-phase transition. However, unlike in mitosis, their function is essential in meiosis. Deletion of *clb5* confers a sporulation defect that is worsened by simultaneous deletion of *clb6*. The spores that do form in the double mutant are not viable (Stuart and Wittenberg, 1998). In addition to this, FACS analysis revealed that the *clb5* mutant exhibits a delay in the onset of DNA replication. The *clb5 clb6* double mutant leads to a more severe defect, with no DNA replication detected even after 24 hours (Stuart and Wittenberg, 1998). The role of these genes in both mitosis and meiosis is in the activation of the cyclin-dependent protein kinase Cdc28. The importance of Cdc28 was shown using the Clb-specific cyclin-dependent protein kinase inhibitor Sic1. The presence of Sic1 resulted in cell cycle arrest with cells exhibiting a G1 content. This is attributed to the inability of these cells to progress into the pre-meiotic S-phase (Stuart and Wittenberg, 1998).

Other cyclin-dependent protein kinases that are required for both mitotic and meiotic checkpoints are Cdc9 and Cdc13 (Weber and Byers, 1992). During mitosis, *cdc9* cells have been shown to arrest at G2 under control of Rad9. This cell cycle arrest is in response to the presence of DNA lesions which need to be repaired for mitosis to successfully occur (Hartwell and Weinert, 1989). Cdc13 is also required to arrest cells that have undergone DNA damage prior to meiosis. *cdc13* cells do not form visible spores (Weber and Byers, 1992). Electron microscopy of the arrested cells showed the absence of the spindle and synaptonemal complex (SC) in these mutants, confirming that the defects were seen early during meiosis (Weber and Byers, 1992). In agreement with this, flow cytometry

showed that most arrested cells had undergone pre-meiotic DNA replication. The unbudded nature of these cells indicates that the cycle of DNA replication that took place represents entry into meiosis (Weber and Byers, 1992). The actions of Cdc13 in this checkpoint are dependent on Rad9, as in mitosis. *cdc13 rad9* cells produce inviable spores which Weber and Byers (1992) attributed to the continued presence of lesions that could not be repaired. Another gene shown to act in this meiotic DNA replication checkpoint, as well as in mitotic checkpoints, is the Mec1 kinase. Under normal conditions, exposure to hydroxyurea (HU) leads to a cell cycle delay to allow for repair. This delay is abolished in *mec1* diploids (Stuart and Wittenberg, 1998). These studies therefore highlight the importance of maintaining the fidelity of replication, by activation of cell cycle checkpoints in response to damage, to ensure successful meiotic, as well as mitotic, divisions through the process of homologous recombination.

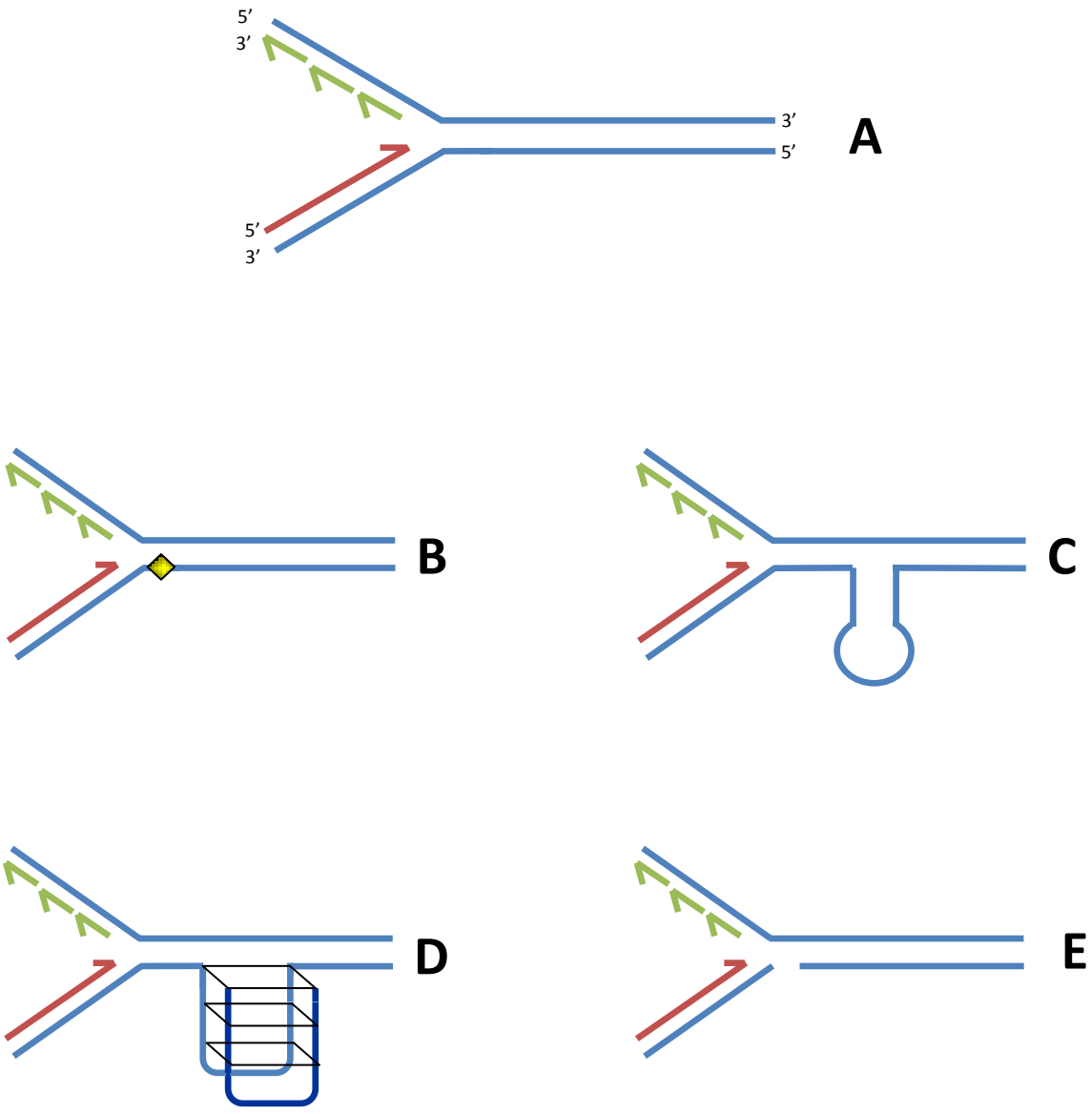


Figure 1.1: Potential factors that lead to the stalling or collapsing of replication forks

Modified from Oakley and Hickson (2002)

Replication forks (**A**) can stall due to the presence of a DNA adduct (**B**), a hairpin (**C**) or a G-quadruplex structure (**D**), whereas replication forks may collapse due to the presence of a single stranded gap (**E**).

1.2 Homologous Recombination

The process of homologous recombination (**Figure 1.2**) is critical in ensuring accurate chromosome segregation during meiosis. During mitosis, homologous recombination is a mechanism used to repair DSBs that have formed due to DNA damage or due to the stalling of replication forks (**Figure 1.1**). In contrast to meiosis, mitotic repair is carried out using the sister chromatid as opposed to the homologous chromosome.

Meiotic homologous recombination is initiated by a double-strand break (DSB). The formation of this break is catalysed by Spo11 via a transesterification reaction (Keeney et al., 1997). It is suggested that the crossover / non-crossover fate is determined at this point (Allers and Lichten, 2001). However, some speculate that the crossover / non-crossover fate is decided even earlier, before the DSB occurs, or immediately after this step (Storlazzi et al., 1996). Physical studies by Allers and Lichten (2001) showed that crossovers and non-crossovers are formed by different pathways. They mutated the meiosis-specific transcription factor *ndt80*, which is required for exit from the pachytene stage of meiosis (Xu *et al*., 1995). These *ndt80* mutants were shown to cause defects in the production of crossovers but still exhibited normal levels of non-crossovers (Allers and Lichten, 2001).

Following the formation of the DSB, 5' strand resection takes place (**Figure 1.2**). This involves the proteins Sae2 (Clerici et al., 2005, McKee and Kleckner, 1997, Prinz et al., 1997), Mre11 and Rad50 (Johzuka and Ogawa, 1995, Tsubouchi and Ogawa, 1998) and Exo1 (Tishkoff et al., 1997, Tsubouchi and Ogawa, 2000). 5' strand resection generates 3' single-stranded overhangs which are then able to invade the homolog (Sun et al., 1991). This strand invasion is facilitated by the homologous-pairing and strand-exchange proteins Rad51 and Dmc1 (Shinohara and Shinohara, 2004), which form nucleoprotein filaments together with the 3' single-stranded overhangs. This strand invasion leads to the formation of a Single End Invasion (SEI) structure (Hunter and Kleckner, 2001). It is proposed that the SEI that forms for the crossover pathway is different to that formed for the non-crossover pathway. This is based on the observation that all detectable SEIs lead to the formation of double Holliday junctions (dHJ) (Hunter and Kleckner, 2001).

For the crossover pathway, the second DSB end is captured by the D-loop following invasion (Lao et al., 2008). This is followed by DNA synthesis and the formation of a double Holliday junction (dHJ) (Hunter and Kleckner, 2001, Schwacha and Kleckner, 1995). Szostak *et al* (1983) proposed that dHJ structures could be resolved two ways, which would yield either a crossover product or a non-

crossover product. This model has since been revised, due to the findings of Allers and Lichten (2001) and also Hunter and Kleckner (2001). The revised model is shown in **Figure 1.2**. In this model, the resolution of the dHJ will yield only crossover products. These crossover products exhibit interference (Egel, 1978), which ensures that a crossover will not form in the vicinity of an existing crossover. The ZMM proteins, as well as some mismatch repair (MMR) proteins, have been shown to promote crossovers that exhibit interference (**Table 1.1**).

Non-crossover products arise from the Synthesis Dependent Strand Annealing (SDSA) pathway (Paques and Haber, 1999). Strand invasion is proposed to occur only on one side of the DSB. This invading strand is not captured as in the DSBR pathway, but is instead displaced. This is followed by strand annealing to complementary sequences on the second DSB end and DNA synthesis, which leads to the formation of a non-crossover product.

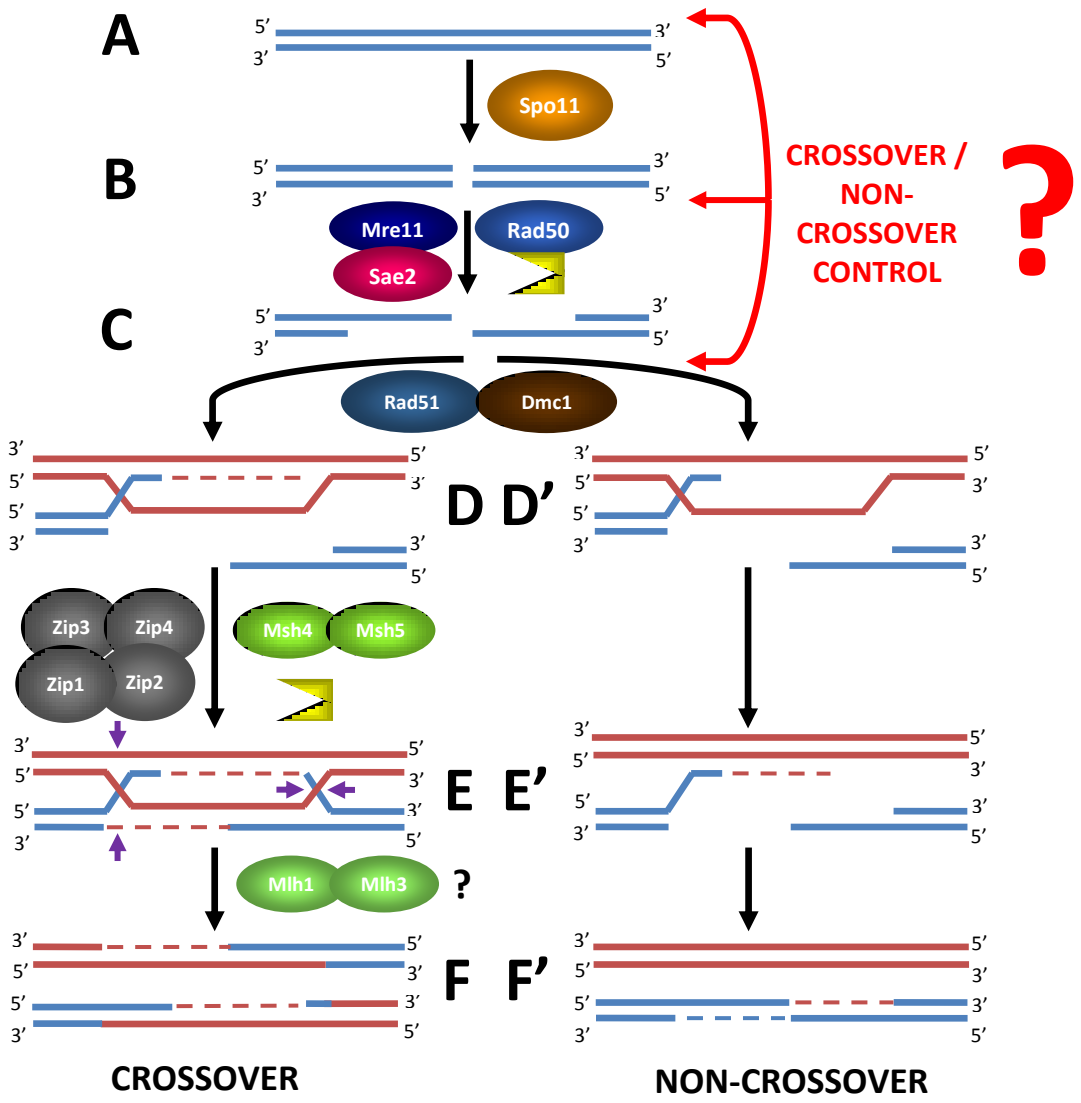


Figure 1.2: Meiotic homologous recombination
Modified from Allers and Lichten (2001).

Meiotic homologous recombination is initiated by a double-strand break (DSB) in the DNA (**A**), catalysed by Spo11. This is followed by 5'-to-3' strand resection (**B**) which leads to the formation of 3' single-stranded overhangs. The 3' single-stranded overhang invades the homolog (**C**). As indicated by the red arrows, it is suggested that the crossover / non-crossover fate is either determined here, immediately after this step, or even before the DSB forms.

For the crossover pathway (**D**, **E** and **F**) – strand capture, DNA synthesis and ligation follows (**D**). This leads to the formation of the double Holliday junction (dHJ) structure (**E**) facilitated by the ZMM proteins, which are pro-crossover factors (*see text for details*). The resolution of the dHJ leads to the production of a crossover product that exhibits interference (**F**).

In the Synthesis Dependent Strand Annealing (SDSA) (non-crossover) pathway (**D'**, **E'** and **F'**) – strand displacement occurs (**D'**). This is followed by strand annealing, DNA synthesis and ligation (**E'**), leading to the formation of a non-crossover product (**F'**).

1.2.1 Crossover / non-crossover control

1.2.1.1 The Leptotene / Zygotene Transition

The model that the crossover / non-crossover fate is determined early in homologous recombination was based on initial observations showing the existence of recombination nodules (Carpenter, 1975, Carpenter, 1979) (also reviewed in Page and Hawley (2003) and Zickler and Kleckner (1999)). Two types of recombination nodules are seen in cells, both showing associations with the SC. The SC forms between homologous chromosomes, holding them in close proximity to each other, thereby allowing chromosome pairing and crossing over to occur. The first class of these recombination nodules, known as Early Nodules (ENs), are seen from the late leptotene / early zygotene stage of meiosis to early mid-pachytene (**Figure 1.3**). The second class, known as Late Nodules (LNs), arise during the pachytene stage of meiosis.

Electron microscopy has defined the structures of these recombination nodules and immunofluorescent studies have elucidated their components. A greater number of ENs are seen in the cell when compared to the number of LNs. ENs are approximately 100nm in diameter and are observed along unsynapsed axes along the euchromatin. Microscopy studies by Anderson *et al* (2001) and immunofluorescent studies by Moens *et al* (2002) showed that ENs are made up of the RecA homologs Rad51 and Dmc1. As these proteins are involved in the search for homology (**Figure 1.2**), it has been suggested that ENs have roles in the synapsis of homologs. In agreement with this, the absence of Rad51 and Dmc1 foci in $Spo11^{-/-}$ mice has been suggested to indicate defects in synapsis (Baudat et al., 2000, Romanienko and Camerini-Otero, 2000). Co-localisation studies in mouse spermatocytes showed that these Rad51 and Dmc1 foci also associate with Replication Protein A (RPA), Msh4 and the RecQ helicase BLM (discussed in the subsequent sections) (Moens et al., 2000, Moens et al., 2002, Walpita et al., 1999).

LNs correlate with the number of crossover events and chiasmata. They are said to provide cytological evidence of crossover control (Borner et al., 2004, Carpenter, 1987, Hunter and Kleckner, 2001, Storlazzi et al., 1996). LNs range in size from 100nm to 200nm and are seen to associate with Mlh1 and Mlh3 (Anderson et al., 1999, Baker et al., 1996, Marcon and Moens, 2003). The number of chiasmata are reduced in $Mlh1^{-/-}$ mice spermatocytes which further supports the role of LNs in crossing over (Baker et al., 1996, Woods et al., 1999). Consistent with this, mutation of *mlh1* in yeast also leads to defects in crossing over (Hunter and Borts, 1997).

Immunofluorescent staining suggests that when the ENs acquire RPA, Msh4 and BLM, they lose Rad51 and Dmc1 (Moens et al., 2002). As meiosis progresses, ENs then lose RPA but they retain BLM (Moens et al., 2002). At this time, LNs, seen as Mlh1 foci, appear. However, their distribution does not coincide with the number of BLM foci (Moens et al., 2002). Co-immunoprecipitation studies have shown that Msh4 interacts with Mlh1 (Santucci-Darmanin et al., 2000). In addition to this, Mlh1 is seen to co-localise with RPA (Moens et al., 2002). These data suggest that some, but not all, ENs (consisting of RPA-BLM-Msh4) may become LNs. Moens *et al* (2002) proposed that the role of Rad51 and Dmc1, as ENs, in synapsis leads to several DNA-DNA interactions. Some of these ENs become LNs caused by recruitment of Mlh1 via interactions with RPA and Msh4. These LNs ultimately result in the formation of crossovers. The DNA-DNA interactions present from these ENs that do not become LNs are proposed to be disrupted by the anti-recombination activity of BLM (discussed in detail in the subsequent sections of this chapter) which prevents an excessive amount of recombination events.

Further support for this comes from electron microscopy and co-localisation studies have also implicated Zip2 and Zip3 as components of LNs (Agarwal and Roeder, 2000, Chua and Roeder, 1998, Fung et al., 2004). Zip2 and Zip3 are components of the Synapsis Initiation Complex (SIC) (**Section 1.2.1.2**). The numbers and distribution of SIC sites correlate with the number of LNs. These SIC sites are proposed to mark the sites of crossovers, as mutations in protein components of the SIC result in a decrease in the number of crossovers, without affecting the levels of non-crossovers (Agarwal and Roeder, 2000, Chua and Roeder, 1998, Fung et al., 2004, Novak et al., 2001, Rockmill et al., 2003, Ross-Macdonald and Roeder, 1994). Like crossovers, SIC sites have been shown to exhibit interference (Fung et al., 2004). However, Fung *et al* (2004) showed that the interference exhibited by Zip2 foci is not disrupted in mutations that disrupt genetic interference. These observations provide evidence for crossover control. In agreement with this, SIC sites are seen during both the zygotene and pachytene stages of meiosis. Therefore, it is suggested that SIC sites represent a subset of ENs that become LNs as meiosis progresses, as discussed above. As Zip2 and Zip3 have been shown to interact with Msh4, they are suggested to also provide a link between ENs and LNs (Agarwal and Roeder, 2000). Therefore, the decision as to which SIC sites will become crossovers is suggested to be made early during meiosis, either at or before zygotene (**Figure 1.3**).

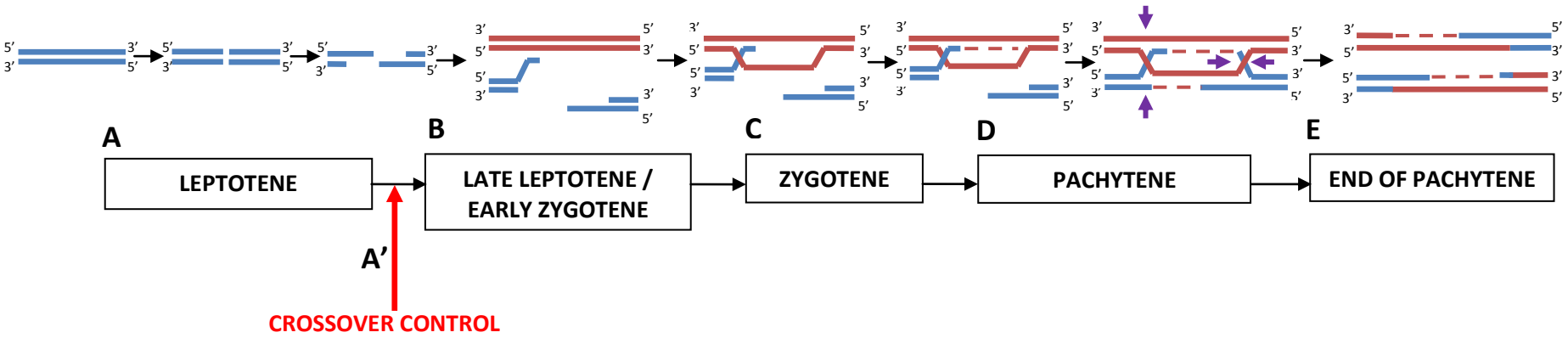


Figure 1.3: The stages of meiotic recombination

Interpreted from Borner *et al* (2004) and Hunter and Kleckner (2001)

The leptotene stage of meiotic recombination involves the formation of the DSB (**A**). This is followed by the onset of SC formation as well as the initiation of SEI events in late leptotene / early zygotene (**B**). During zygotene, the SEI events have been formed whereas the formation of the SC is still continuing (**C**). The SC formation is completed during pachytene (**D**) where the SC acts to link the axes of homologs. SC-associated nodules mark the sites of crossovers at this stage of meiotic recombination. Pachytene also involves the onset of dHJ formation, which is completed by the end of pachytene (**E**). Crossover control is suggested to occur at the leptotene / zygotene transition (**A'**) due to the presence of recombination nodules which are visible as early as zygotene.

1.2.1.2 Defects in pro-crossover factors provide further evidence that the crossover / non-crossover decision is made at an early stage

There are at least 11 genes that have been implicated in crossover formation (**Table 1.1**).

Table 1.1: Proteins involved in meiotic crossing over

Class	Protein	Function	Reference
ZMM	**Mer3**	DNA helicase	Mazina *et al* (2004) Nakagawa and Ogawa (1999)
	Msh4/5	Promote crossing over in meiosis	Hoffmann and Borts (2004) Hollingsworth *et al* (1995) Ross-Macdonald and Roeder (1994) Snowden *et al* (2004)
	Zip1	Transverse filament protein of the SC	Sym *et al* (1993) Sym and Roeder (1994)
	Zip2 **Zip3** **Zip4**	Part of the synapsis initiation complex Zip3 is a SUMO E3 ligase involved in ubiquitination Zip2 and Zip3 foci mark sites of crossovers Zip4 acts with Zip2 in promoting the formation of the SC	Agarwal and Roeder (2000) Cheng *et al* (2006) Chua and Roeder (1998) Fung *et al* (2004) Hooker and Roeder (2006) Perry *et al* (2005) Tsubouchi *et al* (2006)
	Spo16	*unknown function*	Shinohara (2008)
MMR	**Exo1**	DNA exonuclease	Khazanehdari and Borts (2000) Kirkpatrick *et al* (2000) Tsubouchi and Ogawa (2000)
	Mlh1/3	Crossover resolution	Hoffmann and Borts (2004) Hunter and Borts (1997) Wang *et al* (1999)

The ZMM proteins, also known as SIC proteins, are expressed exclusively during meiosis. Deletion of these ZMM genes leads to a marked decrease in crossing over without a reciprocal increase in the levels of non-crossovers (Bishop and Zickler, 2004, Borner et al., 2004). Borner *et al* (2004) showed that *zmm* mutants that were sporulated at 33°C also show defective SEI and dHJ formation. These observations provide further evidence that the crossover / non-crossover decision is made early. This is because if the decision was made at a later stage, preventing the formation of crossovers could result in the DSB becoming repaired as non-crossovers. This would mean that the decrease in crossovers would be accompanied by a reciprocal increase in the number of non-crossovers, which is not seen (Storlazzi et al., 1996).

zmm mutants also show defects in SC formation (Borner et al., 2004). Zip1 is the major component of the SC. In the prophase stage of meiosis I, Zip1 is present between homolog axes (Sym et al., 1993). These homolog axes are not tightly paired in *zip1* mutants (Sym et al., 1993). Homologs are seen to only associate with each other at sparse locations along the chromosome (Sym et al., 1993). These sites of associations are marked by Zip2 and Zip3 foci (Agarwal and Roeder, 2000, Chua and Roeder, 1998, Fung et al., 2004). These foci, which also contain Msh4 and Msh5 (Novak et al., 2001, Ross-Macdonald and Roeder, 1994), are suggested to mark the sites of the initiation of Zip1 polymerisation as well as the sites of crossovers (Agarwal and Roeder, 2000, Chua and Roeder, 1998, Fung et al., 2004, Henderson and Keeney, 2004, Rockmill et al., 2003). As discussed in **Section 1.2.1.1**, these foci are predicted to correspond to LNs.

The remaining genes (*EXO1*, *MLH1* and *MLH3*) have roles in mitotic and meiotic mismatch repair (see **Section 1.3**) as well as promoting meiotic crossovers (Hoffmann and Borts, 2004). The actions of the Mlh1-Mlh3 heterodimer are postulated to be dependent on the Msh4-Msh5 heterodimer. It is suggested that the binding of the Msh4-Msh5 heterodimer to dHJs leads to a recruitment of the Mlh1-Mlh3 heterodimer which aid in resolving these structures as crossovers (Hoffmann and Borts, 2004). Mutation in *exo1* leads to a delay in SC formation caused by a delay in the repair of DSBs (R. Keelagher, *personal communication*).

1.2.2 Crossing over ensures the accurate segregation of chromosomes during meiosis

Crossovers establish the chiasmata, which are chromatin bridges that form between homologs. These provide physical connections during the meiotic prophase which leads to the accurate segregation of homologous chromosomes. This is achieved by ensuring that the chromosomes are correctly orientated on the meiosis I spindle. The recognition that the chromosomes are in the correct orientation is dependent upon the mechanical tension that is created when the homologs are pulled to opposite poles of the spindle. This tension is created by resistance from the chiasmata. Therefore, the absence of crossovers, which results in the absence of chiasmata, increases the probability of homologous chromosomes being pulled towards the same pole during meiosis, leading to aneuploidy (Page and Hawley, 2003).

1.2.2.1 Defects during meiosis

Defects that arise during either meiosis I or meiosis II can lead to the missegregation of homologous chromosomes or sister chromatids (**Figure 1.4**). These events will cause a decrease in spore viability, leading to the death of one, two, three or all four spores.

1.2.2.1.1 Three viable spores

The presence of three viable spores can be explained by two different missegregation events. The first is known as the precocious separation of sister chromatids (PSSC) (**Figure 1.4B**). PSSC is caused by the early separation of one sister chromatid pair during meiosis I. The resulting tetrad will consist of one disomic spore, two normal spores and one nullisomic spore. The disomic spore contains information from both parents due to the presence of one copy of each homolog. For PSSC events, the two normal spores will be non-sister spores (**Section 2.2.11.6**). They can be identified due to the presence of a centromere marker, such as *TRP1*. As *TRP1* is located close to the centromere on chromosome IV, no crossovers will occur between this region. One of these normal spores will be auxotrophic for tryptophan whereas the other will be prototrophic.

The second type of missegregation event that can lead to the formation of three viable spores is meiosis II non-disjunction (**Figure 1.4C**). For this event, one pair of sister chromatids fails to separate during the second meiotic division. Meiosis II non-disjunction also leads to one disomic spore, two normal spores and one nullisomic spore. In contrast to PSSC, the disomic spore will contain information from only one parent. The two normal spores will be sister spores, meaning that both will either be auxotrophic or prototrophic for tryptophan. This allows their differentiation from PSCC events.

1.2.2.1.2 Two viable spores

Two viable spores are caused by a missegregation event known as meiosis I non-disjunction (**Figure 1.4D**). These events are caused by the absence of crossovers, which are essential for the accurate segregation of homologs (described in **Section 1.2.2**). In the absence of crossovers, the likelihood of missegregation events increases. This increases the probability that both homologs will be pulled to the same pole during the first meiotic division, leading to meiosis I non-disjunction. It will lead to two disomic spores and two nullisomic spores (discussed further in **Section 2.2.11.7**).

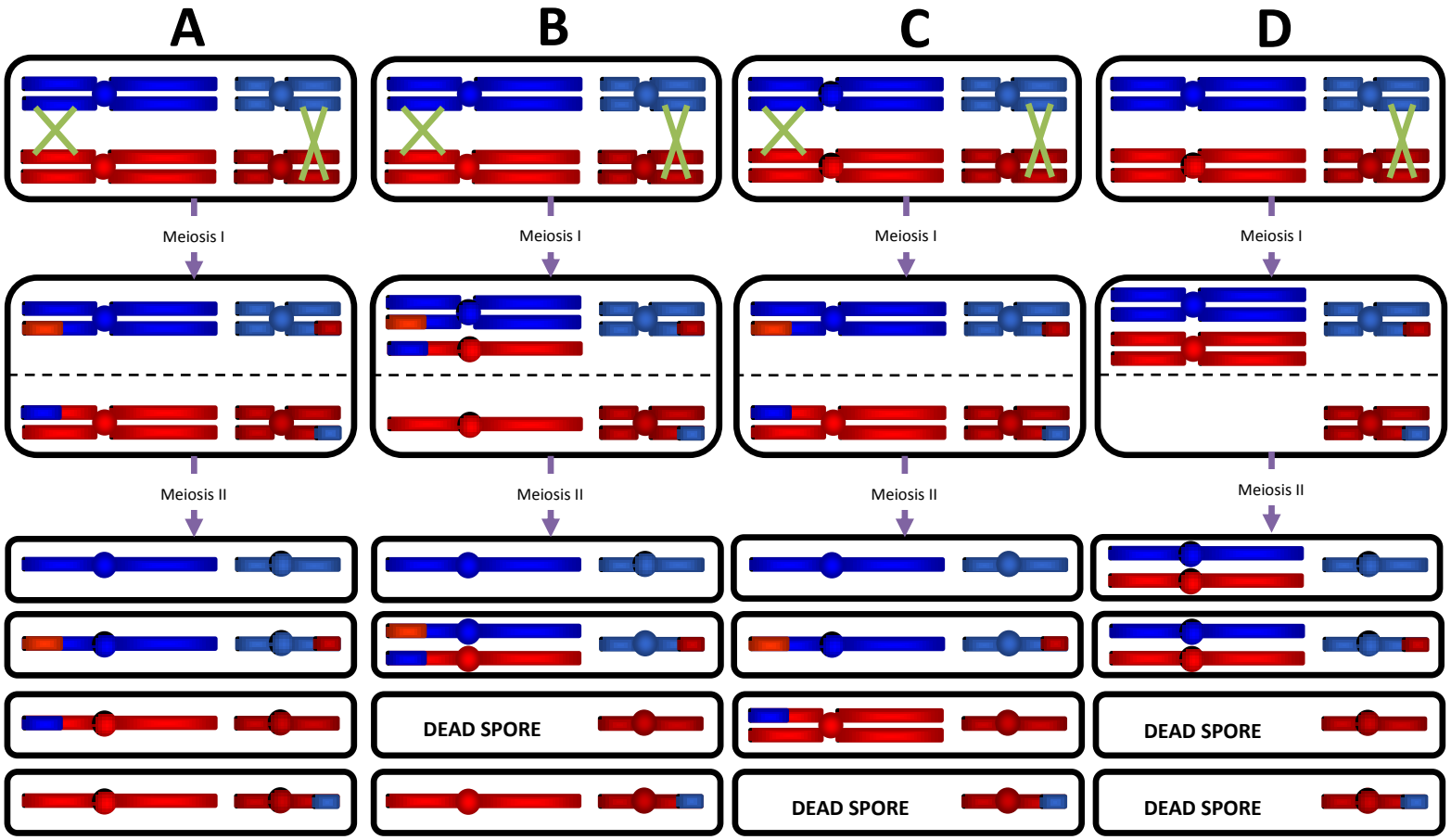


Figure 1.4: Missegregation events during meiosis

Modified from Chaix (2007)

During normal meiosis, crossing over ensures the accurate segregation of homologs during meiosis I, leading to the separation of sisters in meiosis II and the formation of four viable spores (**A**). In PSSC, sister chromatids are separated in meiosis I leading to a nullisomic spore, a disomic spore and two non- sister spores (**B**). In meiosis II non-disjunction, sisters fail to separate at meiosis II, also leading to a nullisomic spore, a disomic spore and two sister spores (**C**). In meiosis I non-disjunction, the absence of crossovers leads to both homologs becoming pulled towards the pole at meiosis I. This leads to two disomic spores (**D**).

1.3 Mismatch Repair

1.3.1 DNA Base Pairing and Mis-Pairing

According to the findings of Watson and Crick (1953), DNA base pairing occurs between adenine (A) and thymine (T) via two hydrogen bonds, and between cytosine (C) and guanine (G) via three hydrogen bonds. However, in some cases, alternative hydrogen bond configurations are known to arise to form what are known as Hoogsteen base pairs (Ghosal and Muniyappa, 2006). These Hoogsteen base pairs have been found to be involved in the stabilisation of guanine quadruplex DNA (Williamson et al., 1989) at telomeres (Patel et al., 2007) leading to the formation of a telomeric D-loop known as a t-loop. The resolution of this structure facilitates the ALT (alternative lengthening of telomeres) pathway which leads to the extension of the telomeric ends of chromosomes in telomerase defective cells (discussed further in **Section 1.6.3.2**).

Usually, base pairs that do not form according to the standard Watson and Crick base pairing may threaten the coding fidelity of replication by affecting the structure of the DNA helix. Various studies have shown that the structure of the DNA helix is affected to different extents by different mismatched bases. The presence of base-base mismatches results in a mismatch-induced stalling of the DNA polymerase during replication (Johnson and Beese, 2004). In addition to single base-base mismatches, an additional type of mismatch, known as insertion/deletion loops (IDLs), also exist. In this case, the presence of a number of extra nucleotides in one of the DNA strands leads to the formation of a loop due to the inability of some of the bases to pair as there are no complementary bases in the opposite strand.

To ensure the integrity of the genetic information, the highly conserved mismatch repair complex acts to correct these mispairs. The activities of the mismatch repair complexes in prokaryotes and eukaryotes are discussed below and are also reviewed in Jiricny (2006), Hsieh and Yamane (2008), Kunz *et al* (2009), Li (2008), Modrich and Lahue (1996) and Schofield and Hsieh (2003).

1.3.2 The Mismatch Repair Complex

1.3.2.1 The *E. coli* MMR Complex

The actions and functions of the mismatch repair complex were first elucidated through studies in *E. coli* (**Table 1.2**).

MutS is a mismatch recognition protein (Modrich and Lahue, 1996) as it has been shown to be able to bind to single base-base mismatches (Su and Modrich, 1986, Su et al., 1988) as well as small IDLs preferentially over homoduplex sequences (Parker and Marinus, 1992). X-ray crystallography studies (Lamers et al., 2000, Obmolova et al., 2000) have revealed how MutS is able to bind to DNA to carry out its mismatch repair functions. These studies, which were carried out on *Thermus aquaticus* (*Taq*) (Obmolova et al., 2000) as well as *E. coli* MutS (Lamers et al., 2000) bound to DNA, showed that MutS binds as a homodimer. However, this homodimer has been described as a 'virtual heterodimer' (Li, 2008) as, although the amino acid composition of the mismatch binding sites for the two subunits are identical, they are composed of structurally and functionally different domains (Lamers et al., 2000, Obmolova et al., 2000). This feature is reflected in eukaryotes where the MutS homologs (MSH) are seen to function as heterodimers (discussed in section **1.3.2.2**). Atomic force microscopy has shown that MutS is capable of binding to both homoduplex and heteroduplex DNA (Wang et al., 2003a). However, when bound to heteroduplex DNA, Wang *et al* (2003a) reported that MutS exists in two forms with DNA – a bent form and an unbent form. They also saw that at the mismatch, the MutS DNA exhibits a 60° kink (Wang et al., 2003a). Taken together with the X-ray crystallography data (Lamers et al., 2000, Obmolova et al., 2000), Wang *et al* (2003a) proposed a model where MutS functions by firstly binding to the DNA in a non-specific manner, and then bending it while searching for the mismatches. They suggest that when MutS encounters the mismatch, it undergoes a conformational change to form a complex named the 'initial recognition complex' (IRC) that contains DNA in a bent state. Mismatch verification leads to the formation of a complex known as the 'ultimate recognition complex' (URC), where the DNA exists in an unbent state. Different mismatches will have different destabilising effects on the DNA. Therefore, Wang *et al* (2003a) argue that the greater the destabilising effect of the mismatch on the DNA, the greater the IRC conformation is favoured. In addition to this, MutS has been shown to interact with the ß-clamp, which is a processivity factor for DNA polymerase III (Lopez de Saro and O'Donnell, 2001). It has also been shown that MutS possesses ATPase activity, and this ATPase activity is critical for mismatch repair (Worth et al., 1998). Once bound to the mismatch, the MutS homodimer recruits the MutL homodimer (Grilley et al., 1989), to form an ATP-dependent ternary complex.

Table 1.2: The protein components of the MMR complex in bacteria and humans.

Bacteria	Eukaryotes	Function
MutS	MutSα (Msh2-Msh6)	recognises base-base mismatches and IDLs of 1 or 2 nucleotides
	MutSß (Msh2-Msh3)	recognises larger IDLs
MutL	MutLα (Mlh1-Pms2)	interacts with downstream effectors and increases the specificity of MutS binding
	MutLß (Mlh1-Pms1)	unknown role
	MutLγ (Mlh1-Mlh3)	aids in the repair of IDLs
MutH	*unknown*	strand discrimination
UvrD	Srs2 ?	helicase
ExoI	ExoI	mismatch excision
ExoVII		
ExoX		
RecJ		
Pol III holoenzyme	Pol δ	DNA resynthesis
	PCNA	
SSB	RPA	single stranded DNA binding to protect single stranded gapped region during excision
	HMGB1	mismatch provoked excision
	RFC	PCNA loading to facilitate resynthesis
DNA ligase	DNA ligase I	nick ligation

The MutL homodimer, which enhances mismatch recognition, associates with the MutS homodimer at the mismatch (Grilley et al., 1989), forming a ternary complex. It has been described as a molecular matchmaker to help assemble the repair complex (Modrich and Lahue, 1996, Sancar and Hearst, 1993), as it is involved in the recruitment MutH and in the activation of its endonuclease activity (Hall and Matson, 1999). MutL also functions as a homodimer (Ban and Yang, 1998a). Like MutS, MutL has been shown to possess ATPase activity (Ban and Yang, 1998a). This ATPase activity is reported to be critical in mediating the activation of MutH by MutS. Mutations in the ATPase domain of MutL, which are defective in ATP hydrolysis but are still able to carry out ATP binding, are able to activate MutH, but unable to stimulate MutH in response to MutS or in response to a mismatch (Aronshtam and Marinus, 1996, Ban and Yang, 1998a). Another function of MutL is highlighted by its association with the clamp loader subunits of DNA polymerase III (Li et al., 2008, Lopez de Saro et al., 2006), which suggests that MutL may aid in the binding of DNA polymerase III to mismatch repair intermediates. As suggested by Li *et al* (2008), this feature of MutL seems to suggest that mismatch repair is coupled to DNA replication.

As mentioned above, MutL aids in the recruitment and activation of MutH (Hall and Matson, 1999). MutH, which functions as a monomer, is involved in strand discrimination. It is able to recognise hemi-methylated *dam* sites that can be found on either side of the mismatch and can be situated at

distances of up to 1kb away (Ban and Yang, 1998b, Lee et al., 2005). These sites refer to the methylation of adenine at position N6 in dGATC sequences. This hemi-methylation allows the parental DNA strand to be differentiated from the daughter strand, and therefore MutH is able to distinguish which strand to repair. Once bound to the mismatch, the complex of MutS, MutL and the activated MutH cuts the unmethylated daughter strand away from the mismatch. This process is dependent on ATP (Junop et al., 2001, Lahue et al., 1989). After this, the UvrD helicase is loaded onto the newly synthesised, unmethylated strand at the nick, and unwinds the duplex DNA that contains the mismatch (Dao and Modrich, 1998). This process is carried out in the presence of MutL and will generate single stranded DNA. The single stranded DNA is bound by Single Stranded Binding protein (SSB) in order to protect it from any potential nuclease activity (Ramilo et al., 2002). Exonucleases are then recruited to digest the unwound DNA up to and slightly past the mismatch. Different exonucleases are required depending on the position of the strand incision relative to the mismatch. If the break is initiated 5' to the mismatch, the 5'-to-3' exonucleases ExoVIII and RecJ are recruited for strand digestion. However, if the break is 3' to the mismatch, then the 3'-to-5' exonucleases ExoI and ExoX are required. The action of the various exonucleases will generate a single stranded gap in the DNA that is subsequently filled by the combined action of DNA polymerase III, which fills the gap, and DNA ligase, which seals the nick (Modrich and Lahue, 1996).

1.3.2.2 The Eukaryotic MMR Complex

In *S. cerevisiae*, six MutS homologs (*MSH1-6*) have been identified. Msh1 and Msh2 were identified by Reenan and Kolodner (1992a, 1992b). *MSH1* has been shown to be important for the maintenance of the mitochondrial genome (Reenan and Kolodner, 1992b). Miret *et al* (1993) showed an *MSH2*-dependent mismatch binding activity using yeast crude extracts. They further showed that Msh2 is required for repair of all types of mismatches. Msh2 has been shown to form heterodimer with Msh3 to aid in the repair of large IDLs (New et al., 1993). This activity was shown by Sia *et al* (1997), who reported that *msh2* and *msh3* mutants were equally defective in the repair of loops that are greater than four nucleotides long. In mammalian cells, Msh2 and Msh3 form a heterodimer known as MutSß (Genschel et al., 1998). MutSß has been shown to also recognise large IDLs by studies that investigated the mutator phenotypes of *msh2*, *msh3* and *msh6* mutations (Greene and Jinks-Robertson, 1997, Marsischky et al., 1996). Msh2 has also been shown to form a heterodimer with Msh6 (Alani, 1996). Studies by Kramer *et al* (1996) showed that the expression of Msh2 and Msh6 is maximal during S phase. Sia *et al* (1997) showed that the Msh2-Msh6 heterodimer does not function in repairing large IDLs, as *msh6* mutants have no effect on the repair efficiency of loops that are greater than 4 nucleotides long. Instead, the Msh2-Msh6 heterodimer

functions by repairing base-base mismatches (Earley and Crouse, 1998, Marsischky et al., 1996). This function is dependent on the binding of ATP to both Msh2 and Msh6 (Junop et al., 2001, Obmolova et al., 2000, Studamire et al., 1998). The model proposes that Msh2 and Msh6 sequentially hydrolyse ATP, and it is the ATPase activity of Msh6 that serves as a sensor to mismatches (Iaccarino et al., 2000, Kijas et al., 2003, Studamire et al., 1998). For smaller IDLs, comprising 1 or 2 nucleotides, it seems as if both heterodimers, Msh2-Msh3 and Msh2-Msh6, actively compete to carry out their repair (Greene and Jinks-Robertson, 1997, Johnson et al., 1996b, Marsischky et al., 1996). Using assays that are able to detect the repair of small IDLs, made using specifically designed oligonucleotides, and also studies that investigated the mutator phenotypes caused by mutations in *msh2*, *msh3* and *msh6*, it was shown that alone *msh3* and *msh6* single mutants display only a weak repair defect. However, when combined, the *msh3 msh6* double mutant is highly defective in the repair of small IDLS, and the repair defect seen is similar to the repair defect in *msh2* single mutants (Greene and Jinks-Robertson, 1997, Johnson et al., 1996b, Marsischky et al., 1996). These data suggest that both Msh3 and Msh6 act in Msh2-dependent pathways. In mammalian cells, the MutS homologs Msh2 and Msh6 act together to form a complex known as MutSα (Drummond et al., 1995) that also functions in the repair of base-base mismatches and small IDLs comprising 1 or 2 nucleotides (Marsischky et al., 1996).

As mentioned above, X-ray crystallography studies show that the *E. coli* MutS homodimer acts as a 'virtual heterodimer' (Lamers et al., 2000, Li, 2008, Obmolova et al., 2000). These studies suggest that MutS subunit A is equivalent to Msh6 whereas MutS subunit B is equivalent to Msh2. For MutS subunit A, it was shown that the phenylalanine residue F39, which resides in domain I, is critical for the binding of mismatches (Obmolova et al., 2000). Whereas, for subunit B, residues found in domain IV, including a lysine at position 471, were reported to be important for forming hydrogen bonds with the sugar phosphate backbone that surrounds the mismatch (Kijas et al., 2003). This domain is important in composing an anti-parallel ß-sheet structure of the protein. By mapping the Msh2-Msh6 heterodimer on to the *Taq* MutS structure, Kijas *et al* (2003) showed that the residue F39 of *Taq* MutS corresponds to F337 of Msh6, and residue K471 of *Taq* MutS corresponds to K564 of Msh2. They showed that *msh6-F337A* and *msh2-K564E* substitution mutations led to a defect in the ability of the Msh2-Msh6 heterodimer to bind mismatches (Bowers et al., 1999, Kijas et al., 2003). Kijas *et al* (2003) were able to detect weak binding using gel mobility shift assays for the *msh2K564E-MSH6* mutation. In the mammalian MutSα complex, Msh6 has been shown to make direct contact with the mismatched base pairs (Warren et al., 2007). It has been shown that there are two critical phenylalanine and glutamate residues that make up a *G-X-FYE* motif that is highly

conserved in Msh6 (Bowers et al., 1999, Dufner et al., 2000). Mutations in either of these two phenylalanine and glutamate residues have been shown to result in a marked decrease in mismatch repair activity carried out by the MutSα complex both *in vivo* and *in vitro* (Bowers et al., 1999, Dufner et al., 2000, Warren et al., 2007). In addition to this, germline mutations in *msh6* and *msh2* have been linked to HNPCC (Hereditary Non-Polyposis Colon Cancer) (Miyaki et al., 1997). The other two MutS homologs, Msh4 (Novak et al., 2001, Paquis-Flucklinger et al., 1997, Ross-Macdonald and Roeder, 1994) and Msh5 (Hollingsworth et al., 1995) are not involved in mismatch repair, but instead have a role in promoting crossovers during meiosis (Hollingsworth et al., 1995, Ross-Macdonald and Roeder, 1994).

The MutL homologs (MLH – *MLH1-3* and *PMS1*) have been shown to increase the mismatch binding specificity and activity of the MutS homologs (Habraken et al., 1997). Pms1 has been shown to form a heterodimer with Mlh1 (Prolla et al., 1994) to aid in the repair of mismatches. Prolla *et al* (1994) showed that the disruption of either *pms1* or *mlh1* or both leads to a mutator phenotype similar to that seen in *msh2* cells. These data led Prolla *et al* (1994) to suggest that the Mlh1-Pms1 heterodimer interacts with both the Msh2-Msh3 and Msh2-Msh6 heterodimers. Hunter and Borts (1997) showed that mutations in *mlh1* lead to a marked decrease in crossing over but the levels of gene conversion remain unaffected. They also showed that the levels of chromosome non-disjunction are increased and the spore viability is decreased in *mlh1* mutants (Hunter and Borts, 1997). Studies in mice by Baker *et al* (1996) showed that *mlh1*$^{-/-}$ mice are defective in the formation of crossovers due to an inability to form chiasmata. Much like the defects in the *pms1* gene, mutations in *mlh1* lead to a mutator phenotype that has been implicated in HNPCC (Bronner et al., 1994). Mlh1 also forms a heterodimer with Mlh3, and both of these genes have been found to be constitutively expressed throughout the cell cycle (Kramer et al., 1996). This heterodimer acts in the crossover pathway (Wang et al., 1999) and its functions in this pathway have been shown to be dependent on their DNA binding and ATP binding activities, but not their ATP hydrolysis activities (Cotton, 2007, Hoffmann et al., 2003). The Mlh1-Mlh3 heterodimer has also been shown to act with Msh3 in the suppression of frameshift mutations (Cotton, 2007, Flores-Rozas and Kolodner, 1998).

In mammalian cells (**Figure 1.5**), the MutLα complex is made up of Mlh1 and Pms2 (Li and Modrich, 1995) and is involved in aiding the MMR activity of the MutSα and MutSß complexes. MutLα possesses ATPase activity and defects in this activity have been shown to inactivate MMR in mammalian cells. MutLα also has been shown to possess endonuclease activity, which is found in the Pms2 subunit of the complex. This endonuclease activity becomes activated in an ATP-

dependent manner by MutSα, PCNA and RFC (Kadyrov et al., 2006). This endonuclease activity is responsible for introducing single stranded breaks in the DNA, suggesting that MutLα is able to create a point of entry for 5'-to-3' hydrolysis by ExoI (Kadyrov et al., 2006). The mammalian MutLß complex is composed of Mlh1 and Pms1 (Prolla et al., 1994, Raschle et al., 1999) but its role is currently unknown. A third mammalian complex, known as MutLγ, is composed of Mlh1 and Mlh3 and has been shown to have a role in meiosis (Lipkin et al., 2000, Lipkin et al., 2002) as well as in the repair of IDLs (Flores-Rozas and Kolodner, 1998, Harfe et al., 2000).

In eukaryotes, no MutH homologs have been found. This is probably because methylation is not the mechanism used for strand discrimination. Instead, it is hypothesised that the 5' ends of Okazaki fragments together with PCNA are used for strand discrimination, as PCNA is found on DNA during lagging strand synthesis (Pavlov et al., 2003). Also, no mammalian homolog to the UvrD helicase has been identified. As discussed above, several exonucleases are implicated in mismatch repair in *E. coli*. However, to date, only one exonuclease has been identified in the eukaryotic mismatch repair system. This is the 5'-to-3' exonuclease ExoI (Fiorentini et al., 1997, Huang and Symington, 1993, Tishkoff et al., 1997). Zhang *et al* (2005b) suggested that strand degradation occurs via an interaction between MutSα and ExoI. They suggest that MutSα and ExoI are continuously loaded onto the DNA until the mismatch is removed by the exonuclease action of ExoI (Zhang et al., 2005b).

It is interesting to note that the *E. coli* MMR system requires the functions of both 5'-to-3' and 3'-to-5' exonucleases, whereas, to date, only the 5'-to-3' exonuclease has been identified in eukaryotes. It is possible that the eukaryotic MMR system does utilise a 3'-to-5' exonuclease which has yet to be identified. Various studies have described the 3'-to-5' exonuclease activities of DNA polymerase ε and DNA polymerase δ, making them potential candidates (Morrison et al., 1991, Simon et al., 1991, Tran et al., 1999). To support this claim, Guo *et al* (2004) showed that PCNA plays a role in 3'-nick directed MMR but not in 5'-nick directed MMR. This may be because the 5'-nick directed MMR functions are efficiently carried out by ExoI.

The resynthesis carried out by DNA polymerase III in *E. coli* is carried out by DNA polymerase δ (Longley et al., 1997) and facilitated by PCNA (Gu et al., 1998, Johnson et al., 1996a, Umar et al., 1996) in the eukaryotic MMR system. DNA polymerase δ functions along with RPA to carry out the resynthesis of DNA (Guo et al., 2006). This synthesis is suggested to be carried out after phosphorylation of the DNA-bound RPA. RPA associates with single stranded DNA, thereby protecting it as it is generated. This phosphorylation reduces the affinity of RPA to the DNA, thereby

leading to its disassociation, therefore allowing the DNA to be resynthesised by the action of DNA polymerase δ (Guo et al., 2006, Ramilo et al., 2002). In addition to this, DNA polymerase δ has been shown to possess 3'-to-5' proofreading activities (Tran et al., 1999).

PCNA has been suggested to play a role in targeting MutSα and MutSß to the mismatch (Lau and Kolodner, 2003, Shell et al., 2007) and has also been shown to interact with MutLα (Gu et al., 1998, Tran et al., 2001). It has been shown that PCNA is able to interact with Msh2, Mlh1 (Gu et al., 1998), Msh6 (Flores-Rozas et al., 2000) and Msh3 (Kleczkowska et al., 2001) via an interaction motif known as the PIP (PCNA Interacting Protein) box (Warbrick, 2000). Flores-Rozas *et al* (2000) showed, using gel mobility shift assays, that PCNA is able to increase the binding specificity of Msh2-Msh6 to mismatches. Studies by Lau and Kolodner (2003) suggest that MutSα binds to PCNA on newly synthesised DNA strands and MutSα is subsequently transferred from PCNA onto the DNA template at the mismatch.

The functions carried out by SSB in *E. coli* are carried out by RPA (Lin et al., 1998), HMGB1 (Yuan et al., 2004) and RFC (Fien and Stillman, 1992) in eukaryotic MMR. RPA binds to single stranded DNA in order to protect the single stranded gap region generated during the excision steps of MMR from the action of MutSα-ExoI (Genschel and Modrich, 2003), and the phosphorylation of RPA reduces its affinity to DNA (Guo et al., 2006, Ramilo et al., 2002). In terms of its phosphorylation status, both research groups (Guo et al., 2006, Ramilo et al., 2002) showed that the unphosphorylated form of RPA is able to stimulate DNA excision in response to mismatches at a greater efficiency than the phosphorylated form. They also showed that the phosphorylated form is more efficient at facilitating DNA resynthesis when compared to the unphosphorylated form (Guo et al., 2006, Ramilo et al., 2002). RPA has been proposed to control the rate of resection that is carried out by MutSα-ExoI, as it has been shown to restrict this resection to approximately 250 nucleotides (Genschel and Modrich, 2003).

The high mobility group box 1 (HMGB1) is a protein that has been shown to bind to mismatches (Fleck et al., 1998) and also has been shown to possess DNA unwinding activity (Javaherian et al., 1978, Javaherian et al., 1979). Studies have shown that it interacts with Msh2 and Msh6 *in vitro* (Yuan et al., 2004) and is able to substitute for RPA in an *in vitro* MMR system made by Zhang *et al* (2005b). Zhang *et al* (2005b) also showed that the 5'-to-3' strand degradation by MutSα-ExoI is carried out at maximal levels in the presence of HMGB1. Replication factor C (RFC) is required to aid in the loading of PCNA and is also required for activation of the endonuclease activity of MutLα

(Kadyrov et al., 2006). Finally, DNA ligase I (DNA ligase in *E. coli*) is required for nick ligation, which is the last step of MMR (Zhang et al., 2005b).

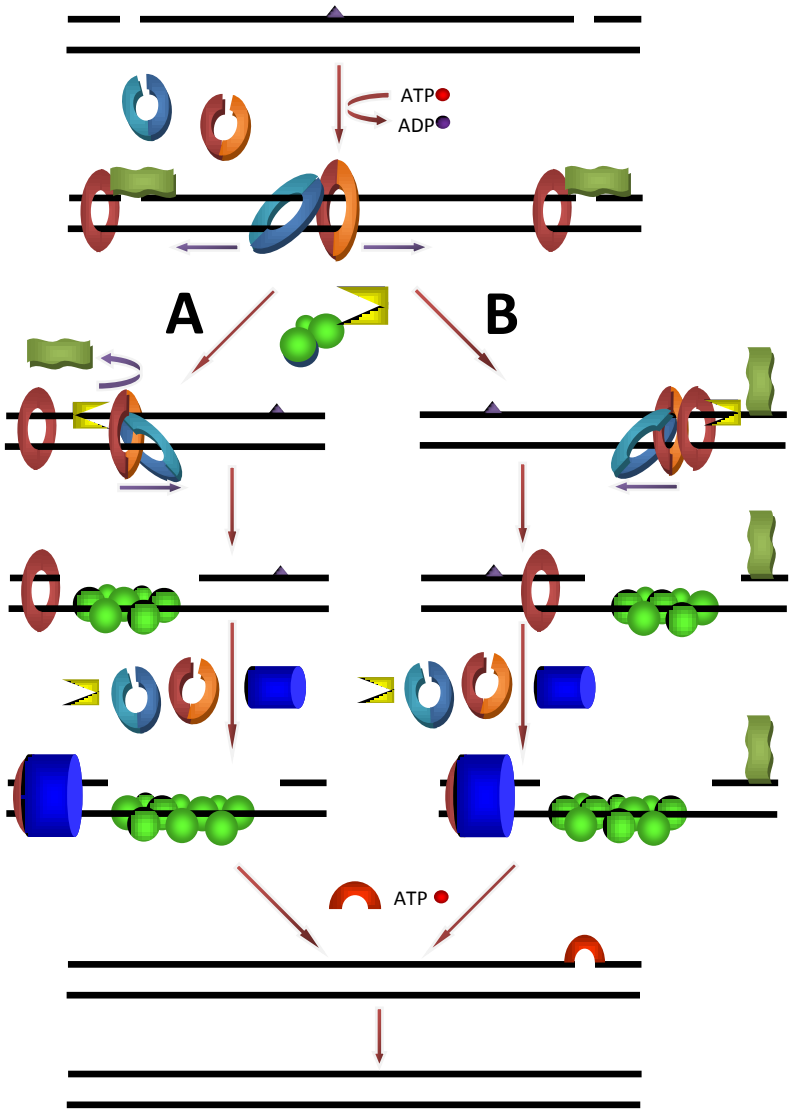


Figure 1.5: Model to show the mode of action of the mammalian MMR system.
Modified from Jiricny (2006)

MutSα recognises the mismatch and recruits MutLα. This causes a conformational change which will lead to the release of the MutSα sliding clamp from the mismatch.

A: The clamp moves upstream, in a 5'-to-3' direction, until it comes across RFC that is bound to the DNA. The clamp will displace RFC and then facilitates the loading of ExoI, which is involved in 5'-to-3' strand degradation. RPA binds to the single stranded gap, protecting it against any potential nuclease attack. When the mismatch is repaired, MutLα inhibits the activity of ExoI. PCNA and DNA polymerase δ fill the single stranded gap and DNA ligase seals the nick.

B: If the clamp moves in downstream, in a 3'-to-5' direction, it will encounter PCNA that is bound to the DNA. ExoI is recruited to carry out strand degradation and this activity is thought to be carried out by multiple loading events of ExoI. The presence of RFC ensures that 5'-to-3' degradation away from the mismatch is not carried out by ExoI. When the mismatch is repaired, ExoI is inhibited by both MutLα and RPA. PCNA and DNA polymerase δ fill the single stranded gap and DNA ligase seals the nick.

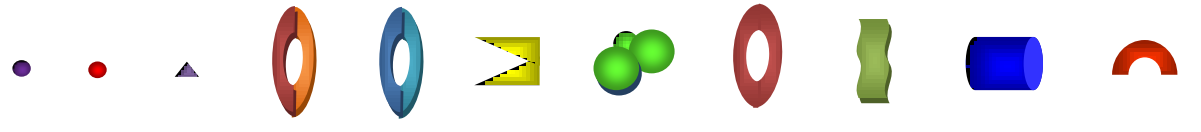


Figure 1.5 is of the molecular switch model (Gradia et al., 1997). This is one of two moving models, which, in addition to a stationary model, have been proposed to account for the mode of action of the eukaryotic MMR system (reviewed by Li (2008) (**Figure 1.6**).

The stationary model (Guarne et al., 2004, Junop et al., 2001) (**Figure 1.6A**) proposes that the interactions between MutSα and MutLα induce bending of the DNA. This allows the mismatch and the strand-specific nick to be brought close together. It is suggested that MutSα remains bound to the mismatch and its ATPase activity is involved in verifying the mismatch. Upon verification, downstream excision is carried out (Junop et al., 2001). Junop *et al* (2001) showed that once MutS recognised the mismatch, it activated the cleavage of a GATC by MutH. Further support of this model came from work by Wang and Hays (2004), who showed that the addition of a biotin-streptavidin block between a mismatch and a pre-existing nick lead to the production of a new break in the heteroduplex DNA, but not in the homoduplex control.

Both moving models (**Figures 1.6B** and **1.6C**) suggest that MutSα/ß and MutLα are loaded at the mismatch. The MMR complexes are then thought to move away from the mismatch in order to search for the nick. On finding the nick, they recruit ExoI so that excision can be carried out. Studies by Pluciennik and Modrich (2007) appear to favour these moving models over the stationary model. Their work showed that the placement of a protein block or a double stranded DNA break between the mismatch and the nick resulted in the inhibition of MMR (Pluciennik and Modrich, 2007). Although the data seemingly contradicts the data obtained by Wang and Hays (2004), it is suggested the difference in the observations comes from the organisms used in these studies (Pluciennik and Modrich, 2007). While Wang and Hays (2004) studied MMR in eukaroyotes, Pluciennik and Modrich (2007) studied prokaryotes. Therefore, Pluciennik and Modrich (2007) suggest that the differences observed may reflect differences in the modes of action for the *E. coli* and human MMR systems. For example, as discussed above, in *E. coli*, the binding of MutL to the MutS-bound mismatch leads to the activation of downstream activities, such as excision. In contrast, in humans, MutLα has been shown to possess endonuclease activities that act in a mismatch-, MutSα-, RFC- and PCNA-dependent manner. *E. coli* MutL does not possess this endonuclease activity. This would suggest that the MMR system has evolved in higher eukaryotes and may therefore act in a different, more complex manner to simpler organisms. Therefore, the models proposed in **Figure 1.6**, may potentially reflect the modes of action of the MMR system in difference organisms.

The Translocation Model (Allen et al., 1997, Blackwell et al., 1998) (**Figure 1.6B**) suggests that repeated cycles of hydrolysis of ATP leads to the translocation of MutSα/ß along the DNA helix until it reaches the strand discrimination signal (Blackwell et al., 1998). The DNA is threaded through the MMR complex in order for this to be carried out, and this process leads to the formation of a loop which is acted on by the exonuclease activity of ExoI to excise the mismatch. The Molecular Switch Model (Gradia et al., 1997) (**Figures 1.5** and **1.6C**) suggests that the MMR complex binds to the DNA in an ADP-bound state. This binding causes a conformational change which induces the exchange of ADP to ATP. This reaction causes yet another conformational change that results in the MMR complex forming a sliding clamp that allows it to slide away from the mismatch (Gradia et al., 1997, Iaccarino et al., 2000, Jiang et al., 2005, Mendillo et al., 2005). Whereas the Translocation Model involves the hydrolysis of ATP, this model suggests that it is the binding of ATP that critical for the execution of downstream events, i.e. the formation of the MutS-MutL-MutH complex and the sliding of this complex in order to locate the break (Gradia et al., 1997, Jiang et al., 2005, Mendillo et al., 2005).

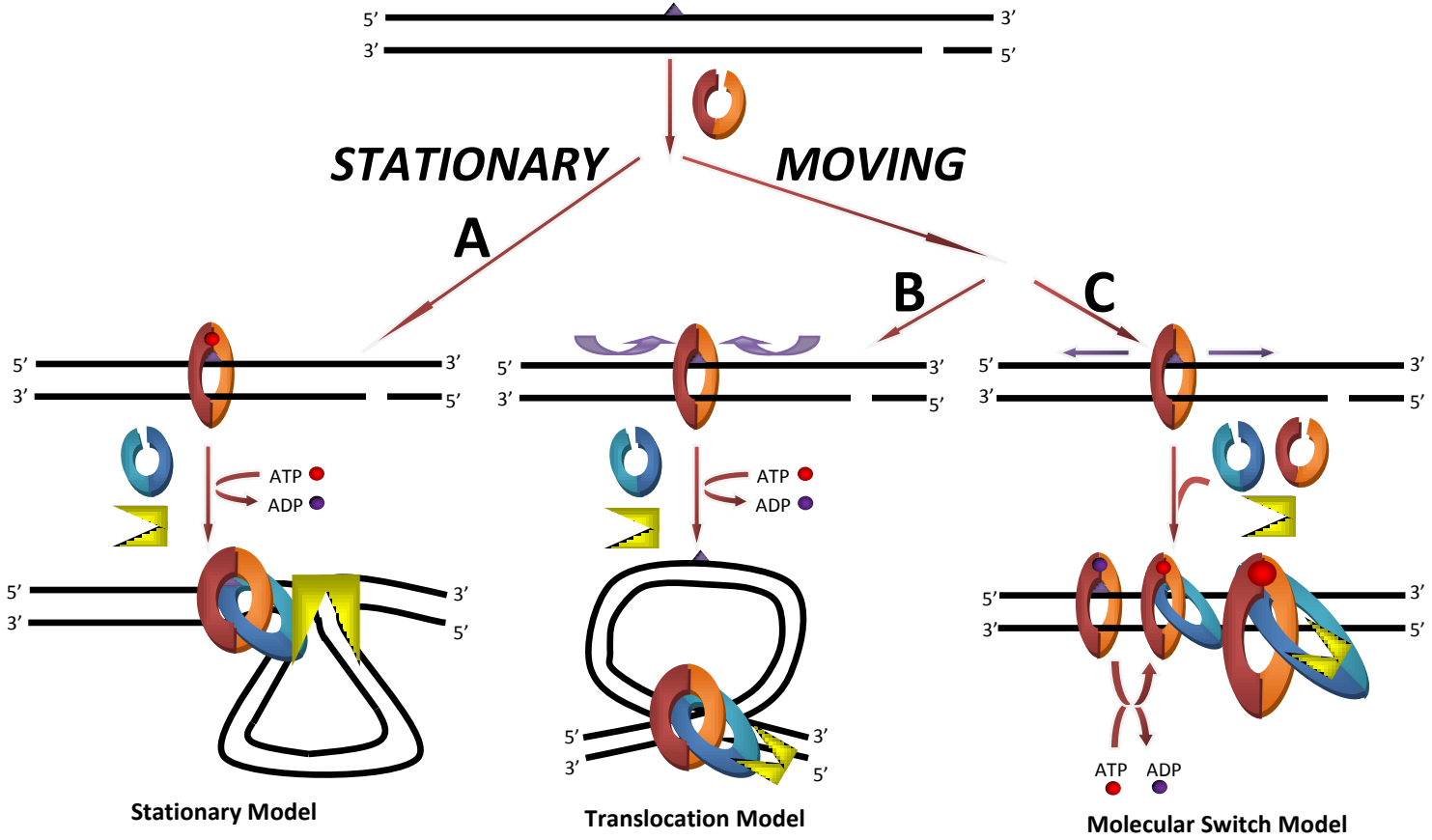


Figure 1.6: Proposed models to account for the processing of mismatches.

Modified from Li (2008).

In all models strand discrimination occurs due to the presence of a strand-specific nick. Taking this into account, three models have been proposed to account for how MMR proteins mediate downstream events after mismatch and strand verification. The Stationary Model (**A**) proposes that interactions between MutSα and MutLα lead to the bending of the DNA. This brings the strand specific nick and the mismatch in close proximity which facilitates downstream excision. The Translocation Model (**B**) and the Molecular Switch Model (**C**) describe Moving Models which suggest that after being loaded at the mismatch, MutSα and MutLα move away from it to search for the strand nick.

1.4 Homeologous Recombination

The process of homologous recombination allows the transfer of genetic information amongst identical stretches of DNA. In contrast, homeologous recombination is the transfer of genetic information between sequences that are slightly diverged. In order to maintain the integrity of the chromosome, the formation of a double Holliday junction requires almost perfect homology (Holliday, 1964, Rayssiguier et al., 1989). Rad51 has been proposed to play a role in this search for perfect homology. Its role involves the formation of nucleoprotein filaments that bind to the single-stranded overhangs caused by strand resection. These Rad51 nucleoprotein filaments are suggested to scan the genome and assess homology (Sung et al., 2003, West, 2003). On finding a homologous strand, invasion can occur which facilitates repair using this homologous strand as a template.

As discussed by Ouyang *et al* (2008), interactions between tandem and repeat sequences that are seen in higher eukaryotes, such as the Alu family members found in the mammalian genome, must be regulated in order to maintain the integrity of the genome (Britten et al., 1988, Rossetti et al., 2004). For plants and humans, chromosomal rearrangements, caused by recombination between these sequences, may be important for driving evolution, adaptation and speciation but may also be responsible for causing disease (Coghlan et al., 2005, Rieseberg et al., 2003). For example, the process of introgressive hybridisation in the Mexican cotton *Gossypium gossypioides* has been shown to be facilitated by homeologous recombination (Cronn et al., 2003). Introgressive hybridisation describes the incorporation of genes from one species into the gene pool of another species. It is caused by repeated backcrossing of an interspecific hybrid to one of its parents (Cronn et al., 2003). It has also been shown that increases in genetic variation amongst plant species is an important factor for various traits, such as flowering time (Pires et al., 2004) and seed yield (Osborn et al., 2003). However, in some cases crosses between certain plants leads to interspecific hybrids that are viable but sterile. This sterility is due to the high level of divergence between the homeologous chromosomes, which results in an inability to recombine. For example, Canady *et al* (2006) saw that recombination between the tomato plants *Solanum lycopersicoides* and *Lycopersicon penellii* was dramatically reduced. They attributed this reduction to the high degree of divergence between the genomes of these two plant species.

Despite potential evolutionary advantages, preventing recombination between diverged repeats is important in maintaining the stability of the genome (Radman, 1989). This is because this type of 'illegitimate' recombination can also potentially give rise to translocations, deletions or inversions (Myung et al., 2001a). The presence of up to 10-20% sequence divergence can greatly affect the

fidelity of DSB repair (Borts and Haber, 1987, 1989, Schneider et al., 1981, Shen and Huang, 1986). This is suggested to be partly due to the role of RecA, which catalyses the formation of heteroduplex DNA by facilitating strand invasion. DasGupta and Radding (1982) showed the 3ϕX174 and G4 DNAs, which differ by approximately 30%, were able to form joint molecule structures in the presence of RecA.

In addition to this, it has been shown that recombination between homeologous sequences can be carried out if the diverged sequences are surrounded by sequences that share a high degree of sequence identity for initiation and resolution (Shen and Huang, 1986, Shen and Huang, 1989, Waldman and Liskay, 1988, Yang and Waldman, 1997). This led to Shen and Huang (1986, 1989) to propose a value that gives an indication of the minimal length of perfect homology that is required for the initiation of recombination. They called this the MEPS (Minimal Efficient Processing Segment) value. Shen and Huang (1986) assessed phage-plasmid recombination and showed that the MEPS value in *Escherichia coli* (*E. coli*) is approximately 30 base pairs. Using the mouse thymidine kinase gene containing varying numbers of mismatches, Waldman and Liskay (1988) showed that the MEPS value in mammalian cells is approximately 134-232 base pairs. Following this study, the same research group showed that the introduction of a single base-base mismatch is sufficient to disrupt the MEPS value and cause a 7- to 175-fold decrease in the rate of recombination (Lukacsovich and Waldman, 1999). By testing a variety of single base mismatches in the thymidine kinase gene, Lukacsovich and Waldman (1999) showed that the G-G and C-C mismatches had the greatest effect on this decrease. Additional studies showed that the number of MEPS exponentially decreases with increasing sequence divergence, indicative of a log-linear relationship between the rate of crossing over and the presence of sequence divergence (Datta et al., 1997, Majewski and Cohan, 1998).

Studies by Yang *et al* (2006) in mouse cells also show that homeologous recombination can be carried out when the diverged sequences are surrounded by regions of high homology. Their assay involved DNA substrates containing the thymidine kinase gene that was disrupted by the insertion of an *XhoI* linker. This was referred to as the 'recipient' gene. They also had a 'donor' thymidine kinase gene that could be used to repair the 'recipient' strain via recombination. This 'donor' gene had either 80% or 100% sequence identity to the 'recipient' gene. The mismatched region was placed either in the middle of the 'donor' so that it is surrounded by regions of perfect homology. Alternatively, the mismatched region was placed at one end of the construct, so that it was situated adjacently to the homologous sequences.

They noted the absence of homeologous sequences in gene conversion tracts when the mismatched sequences were situated adjacently to the homologous sequences. This was assayed by sequencing as well as Southern Blot analysis using probes complementary to either the homologous or homeologous sequences. The homologous probe hybridised to each recombinant, while no signal was achieved using the homeologous probe. However, sequencing showed that homeologous sequences were included in these gene conversion tracts when the mismatched sequences were surrounded by homologous sequences (Yang et al., 2006).

Therefore, Yang *et al* (2006) suggest that homology is important for the initiation and resolution of recombination. They also suggest that the 'dismantling' of mismatched heteroduplex DNA is a caused by a requirement for better sequence identity in order for efficient resolution of recombination to be carried out. This heteroduplex rejection model has been supported by other studies in other organisms, and is discussed further in subsequent sections.

1.4.1 Recombination between diverged sequences is suppressed by the mismatch repair system

Several studies have shown that the eukaryotic and prokaryotic mismatch repair (MMR) systems (discussed in detail in **Chapter 4**) are involved in the regulation of homeologous recombination.

1.4.1.1 The prokaryotic MMR system suppresses homeologous recombination

One of the earliest studies on the control of homeologous recombination was by Rayssiguier *et al* (1989) who investigated recombination between *E. coli* and *Salmonella typhimurium*. These two bacteria are approximately 16% divergent (Sharp, 1991). Rayssiguier *et al* (1989) showed that the MMR genes MutS and MutL, but not MutH and MutU, acted in the prevention of homeologous recombination between *E. coli* and *S. typhimurium*, as a 1000-fold increase was observed when these genes were mutated. The greatest effect reported was for *mutS* and *mutL* mutations, which is consistent with a role for these genes early in mismatch recognition. Based on this study, it was suggested that the MMR system imposes a barrier to recombination between diverged species (Rayssiguier et al., 1989). It was also found that this increase in recombination was dependent on RecA (Matic et al., 1995), which further support the data from DasGupta and Radding (1982). Matic *et al* (1995) showed that the attempted inter-species recombination between *E. coli* and *S. typhimurium* activates a RecBC-dependent SOS response. As reviewed by Michel (2005), the two key genes in this SOS response are the repressor, LexA, and the inducer, RecA. Normally, when no damage is present, the repressor LexA acts by binding to specific sequences in the promoter regions

of SOS genes, repressing their expression. When damage is sensed, the LexA repressor undergoes a self-cleavage reaction, induced by the RecA, which results in expression of the SOS genes. RecA also functions by facilitating strand exchange as described above. This leads to an overproduction of RecA, which facilitates recombination between these two species. This RecBC-dependent SOS signal is strong during inter-species mating, but weak during mating between the same species. Therefore, these data suggest that while the MMR system acts as a negative regulator of inter-species mating, the SOS response in bacteria acts to facilitate it. In agreement with this, Petit *et al* (1991) showed that either the inactivation of the MMR system or the activation of the SOS response causes an increase in intrachromosomal recombination between the diverged repeat sequences *rhsA* and *rhsB*. The importance of RecA in facilitating strand exchange between homeologous sequences was further highlighted in a study by Westmoreland *et al* (1997). This study involved transforming plasmids that either contained or did not contain mismatches into WT or *mutSLHU E. coli* strains (Westmoreland et al., 1997). Westmoreland *et al* (1997) noted that the presence of mismatches in the plasmids led to a 20- to 30-fold decrease in transformation efficiency for wild type strains and that this decrease was enhanced a further 2- to 3-fold when *recA* was deleted (Westmoreland et al., 1997). On the other hand, the transformation efficiency was notably improved when the mismatch-containing plasmids were transformed into *mutSLHU* strains (Westmoreland et al., 1997).

The MMR system of *S. typhimurium* is also important in the prevention of intrachromosomal homeologous recombination. This was shown by Adbulkarim and Hughes (1996) who noted that mutation of the MMR genes *mutSLHU* lead to an increase in recombination between the 99% identical TufA and TufB translation factors. Zahrt *et al* (1994, 1997) showed that MutS and MutL also inhibit recombination between *S. typhimurium* and *Salmonella typhi*. They showed recombination between these two bacterial species, which share approximately 99% identity, is increased when *mutS* and *mutL* are mutated. They also noted that recombination between these two species was increased by mutating the exonuclease activity of *recD*, which inhibits the loading of RecA (Amundsen et al., 2000). When both *mutS* and *recD* are mutated, Zahrt *et al* (1994, 1997) noted a synergistic increase in the levels of homeologous recombination, suggesting that they act in independent pathways. Therefore, inactivating components of both the MMR system and the RecD exonuclease, alleviates the barrier to inter-species recombination for these bacteria. Due to the high degree of homology between *S. typhimurium* and *S. typhi*, these authors suggested that the inhibition of homeologous recombination may not be caused by the inability of RecA to invade the 'homologous' strand, as suggested by Matic *et al* (1995). Instead, they favour a model where the

concerted efforts of the MMR system and the RecBCD exonucleases act at a subsequent step, slowing the process of branch migration in order to correct mismatches.

This hypothesis is supported by studies by Worth *et al* (1994) and Fabisiewicz and Worth (2001) who showed that MutS and MutL act to block RecA-mediated strand invasion during homeologous recombination in bacteria. Worth *et al* (1994) studied the effects of MutS and MutL using M13 and fd phage DNAs, which are diverged by 3%. They noted that MutS acts to inhibit strand exchange reactions between M13-fd DNAs by 60%, and that this inhibition is greatly enhanced by the presence of MutL (Worth et al., 1994). However, it was also shown that the MMR proteins had no effect on homologous M13-M13 or fd-fd interactions, suggesting that both of these proteins act in the inhibition of branch migration when sequences are diverged (Worth et al., 1994). To further extend this study, Worth *et al* (1998) and Fabisiewicz and Worth (2001) went on to show that this activity of MutS is dependent on its ability to hydrolyse ATP. Worth *et al* (1998) showed that two point mutations that are found in the Walker A motif of MutS, *mutS-501* and *mutS-506*, which lead to a 60-100 fold decrease in ATPase activity but a retention of the mismatch binding activity, were unable to block RecA-dependent strand transfer even when MutL was present. Fabisiewicz and Worth (2001) also showed that the MMR proteins act to inhibit RuvAB-dependent activities which promote the branch migration of Holliday junctions on homeologous substrates. This is important, as although RecA is able to catalyse strand transfer on divergent sequences, it has been shown that the efficiency of this activity decreases with increasing divergence (Bianchi and Radding, 1983, Worth et al., 1994). As a result, for larger heterologous sequences, RecA acts with RuvAB, which has been shown to act specifically after strand exchange (Iype et al., 1994). The work described here seems to suggest that MMR proteins act in the destabilisation of RecA and/or RuvAB-DNA complexes, depending on the size of the heterology present, in order to suppress homeologous recombination (Fabisiewicz and Worth, 2001).

The *Helicobacter pylori* MutS homolog HpMutS2 (Alm et al., 1999, Tomb et al., 1997) has also been implicated in the suppression of homeologous recombination (Pinto et al., 2005). *H. pylori* is found in the stomach mucosa in humans. In order to survive, it is able to adapt to the changing gastric environment within the host. Recombination is thought to be an important factor in this adaptation due to the presence of non-randomly distributed repetitive sequences. Recombination between these repeats is suggested to be a major mechanism that allows genome diversification in *H. pylori* (Aras et al., 2003). This suggestion is based on evidence from studies that show that selection favours bacterial strains that show increasing recombination (Radman et al., 2000). HpMutS2 is not

involved in MMR. This was shown by Pinto *et al* (2005) who saw that the rates of spontaneous mutations leading to rifampicin resistance were not different for strains in which the HpMutS2 ORF was deleted when compared to wild-type. As exogenous DNA sequences are commonly integrated into the bacterial chromosomes of *H. pylori* by homologous recombination, Pinto *et al* (2005) monitored the rates of homeologous recombination by assessing the incorporation of several selectable markers into a non-essential locus. These markers ranged in divergence from 0% to 26%. They noted that in *mutS2* mutant strains, the rates of recombination were markedly increased, showing that HpMutS2 is involved in the regulation of homeologous recombination in *H. pylori*. In addition to this, Pinto *et al* (2005) showed that HpMutS2 inhibits RecA-mediated strand transfer in the presence of 8% sequence divergence. Therefore, although it lacks MMR activity, HpMutS2 resembles *E. coli* MutS in its ability to suppress homeologous recombination and block RecA-mediated strand transfer.

The work described above (Fabisiewicz and Worth, 2001, Matic et al., 1995, Pinto et al., 2005, Worth et al., 1994, Worth et al., 1998) show that although the *E. coli* MMR system acts to prevent homeologous recombination, genes involved in the SOS response (as reviewed by Michel (2005)) acts as a positive regulator by causing the excessive production of RecA. The fact that the SOS response acts to facilitate homeologous recombination suggest that recombination between diverged sequences can be beneficial. For example, homeologous recombination may promote survival in response to severe DNA damage when perfectly homologous sequences are unable for repair. Alternatively, homeologous recombination may drive evolution by allowing cells to acquire new genes.

1.4.1.2 The eukaryotic MMR system suppresses homeologous recombination

Various studies have been carried out to investigate the role of MMR proteins in single strand annealing (SSA) in *S. cerevisiae* (Goldfarb and Alani, 2005, Sugawara et al., 1997, Sugawara et al., 2004). The SSA assay used for these investigations, developed by Sugawara *et al* (1997), is illustrated in **Figure 1.7**. Sugawara *et al* (1997) saw that when a DSB was formed in between two direct repeats, the DSB is processed by exonuclease activity, leading to the formation of 3' single stranded overhangs. The subsequent annealing of the direct repeats will result in an intermediate that contains 3' single stranded tails, which must be removed prior to any synthesis or ligation reactions that need to be carried out for completion of SSA. Msh2-Msh3 plays a role in facilitating this removal when the complementary regions are less than 1kb in length (Sugawara et al., 1997) and when the non-homologous tails are more than 30 nucleotides in length (Paques and Haber,

1997). It is suggested that the role of Msh2-Msh3 in SSA is to recruit the structure specific endonucleases Rad1-Rad10 (Sugawara et al., 1997). This was proposed due to the role of Rad1-Rad10 in 3' single strand cleavage (Bardwell et al., 1994, Sung et al., 1993, Tomkinson et al., 1993) as well as interactions between Msh2-Msh3 and Rad1-Rad10 (Bertrand et al., 1998).

When mismatches are introduced, the efficiency of SSA is reduced in a wild type construct. This has been shown to be due to the mismatch binding activity of the MMR proteins (Goldfarb and Alani, 2005). In order to further define which MMR proteins were required for this process, Goldfarb and Alani *et al* (2005) investigated mutations in the MMR proteins Msh2 and Msh6. These mutations render the MMR proteins defective in either mismatch binding or ATP hydrolysis. The mismatch binding mutations, *msh2-K564E* and *msh6-F337A* were originally identified by Bowers *et al* (1999) and Kijas *et al* (2003) by mapping the structure of Msh2-Msh6 onto the *Taq* MutS structure (Obmolova et al., 2000). Both were shown to be defective in heteroduplex rejection (Goldfarb and Alani, 2005) as well as in mismatch binding. The ATP hydrolysis defective mutations, *msh2-G693D* and *msh6-G987D*, were shown to be also defective in non-homologous tail removal (Studamire et al., 1999). They also found that *msh2-R730W* (Studamire et al., 1999), was also defective in heteroduplex rejection (Goldfarb and Alani, 2005). In addition to this, this *msh2-R730W* mutation has been shown to be defective in mismatch binding but maintains the ability to recruit Mlh1-Pms1 in an ATP-dependent manner (Kijas et al., 2003). Therefore, this *msh2-R730W* mutation separates the functions of Msh2. They also investigated the *msh2-S656P* mutation, which is proposed to disrupt the structure of the ATP binding pocket and disrupt the ability of Msh2 to recruit Mlh1-Pms1 (Kijas et al., 2003). This mutation was also shown to be defective in heteroduplex rejection (Goldfarb and Alani, 2005). These results show that heteroduplex rejection is not only dependent on the binding of mismatches by Msh2-Msh6, but also dependent on their ATP binding and hydrolysis activities (Goldfarb and Alani, 2005). This could mean that mismatch binding results in a conformational change caused by the hydrolysis of ATP, which facilitates the rejection of the heteroduplex (Goldfarb and Alani, 2005).

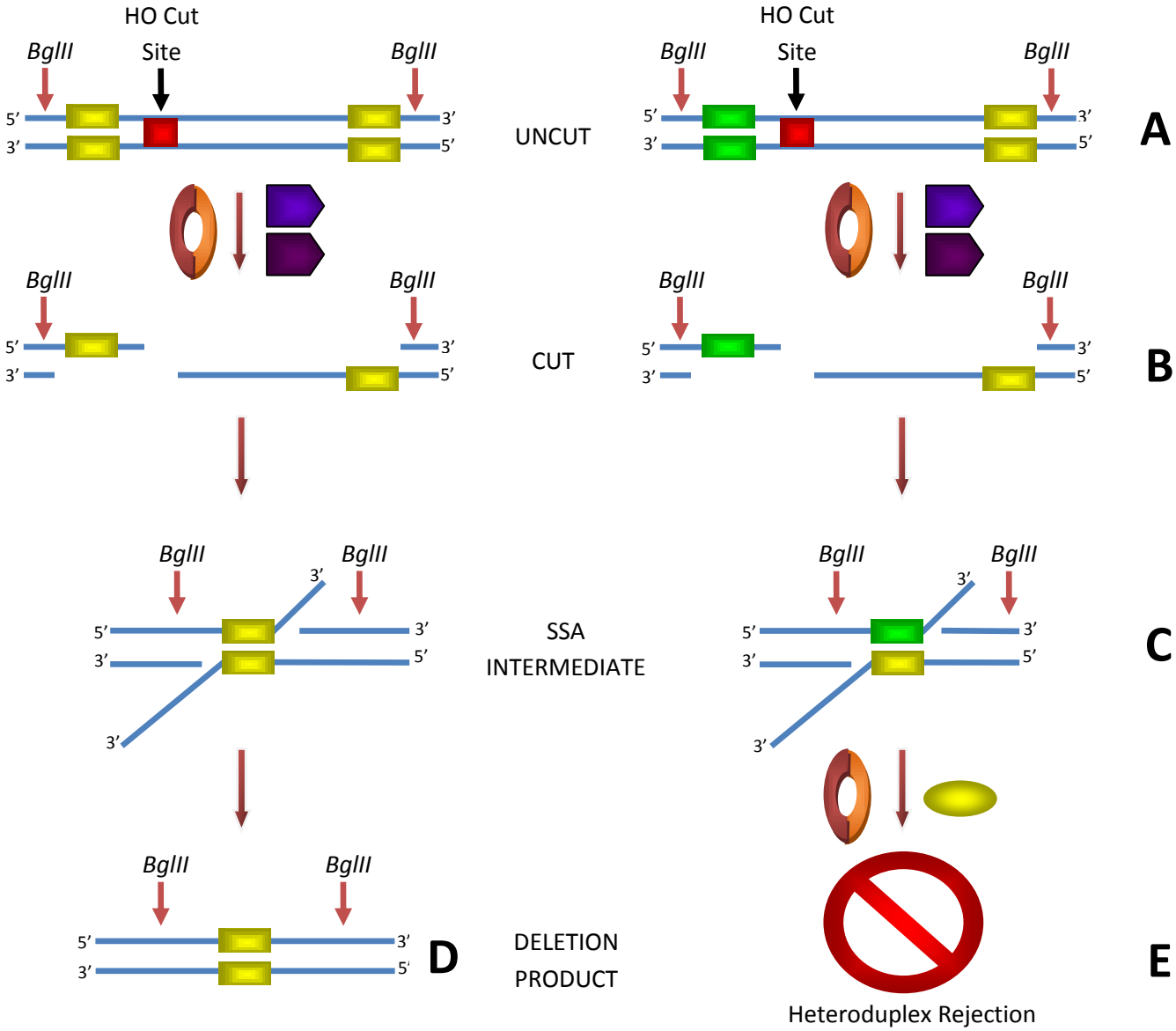


Figure 1.7: The Single Strand Annealing (SSA) Pathway

Modified from Goldfarb and Alani (2005)

Goldfarb and Alani (2005) used a homologous construct, originally developed by Sugawara *et al* (2004), consisting of two *URA3* repeats that were either identical (yellow boxes) or had 3% sequence divergence (green boxes). DSBs are induced via the HO cut site (**A**). Their repair is carried out via the flap endonucleases Rad1-Rad10 (**B**) which are recruited by the MMR proteins Msh2-Msh3 (Sugawara et al., 1997). Once complementary sequences are exposed, via the action of these exonucleases, an annealing reaction occurs which leads to an intermediate that contains 3′ single stranded tails (**C**). These must be removed before the synthesis and ligation steps can be carries out which completes the repair pathway (**D**). In the presence of sequence divergence, the MMR proteins recruit Sgs1 to unwind the heteroduplex DNA, leading to heteroduplex rejection (**E**).

Selva *et al* (1995, 1997) investigated the role of MMR proteins in the suppression of mitotic homeologous recombination using an inverted-repeat assay. This assay used the *S. cerevisiae SPT15* gene at one end of chromosome V that carried a point mutation, which leads to auxotrophy to lysine. At the other end of the chromosome, they (Selva et al., 1995, Selva et al., 1997) inserted a hybrid of the *SPT15* gene and its *S. pombe* homolog *TBP*, which share 73% sequence identity in their 3' regions (Eisenmann et al., 1989, Fikes et al., 1990). The construct is made in such a way that 330bp of the *S. pombe TBP* sequence is surrounded by sequences that are homologous to the *S. cerevisiae SPT15* gene (Fikes et al., 1990). The 330bp *TBP* sequences overlaps with the sequence that contains the point mutation found at the endogenous *spt15* allele. Recombination will lead to prototrophy to lysine and is achieved by either a pop-out (caused by SSA or an unequal or intrachromosomal crossover), or a gene conversion (where the point mutation is replaced using the homologous *SPT15* sequences from the *SPT15-TBP* hybrid) (Selva et al., 1997). Selva *et al* (1995) showed that mutation in *msh2* and *msh3* led to an increase in recombination, respectively, to wild-type, whereas mutation in *pms1* had no significant effect. They also noted that the rates of homologous recombination were not significantly affected by mutations in the MMR genes (Selva et al., 1995). These results suggest that while Msh2 has independent functions of Msh3, the functions of Msh3 are dependent on the functions of Msh2 in the suppression of mitotic homeologous recombination. In a subsequent study Selva *et al* (1997) used Southern blot hybridisation to assess the specific roles of Msh2 and Msh3. They revealed that deletion of *msh2* led to an increase of both crossovers and gene conversions, whereas deletion of *msh3* led to an increase in gene conversions but no increase in interchromosomal crossovers and a decrease in intrachromosomal crossovers.

Using the inverted-repeat assay system developed by Datta *et al* (1996), where recombination between either 100% or 91% identical inverted 5'- and 3'-*his3* substrates results in the formation of full length *HIS3*, Nicholson *et al* (2000) also showed a role for the MMR system in the suppression of mitotic homeologous recombination. They showed that in the wild-type, the rate of homeologous recombination, leading to prototrophy to histidine, was decreased by 33-fold (Nicholson et al., 2000). Deleting the MMR genes *msh3*, *msh6*, *mlh1* and *pms1* lead to 3-fold, 7-fold, 8-fold and 11-fold increased in homeologous recombination, respectively (Nicholson et al., 2000). The *mlh1 pms1* double mutant led to a 5-fold increase in homeologous recombination, which was not significantly different to the single mutants (Nicholson et al., 2000) and the fact that their mutation leads to a similar increase in homeologous recombination provides further evidence that these two proteins function as a heterodimer (Datta et al., 1996, Nicholson et al., 2000). The *msh2 msh3* double mutant and the *msh2 msh3 msh6* triple mutant both led to a 20-fold increase in homeologous

recombination (Nicholson et al., 2000). For base-base mismatches, it was shown that the data obtained for the *msh3 msh6* double mutant were similar to that obtained for the *msh2* single mutant (Nicholson et al., 2000). It was also shown that deletion of *msh2* or *msh3* led to an increase in homeologous recombination for substrates that contained 4 or 12 nucleotide loops or 18 nucleotide palindromes, whereas deletion of *msh6* had no effect (Nicholson et al., 2000). This suggests that Msh2 and Msh3, but not Msh6, are able to recognise and prevent recombination between these mismatch-containing loops (Nicholson et al., 2000). As well as investigating the effects of the MMR system of homeologous recombination, Nicholson *et al* (2000) also showed that mutation of the endonuclease *rad1* and the exonuclease *exo1* (**Table 1.1**) also caused an increase in the levels of mitotic homeologous recombination. However, they saw no effect on the rates of homeologous recombination when they mutated the other MutS homologs *msh4* and *msh5*, which is consistent with their meiosis-specific role in the promotion of crossovers (**Table 1.1**).

The effects of Msh2 in the suppression of homeologous recombination were also shown in mouse models by Elliot *et al* (1998, 2001) who investigated whether gene conversions at a DSB site were repaired by gap repair or by MMR of the mismatched DNA. Elliot *et al* (1998) showed that increasing divergence leads to a decrease in the efficiency of repairing DSBs by homologous recombination, as the presence of 1.5% divergence resulted in a 17-fold decrease in DSB repair efficiency. In a separate study, Elliot and Jasin (2001) showed that in *Msh2*$^{-/-}$ mice cells there was a 1.7-fold increase in DSB-induced recombination, suggesting that the gene conversions are repaired by MMR activities. Msh2 has also been shown to play a role in the suppression of homeologous recombination in *Arabidopsis thaliana* (Li et al., 2006). Li *et al* (2006) measured the frequency of recombination by inserting two copies of an intron inserted in the ß-glucuronidase gene. These introns were either perfectly homologous or shared varying degrees of divergence. In a wild type construct, the rates of recombination decreased as the degree of divergence increased (from a 4.1-fold decrease with 0.5% divergence to a 20.3-fold decrease with 9% divergence) (Li et al., 2006). Deletion of *msh2* led to a 2- to 9-fold increase in homeologous recombination, but this increase did not correlate with the degree of divergence (Li et al., 2006). In addition to this, Li *et al* (2006) showed that the rate of recombination in *msh2* cells could be decreased by the addition of wild type *Arabidopsis thaliana MSH2*.

To further investigate the role of Msh2, Chen and Jinks-Robertson (1998) measured the lengths of mitotic gene conversion tracts using an inverted-repeat assay. Their construct utilised 5' portion of the *HIS3* intron along with a recombination substrate, cß2 in combination with the 3' region of the

HIS3 intron along with a different substrate, cß7. The substrates shared 94% identity. The 5' and 3' *HIS3* introns are in opposite directions, leading to auxotrophy to histidine. Crossover events that occur between the two cß sequences will lead to a reorientation of the *HIS3* introns, which leads to prototrophy to histidine. PCR and sequencing were then carried out to confirm gene conversions, as gene conversion events will lead to same nucleotide sequences present in both cß sequences (Chen and Jinks-Robertson, 1998). They noticed that conversion gradients, assessed by the length of heteroduplex DNA, were dependent on the activity of MMR proteins. This was because an *msh2 msh3* double mutant and a *pms1* single mutant lead to longer gene conversion tracts compared to wild type. The data agree with studies by Negritto *et al* (1997) who showed that the anti-recombination activity of the MMR proteins leads to a shortening of gene conversion tracts. This is indicative of a role for MMR proteins in impeding the extension of the heteroduplex through mismatched regions. Msh2 displays a stronger anti-recombination activity than Pms1, as deletion of *msh2* leads to a 40-fold increase in recombination, whereas deletion of *pms1* only leads to a 15-fold increase (Chen and Jinks-Robertson, 1998, Datta et al., 1996, Selva et al., 1995). Based on these results, Chen and Jinks-Robertson (1998) went on to propose that the impeding actions of the MMR proteins on the heteroduplex leads to a 'helicase-catalysed reversal of heteroduplex formation'. This model is similar to that suggested by Zahrt and Maloy (1997) in *E. coli* (**Section 1.4.1.1**).

In a subsequent study, Chen and Jinks-Robertson (1999) went on to show a role for the MMR proteins in meiotic homeologous recombination. An increase in the length of gene conversion tracts in MMR-defective strains was observed using random spore analysis (Chen and Jinks-Robertson, 1999). However, for lower levels of sequence divergence, the MMR proteins seem to have a more prominent role in mitosis than in meiosis (Chen and Jinks-Robertson, 1998, 1999). This bias is not seen for higher levels of divergence (Chen and Jinks-Robertson, 1998, 1999) which may suggest the existence of a threshold to the number of mismatches. When this threshold is exceeded, the anti-recombination activity of the MMR proteins is no longer increased. This may be caused by saturation of the system, caused by insufficient amounts of Msh2, when this threshold is exceeded. Alternatively, exceeding this threshold may result in an inability to carry out strand invasion which is seen by the constant level of anti-recombination activity of the MMR proteins.

Karyotyping experiments have shown *S. cerevisiae* and *S. paradoxus* contain the same number of chromosomes (Naumov et al., 1992). Genetic studies showed that these genes are located in the same order along the chromosomes (Naumov et al., 1992). Sequence analysis has shown that the genomes of both *S. cerevisiae* and *S. paradoxus* are approximately 85% similar (Cliften et al., 2001,

Kellis et al., 2003, Liti et al., 2009, Wei et al., 2007) (**Figure 1.8**). Both species of yeast have been found in similar locations in nature (Sampaio and Goncalves, 2008, Sniegowski et al., 2002). Hunter *et al* (1996) showed that an inter-specific yeast hybrid using *S. cerevisiae* and *S. paradoxus* leads to a decrease in the rate of homeologous recombination, along with an accompanying increase in meiosis I non-disjunction. There is also a major viability defect, with only 1% of the spores being viable. By deleting *msh2* and *pms1*, the spore viability was improved 8.7-fold and 6.1-fold, respectively (Hunter *et al*., 1996). In addition to this, deletion of these two MMR proteins resulted in an increased frequency of homeologous recombination with an accompanying decrease in meiosis I non-disjunction events, with *MSH2* appearing to play a more prominent role than *PMS1* (Hunter *et al*., 1996). These data further support a role for the MMR system in the suppression of recombination between diverged sequences.

1.4.2 Chromosome transfer experiments allow assessments of the effects of homeology using partial hybrids in yeast

Chromosome transfer between different yeast species can be achieved by mutations in the *kar1* gene (Dutcher, 1981). *KAR1* plays an important role in karyogamy. Karyogamy is the process of bringing the nuclei of both parents close together to facilitate their fusion during conjugation. Conjugation (i.e. normal mating) involves the formation of a heterokaryon, where both parents are connected via cytoplasmic connections. The *kar1-1* mutation (Conde and Fink, 1976) prevents fusing of the nuclei in these heterokaryons. In *kar1-1* mutations, the heterokaryons persist for longer and have a probability of approximately 0.38 of losing one of more chromosomes (Dutcher, 1981). Chromosomes are also transferred from one parental nucleus to the other during the attempted nuclear fusion (Dutcher, 1981). This transfer only occurs when one parent has the *kar1-1* mutation and is never seen in *KAR1* x *KAR1* heterokaryons (Dutcher, 1981). In addition to this, it was also shown that the frequency of transfer is inversely correlated to the size of the chromosome, i.e. the frequency of transfer is increased with decreasing chromosome size (Dutcher, 1981). The transfer of a chromosome will either lead to the recipient chromosome being replaced or will co-exist with this chromosome in a disomic state (Nilsson-Tillgren et al., 1980). Using the *kar1-1* mutation, Nilsson-Tillgren *et al* (1981, 1986) transferred chromosomes III and V from *Saccharomyces carlsbergensis* to *S. cerevisiae*. Their analysis revealed that the chromosomes of *S. carlsbergensis* are made up of different yeast species, including portions that are homologous to the *S. cerevisiae* genome. These data are indicative of how recombination is important in the evolution of a species. However, this mosaicism means that this strain is not ideal for the analysis of the effects of homeology on recombination.

Using a similar approach involving a *kar1Δ13* mutation, Chambers *et al* (1996) made a partial hybrid strain in which the chromosome III from *S. cerevisiae* was replaced by chromosome III from *S. paradoxus* (**Figure 1.8**). Chromosome transfer was initially screened using the cycloheximide marker on chromosome VII. The *S. cerevisiae* strain contained a *cyh2-1* mutation which renders the strains resistant to cycloheximide. The *S. paradoxus* strain was wild-type for *CYH2* which confers cycloheximide sensitivity, which is the dominant trait. After mating the *S. cerevisiae* and *S. paradoxus* strains for a short time, the mating mixture was plated on medium supplemented with cycloheximide. As *kar1* mutations lead to chromosome transfer, any cycloheximide papillations are indicative of the *S. cerevisiae* strain retaining its own copy of chromosome VII. To select for the transfer of chromosome III, Chambers *et al* (1996) genetically screened for prototrophic markers that were present on chromosome III of *S. paradoxus* (**Figure 1.8**). To eliminate any strains in which additional chromosomes had also been transferred, the transfer of only the *S. paradoxus* chromosome III was then confirmed using CHEF Gels (**Section 2.2.2**). A doubly intense band for chromosome III represents disomy for that chromosome only. The resulting strain therefore contained a copy of *S. paradoxus* chromosome III as well as a copy of *S. cerevisiae* chromosome III. To lose the *S. cerevisiae* copy of chromosome III, the *msh3* gene (on chromosome III) was disrupted with a *URA3* cassette. This strain was plated onto 5-Fluororotic Acid (5-FOA) media which is counter-selective for uracil. Therefore, 5-FOA resistant papillations were indicative of the loss of the *S. cerevisiae* copy of chromosome III. The resulting partial hybrid strain gives a more accurate representation of the phenotypes caused by the presence of homeology than the chromosome transfer experiments carried out by Nilsson-Tillgren *et al* (1981, 1986).

Chambers *et al* (1996) investigated the role of these MMR proteins using a homeologous strain where chromosome III of *S. cerevisiae* was replaced by chromosome III of *S. paradoxus*. They saw an overall 25-fold decrease in the rate of meiotic homeologous recombination between the *HML* and *THR4* intervals using this partial hybrid. This decrease in recombination was accompanied by an increase in the levels on meiosis I non-disjunction. However, deletion of *msh2* and *pms1* led to a 5.5-fold and 2.5-fold increase in the rates of recombination, respectively, as well as a decrease in the rates of meiosis I non-disjunction (Chambers et al., 1996). The data suggest that both Msh2 and Pms1 act to prevent homeologous recombination, confirming observations made by Hunter *et al* (1996). Recombination was further improved in the *msh2 pms1* double mutation, suggesting that Msh2 and Pms1 act independently in the regulation of homeologous recombination. Chambers *et al* (1996) noted the levels of homeologous recombination never reached homologous levels, even

when both *msh2* and *pms1* were deleted. They suggested that this was potentially due to the ability of other mismatch repair proteins to partially substitute for Msh2 and Pms1 in their absence, or because of the high degree of sequence divergence along chromosome III. This led them to propose that the mismatch repair system acts in the assessment of the degree of divergence when the heteroduplex DNA is formed (Chambers et al., 1996) (discussed further in **Section 1.8**).

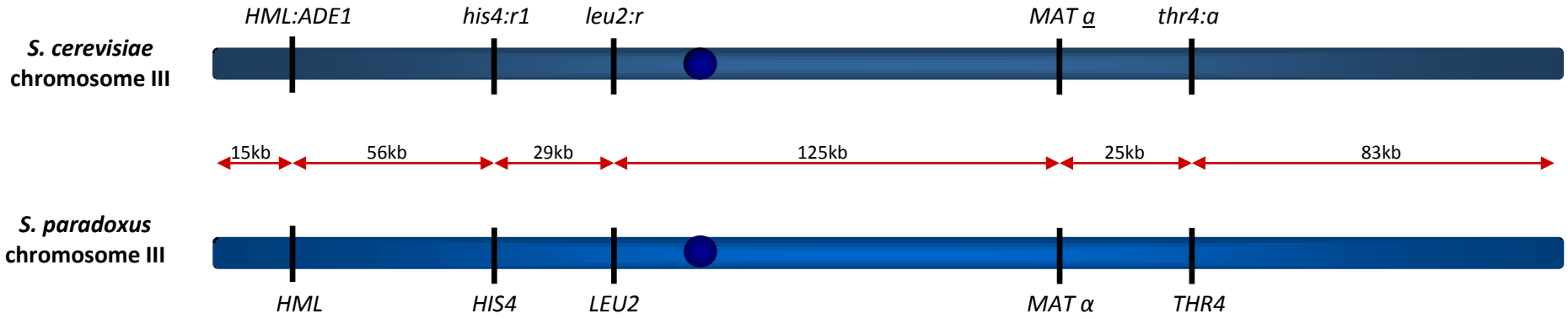


Figure 1.8: Genetic markers on chromosome III of S. cerevisiae and S. paradoxus

Modified from Chambers *et al* (1996)

These markers were used to assess the transfer of chromosome III from *S. paradoxus* to *S. cerevisiae* by Chambers *et al* (1996).

1.5 Topoisomerases

The process of replication generates torsional stress (Liu and Wang, 1987) which leads to positive DNA supercoiling in front of the replication fork and negative DNA supercoiling behind it. Topoisomerases are evolutionary conserved enzymes that act by relieving this supercoiling, as well as separating interlinked DNA molecules (as reviewed by Champoux (2001) and Wang (1996)). Their actions are initiated by the cleaving of DNA. This cleavage is associated with the formation of a phosphodiester bond between a conserved tyrosine residue located in the topoisomerase and one of the ends of the broken DNA strand (Champoux, 2001). Topoisomerases can be separated into two classes depending on their mode of action. The type I topoisomerases act by cleaving only one strand of the duplex DNA whereas type II topoisomerases cleave both strands. The type I class of topoisomerases can be further sub-divided into two further groups. Topoisomerases that become attached via a 5' phosphodiester bond are classed as type IA topoisomerases, whereas those that attach via a 3' phosphodiester bond are classed as type IB topoisomerases (Champoux, 2001, Wang, 1996).

As reviewed by Champoux (2001) and Wang (1996), type IA topoisomerases are able to relax plasmids containing negative, but not positive, supercoils. This relaxation also requires the substrate DNA to have an exposed single-stranded region (Kirkegaard and Wang, 1985). Members of this class of topoisomerases include the *E. coli* topoisomerases Top1 and Top3, the *S. cerevisiae* topoisomerase Top3 and the human topoisomerases TopoIIIα and TopoIIIß. Each of these proteins contain a cleavage / strand passage domain which contains the tyrosine site which is involved in the formation of the phosphodiester bond during DNA cleavage. The type IB topoisomerases are able to relax both positive and negative supercoils. Unlike the type IA class, type IB topoisomerases do not require the presence of single-stranded DNA in the substrate. The eukaryotic topoisomerase I protein, which has roles in DNA replication, belongs to this class of topoisomerases. In the absence of topoisomerase I, mitotic recombination is seen at an increased frequency at repeated sequences (Wang et al., 1990).

Unlike the type I topoisomerases which function as a monomer, the type II topoisomerases function as a dimer. Cleavage occurs when a tyrosine present in each subunit of the dimer becomes covalently attached to the duplex DNA via a 5' phosphodiester bond. This results in a conformational change which leads to the two ends of the cleaved duplex DNA being pulled apart, forming an opening known as the gated-segment DNA. Relaxation is achieved when DNA from the same molecule is passed through this opening. Alternatively, catenation or decatenation is achieved

if the DNA that is passed through the opening is from a different molecule. Type II topoisomerases require ATP hydrolysis and the presence of magnesium ions to carry out these reactions. The eukaryotic topoisomerase II, which is involved in the final stages of chromosome condensation, belongs to this group of topoisomerases (Holm et al., 1985, Uemura et al., 1987).

1.5.1 The yeast topoisomerase III

The gene for topoisomerase III was discovered by Wallis *et al* (1989) in a screen for mutations that led to hyper-recombination between repetitive sequences in *S. cerevisiae*. Originally named *EDR1*, it was found to be Top3 due to its high sequence homology to *E. coli* Top3. Top3 is not absolutely essential for viability in *S. cerevisiae*. However, *top3* mutants do exhibit a slow growth phenotype, which is caused by cell cycle arrest at G2/M, characterised by an accumulation of large budded cells with a single nucleus (Gangloff et al., 1994). The fact that a deletion of *top3* leads to slow growth despite the presence of other topoisomerases in the cell shows that it plays an important role in mitosis. When *top3* deleted, strains also exhibit an increase in the rate of mitotic recombination (Wallis et al., 1989). *top3Δ* cells are sensitive to the replication inhibitors hydroxyurea (HU), methyl methane sulfonate (MMS) and UV irradiation (Chakraverty et al., 2001). This sensitivity is partially attributed to a defect of these *top3Δ* cells in leading to the phosphorylation of Rad53 during S-phase which activates this S-phase specific checkpoint (Chakraverty et al., 2001). In addition to this, they have a sporulation defect, which is indicative of problems during meiosis (Gangloff et al., 1999). The role of topoisomerases in genome integrity is evolutionarily conserved, as the *S. pombe* top3+ has also been shown to play an essential role in cell division (Goodwin et al., 1999, Maftahi et al., 1999). In *S. pombe*, *top3* cells are seen to undergo less than ten divisions and then die whilst carrying out mitosis (Goodwin et al., 1999). These *top3* cells have been shown to display a 'cell untimely torn' (cut) phenotype where the septum divides the nucleus.

1.5.2 The mammalian topoisomerase III

The mammalian topoisomerase III exists in two isoforms – TopoIIIα and TopoIIIß (Hanai et al., 1996, Ng et al., 1999). Deletion of Top3α leads to embryonic lethality in mice (Li and Wang, 1998). This is also seen in *A. thaliana*, as *top3α-1* mutations cause severe developmental defects and result in the lethality of these plants (Hartung et al., 2007). These observations are consistent with those seen in *S. pombe* cells that have *top3* deletions (Goodwin et al., 1999, Maftahi et al., 1999). This lethality is not seen when TopoIIIß is deleted though, as mice develop to maturity (Kwan and Wang, 2001). However, these mice die earlier than normal and are seen to suffer from various organ defects as well as reduced fertility (Kwan and Wang, 2001).

1.6 The RecQ Helicase Family

The RecQ helicase family (**Figure 1.9**) has been implicated in maintaining the fidelity of both mitotic and meiotic recombination (reviewed in Bachrati and Hickson (2003, 2008), Chakraverty and Hickson (1999), Harrigan and Bohr (2003), Karow *et al* (2000b), Khakhar *et al* (2003), Mohaghegh and Hickson (2001), Singh *et al* (2008) and van Brabant *et al* (2000a)).

There are three main regions that are conserved amongst all members of the RecQ helicase family. The first is a helicase domain that is characterised by seven conserved amino acid motifs. This region is responsible for the unwinding of DNA in a 3'-to-5' direction (Ahmad et al., 2002, Bennett et al., 1998, Gray et al., 1997, Karow et al., 1997, Lu et al., 1996, Ozsoy et al., 2001, Ozsoy et al., 2003, Tada et al., 1996, Umezu et al., 1990, Yan et al., 1998). This unwinding is caused by the disruption of hydrogen bonds and is dependent on the presence of magnesium ions and ATP. As discussed in detail in the subsequent sections, the RecQ helicases show a preference for various recombination intermediates such as both single and double Holliday junctions, D-loops and G-quadruplex DNA (Huber et al., 2002, Karow et al., 2000a, Sun et al., 1998, van Brabant et al., 2000b, Wu and Hickson, 2003) and are suggested to have an anti-recombination activity (Bugreev et al., 2007, Jessop et al., 2006, Harmon and Kowalczykowski, 1998, Oh et al., 2007, Oh et al., 2008). The second conserved domain is the Helicase-and-RNaseD-C-terminal (HRDC) domain. This region facilitates the stable binding of nucleic acids (Morozov et al., 1997). The third is RecQ Conserved (RQC) domain which mediates protein-protein interactions. These domains will be discussed in detail with respect to their effects on the relevant protein in the subsequent sections.

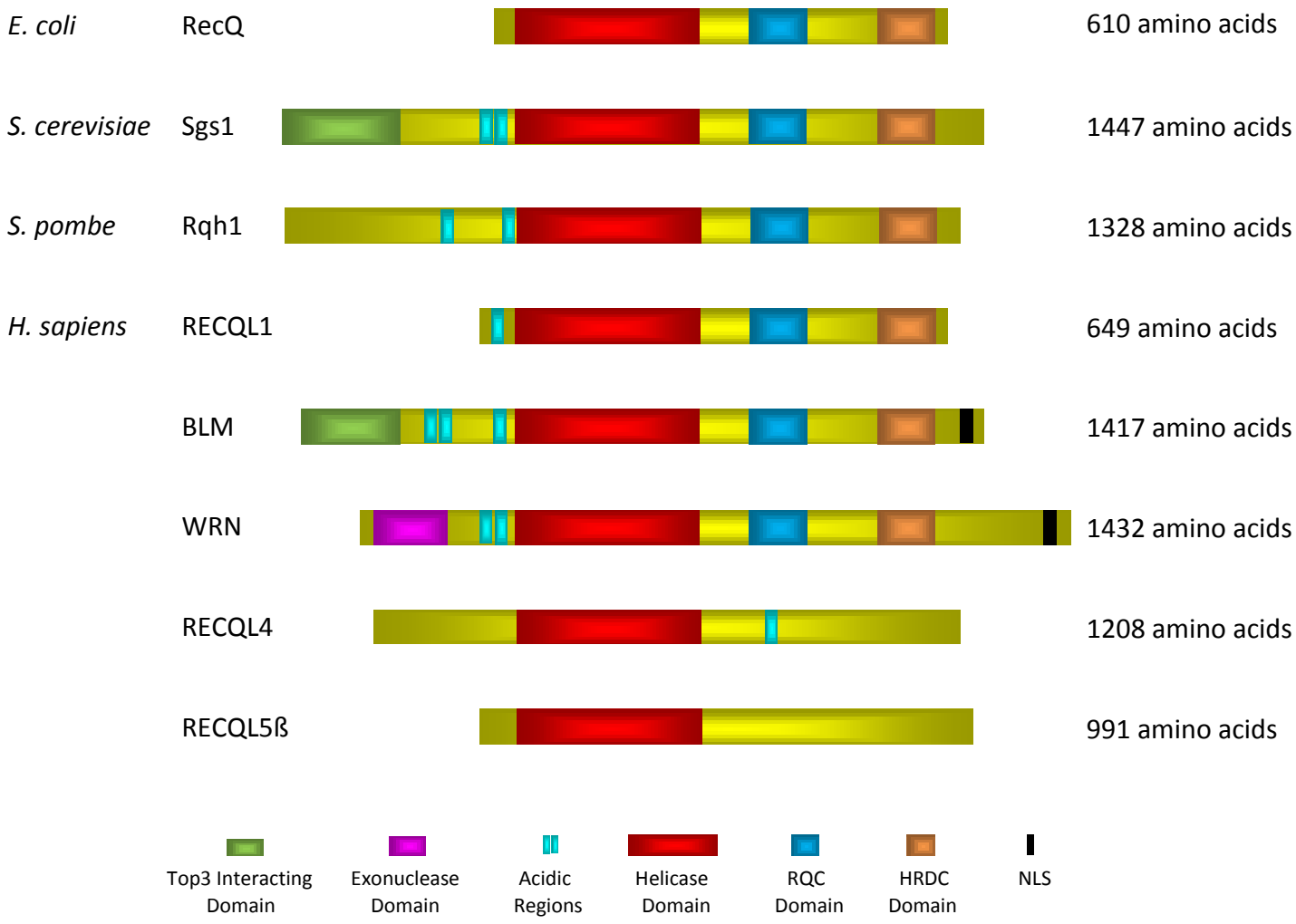


Figure 1.9: Members of the RecQ helicase family

Modified from Harrigan and Bohr (2003)

The RecQ helicase family shares seven conserved helicase motifs responsible for the binding and hydrolysis of ATP. The RecQ Conserved (RQC) domain facilitates protein-protein interactions. The Helicase-and-RNaseD-C-terminal (HRDC) domain is required for DNA binding. BLM and WRN possess Nuclear Localisation Signals (NLS) which localise the proteins to the nucleus. WRN possesses 3'-to-5' exonuclease activity. Some members have acidic regions, whose functions are currently being elucidated.

1.6.1 The *E. coli* homolog RecQ

RecQ was found to act in the RecF pathway, which is involved in recombination in *E. coli* (Kolodner et al., 1985, Nakayama et al., 1984). This pathway has been shown to help restart replication forks which have stalled due to damage caused by UV light (Courcelle and Hanawalt, 1999, Cox et al., 2000).

The helicase activity of RecQ was initially characterised by Umezu *et al* (1990, 1993). It was discovered that both the helicase and RQC domains of RecQ exist as a single structural domain that is referred to as the catalytic domain (Bernstein et al., 2003, Bernstein and Keck, 2003, Liu et al., 2004). This domain contains all seven helicase motifs that are required for the unwinding activity of RecQ. It also contains a zinc finger motif and a winged-helix domain which are responsible for the ability of RecQ to interact with other DNAs and proteins (Bernstein et al., 2003, Bernstein and Keck, 2003, Liu et al., 2004). This was confirmed by Bernstein and Keck (2003) using an electrophoretic mobility shift assay (EMSA) that monitored DNA binding. This assay used a radiolabelled 18 base pair oligonucleotide that was annealed to a 12 base pair 3' single stranded extension as a substrate. This substrate was incubated with full length *E. coli* RecQ and a C-terminal truncation of RecQ that deleted the HRDC domain. The mixture was purified by polyacrylamide gel electrophoresis. Bernstein and Keck (2003) saw that the full length RecQ was able to shift the electrophoretic mobility of the DNA, however the C-terminal truncation could not, showing that the HRDC domain was important for DNA binding. Subsequent EMSA analysis using both double-stranded and single-stranded DNA substrates showed that the HRDC domain of the *E. coli* RecQ preferentially binds single-stranded DNA (Bernstein and Keck, 2005). They also showed that mutation of the tyrosine residue at position 555 to an alanine resulted in the ablation of the HRDC domains activity (Bernstein and Keck, 2005).

RecQ was shown to act alongside RecA and Single Stranded Binding protein (SSB) to unwind various DNA substrates that resemble recombination intermediates, which is indicative of an anti-recombination function of the helicase (Harmon and Kowalczykowski, 1998). These substrates include blunt-end duplexes, duplexes with single-stranded overhangs, three- and four-way junctions and forked DNA substrates (Harmon and Kowalczykowski, 1998). Harmon and Kowalczykowski (1998) also proposed that the unwinding activity of RecQ creates a single-stranded substrate that is used by RecA in the initiation of recombination. The data imply that RecQ has both pro- and anti-recombination activities.

RecQ has also been shown to bind to topoisomerase III (Harmon et al., 1999) (**Section 1.5**). Both act together in the decatenation of double-stranded plasmid DNA. This suggests a potential role in decatenating linked chromosomes during replication leading to the prevention of excessive sister chromatid exchanges (SCEs) (Harmon et al., 1999, Oakley et al., 2002, Wu and Hickson, 2001). This interaction with topoisomerase III is well conserved amongst the RecQ helicase family.

1.6.2 The *S. cerevisiae* RecQ helicase Sgs1

Sgs1 was identified during a screen to find mutations that suppress the slow growth phenotypes conferred by deletion of the topoisomerase Top3 (Gangloff et al., 1994). This mutant phenotype was the basis for the naming of Sgs1, which stands for Slow Growth Suppressor 1 (Gangloff et al., 1994). Yamagata *et al* (1998) showed that expression of wild-type *SGS1* in an *sgs1 top3* double mutant strain leads to slow growth.

Sgs1 has been implicated in various cellular functions (discussed below and in the subsequent sections). The importance of Sgs1 to the cell can be seen when it is deleted. *sgs1* cells are sensitive to various DNA damaging agents, including methyl methane sulfonate (MMS) and HU (Frei and Gasser, 2000, Miyajima et al., 2000a, Onoda et al., 2000, Yamagata et al., 1998). *sgs1* diploids also display moderate levels of sensitivity to UV irradiation and X-rays (Gangloff et al., 2000, Onoda et al., 2001, Watt et al., 1996). This phenotype implicates Sgs1 in the DNA damage response during mitosis (discussed further in **Section 1.7**). In agreement with this, Sgs1 has been shown to interact with Rad51 (Wu et al., 2001) and Rad53 (Frei and Gasser, 2000) in response to replication forks that have stalled due to the presence of DNA damage.

Consistent with a role in mitosis, *sgs1* cells are seen to exhibit a hyper-recombination phenotype. This leads to an increase in both intra- and inter- chromosomal recombination between repetitive sequences, ectopic recombination and unequal sister chromatid exchange (USCE) events (Gangloff et al., 1994, Nag and Cavallo, 2007, Onoda et al., 2000, Watt et al., 1996). This hyper-recombination phenotype is associated with a 10-fold increase in chromosome loss and an increase in chromosome non-disjunction during mitosis (Watt et al., 1995, 1996) (discussed further in **Section 1.7.2**).

Frei and Gasser (2000) have shown that Sgs1 acts early in response to DNA damage and is an integral part of the intra-S checkpoint (discussed further in **Section 1.7.1**). After microarray analysis showed that the levels of Sgs1 were shown to peak during S-phase (Cho et al., 1998), Frei and Gasser (2000) carried out FACS analysis to monitor the rate of progression of *sgs1* cells. When exposed to MMS, *sgs1* progressed through S-phase at a much faster rate than wild-type cells (Frei and Gasser, 2000). Simultaneous deletion of both *rad24* and *sgs1* leads to an even faster progression through S-phase when cells are exposed to MMS. This suggests that the absence of Sgs1 (and Rad24) does not allow for the slowing of the cell cycle in order to repair any MMS-induced damage. However, when these cells were monitored during the G2/M- or G1/S-phases of the cell cycle, the rates of progression were the same for both wild-type and *sgs1*, suggesting that Sgs1 acts during S-phase (Frei and

Gasser, 2000). Further evidence of an S-phase specific role of Sgs1 came from time-course analysis using Western blotting which showed that the levels of Sgs1 increase during S-phase but remain low during other parts of the cell cycle (Frei and Gasser, 2000).

As Sgs1 was seen to localise to the nucleus in S-phase specific foci with Rad53, the actions of Sgs1 in the intra-S phase checkpoint were further assessed by its ability to phosphorylate Rad53 (Frei and Gasser, 2000). This was carried out as phosphorylation of Rad53 is essential for cell cycle arrest in response to DNA damage. Using mobility shift assays, Frei and Gasser (2000) showed that phosphorylation of Rad53 was still achieved in *sgs1* and *rad24* mutations. However, an *sgs1 rad24* double mutation was unable to phosphorylate Rad53 (Frei and Gasser, 2000), suggesting that both Sgs1 and Rad24 act upstream of Rad53, in independent pathways, in response to DNA damage. Chakraverty *et al* (2001) saw that Top3 also acts upstream of Rad53. As reviewed by Oakley and Hickson (2002), and discussed in further detail in **Section 1.7.4**, it is suggested that Sgs1 acts on double Holliday junction-type structures and forms a recombination intermediate that is subsequently acted on by topoisomerase III. This model suggests that the intermediate that is created by Sgs1 is toxic to the cell in the absence of *top3* and its accumulation leads to various defects. It also suggests that Sgs1 acts upstream of Top3 in this pathway. This was inferred by experiments showing that the *top3Δ* phenotypes were suppressed by deletions of homologous recombination genes *rad51*, *rad52* and *rad54* (Oakley *et al*., 2002), suggesting that inactivating the homologous recombination pathway results in the absence of accumulating of these toxic substances. It is therefore suggested that both Sgs1 and Top3 act together to process lesions (**Section 1.7.4**) which then generate an intermediate which acts as a signal activating of the intra-S phase checkpoint (Chakraverty et al., 2001).

In agreement with this, the N-terminal of Sgs1 has been shown to interact with Top3. The interaction between Sgs1 and Top3 was confirmed by yeast two hybrid analysis (Gangloff et al., 1994) which implicated the N-terminal 500 amino acid residues in this interaction (Gangloff et al., 1994). This observation was further confirmed by affinity chromatography experiments by Bennett *et al* (2000) and by co-immunoprecipitation studies by Fricke *et al* (Fricke et al., 2001). This region was further refined to residues 1-106 (Bennett and Wang, 2001). Bennett and Wang (2001) fused the Top3 protein to a truncated Sgs1 protein where the first 106 amino acids were deleted. They showed that this fusion protein was able to complement the MMS and HU sensitivity that is exhibited by *sgs1-NΔ106*. This interesting finding shows that fusion of Top3 to Sgs1 renders the N-terminal region of Sgs1 dispensable. In addition to this, they carried out random mutagenesis and

showed that mutation of the valine residue at position 29 to glutamate affected the interaction between Sgs1 and Top3 (Bennett and Wang, 2001). Fusing Top3 to this *sgs1-V29E* point mutation also lead to the complementation of HU and MMS sensitivities (Bennett and Wang, 2001). By introducing point mutations in Sgs1 into their yeast two hybrid screen, Duno *et al* (2000) showed that the leucine residue at position 9 of Sgs1 was important for interactions with Top3. This was confirmed by Ui *et al* (2001) who showed that, in addition to this leucine residue at position 9, the lysine residue at position 4 and the proline residue at position 5 were also important in Top3 binding. They showed that this *sgs1-K4AP5AL9A* mutation was sensitive to the DNA damaging agents HU and MMS, and also exhibited a slow growth phenotype resembling *top3Δ* cells (Ui et al., 2001).

The C-terminal contains the RecQ Conserved (RQC) domain that is conserved amongst all members of the RecQ helicase family. This domain is suggested to be responsible for mediating protein-protein interactions, as it structurally resembles the winged-helix domain which carries out this function in other proteins (Bachrati and Hickson, 2003, Bernstein et al., 2003, Brosh et al., 2001b, von Kobbe et al., 2002). The C-terminal also houses the Helicase-and-RNaseD-C-terminal (HRDC) domain which facilitates nucleic acid binding (Liu et al., 1999, Morozov et al., 1997). Liu *et al* (1999) described the three-dimensional structure of the HRDC domain of Sgs1 by nuclear magnetic resonance (NMR) spectroscopy and confirmed its ability to bind DNA by EMSA. It comprises five helices, named α1-α5, which share conserved residues present in other HRDC domains, including a hydrophobic loop between helices α1 and α2 (Liu *et al*., 1999). This hydrophobic loop is also present in the HRDC domains of both BLM and WRN, suggesting that this region mediates intra- or inter-molecular interactions amongst the RecQ helicases (Liu *et al*., 1999). However, the only residue that is conserved amongst Sgs1, BLM and WRN for this hydrophobic loop is a lysine residue located at position 69 of the domain (Liu *et al*., 1999). Liu *et al* (1999) also saw that the HRDC domain of Sgs1 is highly positively charged on its surface and this region is suggested to facilitate the binding of DNA (Liu *et al*., 1999). In contrast, the HRDC domain of BLM (described in **Section 1.6.3.1**) is seen to contain a negatively charged region around helix α3 (Liu *et al*., 1999). These variations likely reflect a difference in the molecular interactions carried out by the HRDC domains for Sgs1, BLM and WRN. Yeast-two-hybrid analysis has shown that the Rad51-interacting domain of Sgs1 partially overlaps with the HRDC domain (Wu et al., 2001).

The N-terminal region of Sgs1 possesses two highly acidic regions (ARs) known as AR1 and AR2. Initially predicted to span amino acids 400-474 (AR1) and 510-596 (AR2) (Miyajima et al., 2000b),

protein sequence analysis now suggests that these domains span residues 321-474 (AR1) and 502-648 (AR2) (Bernstein et al., 2009). The activities carried out by the ARs have only recently begun to be characterised. Bernstein *et al* (2009) showed that mutation of AR2 leads to a separation-of-function phenotype. These mutants are able to suppress the slow growth phenotypes conferred by *top3* and *rmi1* mutations, thus resembling *sgs1Δ* cells. They are also seen to accumulate X-shaped structures at damaged DNA forks, like *sgs1Δ* cells. This is indicative of an inability to resolve recombination intermediates. However, these AR2 *sgs1* mutants are resistant to DNA damage caused by exposure to HU and MMS, resembling wild-type Sgs1 (Bernstein et al., 2009). They also do not exhibit a hyper-recombination phenotype that is seen when *sgs1* is deleted (Gangloff et al., 1994, Miyajima et al., 2000b, Mullen et al., 2000, Watt et al., 1996). This suggests that these AR2 *sgs1* mutants retain the ability to aid in restarting stalled replication forks. These data suggest that the functions of Sgs1 (acting together with Top3 and Rmi1) in the repair of DNA recombination intermediates can be uncoupled from the functions of Sgs1 in restarting stalled replication forks. Based on these observations, and studies that have shown that highly acidic regions are involved in mediating protein-protein interactions (Struhl, 1995), Bernstein *et al* (2009) propose that mutation of AR2 may affect the ability of Sgs1 to interact with proteins that have roles in DNA metabolism. Due to the phenotypes caused by mutation of AR2, we suggest that these proteins are likely to be Top3, as well as the type II topoisomerase Top2, which is important in ensuring accurate chromosome segregation. Yeast-two-hybrid analysis has revealed that Sgs1 is able to interact with the topoisomerase Top2 (Watt et al., 1995) and this interaction has been shown to occur in regions that overlap both the ARs and the helicase domain of Sgs1. In the same region, Sgs1 has also been shown to interact with Rad16, which is involved in nucleotide excision repair (Saffi et al., 2001).

The 3'-to-5' helicase activity of Sgs1 was characterised by Bennett *et al* (1998) and Lu *et al* (1996) using a strand displacement assay. The helicase activity of Sgs1 has been implicated in the unwinding of substrates that resemble recombination intermediates. For example, Sgs1 has been shown to branch migrate Holliday junctions (Bennett et al., 1999) and unwind G-quadruplex DNA (Sun et al., 1999), the presence of which may arrest DNA polymerization. Sgs1 can also unwind flayed duplex structures (resembling replication forks) as well as three- and four-way junctions (Bachrati and Hickson, 2003).

Ever since a point mutation that disrupts the helicase activity of Sgs1 was characterised by Lu *et al* (1996), the role of the helicase domain of Sgs1 has been controversial. This point mutation changes the lysine residue at position 706 in the ATP-binding pocket of the helicase domain (Lu *et al*., 1996)

and has been shown to be important for the activity of other helicases (Sung *et al*., 1988). It would be expected that the helicase domain would be responsible for the effects of Sgs1. However, the phenotypes reported by Lu *et al* (1996) led them to propose that the helicase function of Sgs1 was not required *in vivo*. They noted that the helicase defective mutation of Sgs1 behaved like wild-type in its ability to decrease the rate of growth in an *sgs1 top3* strain as well as improving the rate of growth in an *sgs1 top1* strain (Lu et al., 1996). This was also seen in a subsequent complementation study by Mullen *et al* (2000). In addition to this, Miyajima *et al* (2000a, 2000b) showed that, like *SGS1*, *sgs1-K706A* was able to complement the poor sporulation phenotype and decrease the frequency of meiotic recombination that it caused by *sgs1Δ*.

However, other researchers saw that inactivation of the helicase domain does affect the functions of Sgs1 in a manner similar to a null mutation of *sgs1*. *sgs1-K706A* is sensitive to the DNA damaging agents MMS and HU (Frei and Gasser, 2000, Miyajima *et al*., 2000a, Miyajima *et al*., 2000b, Mullen *et al*., 2000, Onoda *et al*., 2000, Saffi *et al*., 2000, Ui *et al*., 2001). The mitotic hyper-recombination phenotype of *sgs1-K706A* is indistinguishable to that seen for *sgs1Δ* (Miyajima *et al*., 2000a, Miyajima *et al*., 2000b, Mullen *et al*., 2000, Onoda *et al*., 2000). Miyajima *et al* (2000a) and Mullen *et al* (2000) showed that *sgs1-K706A* exhibited an increase in homologous recombination between heteroalleles, and Onoda *et al* (2000) showed that this mutant leads to an increase in the frequency of USCE. Using DAPI staining analysis, Weinstein and Rothstein (2008) saw that *sgs1-K706A*, like *sgs1Δ*, was able to suppress the accumulation of large budded cells that contain a single nucleus that is characteristic of *top3Δ* cells. Weinstein and Rothstein (2008) also saw that *sgs1-K706A* behaved like *sgs1Δ* in causing synthetic lethality when combined with a deletion of *srs2*. In contrast to others, Weinstein and Rothstein (2008) also saw that *sgs1-K706A* was not able to complement some of the phenotypes caused by a deletion of *sgs1Δ*. They showed that *sgs1-K706A* was only partially able to rescue the slow growth phenotype exhibited by *sgs1 top1* double mutants by measuring the doubling times of these strains (Weinstein and Rothstein, 2008). They showed that *sgs1-K706A* exhibited a poor sporulation phenotype similar to that observed by *sgs1Δ* cells, however heterozygous *SGS1/sgs1-K706A* diploids were able to sporulate (Weinstein and Rothstein, 2008). Using DAPI staining, they saw that approximately only 20% of the cells sporulated after 3 days (Weinstein and Rothstein, 2008).

One possible reason for these discrepancies is that Weinstein and Rothstein (2008) transplaced the *sgs1-K706A* mutation at the endogenous site within the genome, whereas the observations made by the other studies described above were carried out using plasmid-based complementation studies.

By using plasmid-based complementation studies, expression of the mutated gene on the plasmid may not be carried out by the endogenous promoter at the site of regulation, which may result in altered levels of the protein being produced. This is highly unlikely however, as the plasmids used by Miyajima *et al* (2000a, 2000b) include a centromere element and an autonomously replicating sequence, which should ensure expression of the mutated protein. Another possibility as to why these groups reported different phenotypes for the *sgs1-K706A* mutation is that the plasmid may have popped-out in the studies by Miyajima *et al* (2000a, 2000b). The only way to check whether this has occurred would be to check every tetrad to ensure that the plasmid carrying the *sgs1-K706A* mutation was still present. By transplacing the *sgs1-K706A* mutation at the endogenous site within the genome, Weinstein and Rothstein (2008) proposed that these types of issues are avoided and they therefore suggested that their approach gives a more accurate representation of the effects of this mutation. It remains possible, however, that the mutant phenotypes observed by Miyajima *et al* (2000a, 2000b) were not artefacts caused by plasmid loss. For example, the differences in the reported phenotypes amongst these studies may be because the plasmids used by Miyajima *et al* (2000a, 2000b) led to the overexpression of the helicase-defective Sgs1 protein. If this is the case, then the reported rescued sporulation phenotype would suggest that the *sgs1-K706A* mutant is hypomorphic, meaning that disruption of lysine at position 706 leads to a partial reduction in the activity of the Sgs1 gene. Miyajima *et al* (2000a, 2000b) noted that although *sgs1-K706A* rescues the sporulation defect caused by *sgs1Δ* cells, the rescue was very slow as they saw only 25% sporulation after 12 hours and 60% sporulation after 24 hours. This observation adds further support to the theory that *sgs1-K706A* is a hypomorphic mutation.

Sgs1 is also seen to act during meiosis. Deletion of *sgs1* also confers a sporulation defect (Gangloff et al., 1999, Miyajima et al., 2000b, Rockmill et al., 2003, Watt et al., 1995). Rockmill *et al* (2003) hypothesised that this was due to a checkpoint-induced arrest at the pachytene stage of meiosis. This suggestion was based on observations that nuclei with fully synapsed chromosomes remain present for longer periods of time in *sgs1* cells when compared to wild-type. This suggests that *sgs1* cells are unable to exit the pachytene stage of meiotic prophase when normally required, which would lead to defects in sporulation.

Another role of Sgs1 is to negatively regulate crossovers during meiosis. Jessop *et al* (2006), Oh *et al* (2007) and Rockmill *et al* (2003) showed that deletion of *sgs1* leads to an increase in the rates of crossovers without an increase in the number of gene conversions or non-crossovers. The crossovers that are produced were found to be located in close proximity to each other (Oh et al.,

2007). Studies by Jessop *et al* (2006) and Rockmill *et al* (2003) also noted that *sgs1* cells display an increase in the number of axial associations. These axial associations connect homologous chromosomes to each other. They are suggested to mark the sites where the synaptonemal complex formation initiates and are proposed to mark the sites of crossovers (Agarwal and Roeder, 2000, Fung et al., 2004). In addition to this, Jessop *et al* (2006) and Oh *et al* (2007) saw that deleting *sgs1* leads to an accumulation of inter-homolog joint molecules. They also saw an accumulation of multi-chromatid joint molecules, which are not seen in wild-type. These joint molecules are a direct consequence of the increase in the number of axial associations and closely-spaced double crossovers. These data implicate Sgs1 in the regulation of crossover interference.

As Zip 2 and Zip3 foci are also proposed to mark crossover sites (Borner et al., 2004), Rockmill *et al* (2003) monitored their localisation by tagging them with green fluorescent protein (GFP) in *sgs1* cells. They saw a 1.4-fold increase in Zip2 foci and a 1.5-fold increase in Zip3 foci in *sgs1* cells when compared to wild-type (Jessop et al., 2006, Rockmill et al., 2003). They also saw that in wild-type cells, Sgs1 is seen to co-localise with Zip3 to these meiotic chromosomal foci (Rockmill et al., 2003). Taken together, these data imply that Sgs1 prevents the formation of crossovers that are facilitated by the presence of the ZMM proteins Zip2 and Zip3.

In agreement with this, Jessop *et al* (2006) and Oh *et al* (2007) showed that crossovers are markedly increased when *sgs1* is deleted in *zmm* mutants. For example, deletion of *msh4* leads to 2.5-fold decrease in crossovers. When *sgs1* is mutated in an *msh4* background, crossovers are restored to near wild-type levels (Jessop et al., 2006). Similar results are obtained when *sgs1* is mutated in *msh5* or *mlh3* backgrounds (Oh et al., 2007). Mutation of *sgs1* in *zip1*, *zip2* or *mer3* backgrounds also leads to increases in crossing over, but not to wild-type levels (Jessop et al., 2006). This is most likely due the impairment of synaptonemal complex formation in *zip1* and *zip2* mutations and defects in DSB processing in *mer3* mutations.

The above studies implicate Sgs1 as an anti-crossover factor, whose actions are opposed by the pro-crossover activities of the ZMM proteins. Sgs1 acts to specifically inhibit the formation of closely-spaced crossovers, which would ultimately lead to the formation of inter-homolog and multi-chromatid joint molecules. The presence of these would be detrimental to the cell, as their resolution may lead to chromosome loss due to the inability to carry out nuclear division (Jessop and Lichten, 2008).

1.6.3 The human RecQ helicases

1.6.3.1 BLM

Humans possess five RecQ helicases homologs (**Figure 1.9**). The most closely related to Sgs1, in terms of structure and function, is the BLM protein. BLM, which maps to chromosome 15q26.1 in humans, encodes a 159 kDa 1417 amino acid protein (Ellis et al., 1995). As with other RecQ helicases, BLM possesses the seven conserved helicase motifs which are responsible for its 3'-to-5' helicase activity (Karow et al., 1997). BLM also contains the RQC, HRDC and AR domains that are seen in most RecQ helicases (Liu et al., 1999, Morozov et al., 1997). However, unlike Sgs1, BLM also possesses a nuclear localisation signal (NLS) in the C-terminal, which directs the protein to the nucleus (Kaneko et al., 1997, Neff et al., 1999).

Various studies have shown that Sgs1 and BLM are very closely related. Yamagata *et al* (1998) showed that expression of BLM cDNA in *sgs1 top3* cells in *S. cerevisiae* leads to a slow growth phenotype resembling that seen in *top3* cells. They also showed that BLM is able to partially suppress the hyperrecombination phenotype and the sensitivity to HU that is exhibited in *sgs1Δ* yeast cells (Yamagata et al., 1998). Further to this, the introduction of missense mutations found in BS patients into Sgs1, leads to sensitivity to HU and MMS, an increased frequency of intrachromosomal deletions and an increase in the frequency of USCE in these yeast cells (Onoda et al., 2000). This shows that there is considerable functional and sequence conservation among Sgs1 and BLM.

Mutations in the BLM gene can result in the cancer predisposition syndrome Bloom's syndrome (BS) (Ellis et al., 1995). Studies by German *et al* (1993, 1995, 2007) have characterised this disorder. BS individuals are seen to suffer from a retarded growth rate, impaired fertility, immunodeficiency as well as a predisposition to cancer. The usual onset of cancer is before the age of 24. Individuals who suffer from BS do not usually live longer than 30 years of age. The main population affected by BS are the Ashkenazi Jewish population (German, 1995), who have a carrier frequency of 1% (Straughen et al., 1998). The BLM^{Ash} mutation that is seen in many of these BS cases, is caused by a frameshift mutation within the helicase domain of BLM (Straughen et al., 1998). However, various other mutations have been reported, including nonsense and splice-site mutations, that impair the expression of the BLM protein, its nuclear localisation or its function. Most of these problems arise from mutations that lead to the premature truncation of the BLM protein, often leading to a deletion of the helicase domain (German et al., 2007). The importance of the helicase activity of BLM is further evident by the discovery of missense mutations in Bloom's syndrome patients that

alter sequences within the helicase domain (Barakat *et al*., 2000, Ellis *et al*., 1995, Rong *et al*., 2000). Bahr *et al* (1998) reconstituted two of these mutations in mice, and showed that they resulted in a lack of ATPase and helicase activities *in vitro*.

The helicase activity of BLM has been shown to promote the branch migration of Holliday junction structures, implicating it in homologous recombination (Karow et al., 2000a). Similarly to Sgs1, BLM has been shown to interact with TopoIIIα (human homolog to the *S. cerevisiae* topoisomerase Top3) and RMI1 in the dissolution of double Holliday junctions (Hartung et al., 2008, Johnson et al., 2000, Raynard et al., 2006, Wu et al., 2006, Yin et al., 2005) (discussed further in **Section 1.7.5**). In addition to this, the helicase activity of BLM has also been shown to unwind other structures. These include G-quadruplex DNAs, which can result in the stalling of DNA polymerase during replication (Sun et al., 1998). BLM is also able to disrupt duplex DNA that contains a terminal fork (resembling replication forks) and duplex DNA containing an internal bubble (resembling a D-loop) (Adams et al., 2003, Mohaghegh et al., 2001). In agreement with this, BS cells are seen to suffer from a retarded rate of replication fork progression (Lonn et al., 1990). These data implicate BLM in acting during DNA replication. Like Sgs1, the levels of BLM are seen to peak during the S-phase of the cell cycle (Bischof et al., 2001, Dutertre et al., 2000, Sanz et al., 2000). In addition to this, BS cells are sensitive to various DNA damaging agents such as camptothecin, UV light and mitomycin C (Davalos and Campisi, 2003, Ishizaki et al., 1981). These observations further implicate a potential role for BLM in DNA replication.

Wu *et al* (2005) showed that the HRDC domain of BLM (which spans residues 1210-1290) is required for the dissolution of double Holliday junctions. They deleted residues 1268-1417 of BLM, which lead to a truncation of the HRDC domain and saw that this mutation exhibited a reduced affinity for double Holliday junctions and were unable to unwind these substrates (Wu *et al*., 2005). This led them to suggest that the C-terminal, specifically the HRDC domain, was responsible for conferring substrate specificity (Wu *et al*., 2005). They also confirmed the observations made by Bernstein and Keck (2003), as they saw that a similar truncation in *E. coli* RecQ decreased its ability to dissolve double Holliday junction structures by 10-fold (Wu *et al*., 2005). Using site directed mutagenesis, Wu *et al* (2005) mutated various residues that they hypothesised would disrupt the α-helix structure of the HRDC domain. Mutation of a lysine residue at position 1270 (*K1270V*) resulted in a decreased dissolution activity in the presence of TopoIIIα when compared to wild-type BLM (Wu *et al*., 2005). However, they noted that this *K1270V* point mutation still retained the ability to act on forked DNA substrates, suggesting that additional sites are responsible for the function of the HRDC domain of

BLM (Wu *et al*., 2005). As the *K1270V* point mutation seemingly does not completely disrupt the function of the HRDC domain of BLM, Wu *et al* (2005) did not check whether it affected the suppression of SCEs, which are characteristically increased in Bloom's syndrome.

In addition to a role in replication, studies using metaphase spreads from BS cells show that they exhibit increased chromosomal instability (German et al., 1965). These cells show an increased presence of breaks and gaps in the chromatids, an increase in anaphase bridges, increased telomeric associations as well as an increase in acentric fragments (Chan et al., 2007, German et al., 1965). The increase in anaphase bridges seen in BS cells is proposed to reflect the role of BLM in promoting sister-chromatid decatenation during anaphase (Chan et al., 2007). BS cells also exhibit an accumulation of abnormal replication intermediates (Lonn et al., 1990). A characteristic feature of BS cells is an elevated rate of SCEs which is seen in both humans and in mice (Chaganti et al., 1974). In fact, this is used as a method for diagnosis of the disorder, as BS patients are seen to suffer from approximately a 10-fold increase in SCEs when compared to normal individuals. Metaphase spreads from normal individuals show an average of 5 to 10 SCEs per 46 chromosomes. However, BS individuals are seen to have around 50 to 150 SCEs per 46 chromosomes (Chaganti et al., 1974). Yankiwski *et al* (2001) saw that an internal deletion of BLM that deleted the entire HRDC domain *in vitro* was not able to complement the increased SCE phenotype seen in BS cells, showing that this domain is important for the ability of BLM to suppress SCEs.

Further evidence of a role for BLM in DNA replication comes from its association with promyelocytic leukemia (PML) bodies during late G2/S (Bischof et al., 2001, Sanz et al., 2000, Yankiwski et al., 2000, Zhong et al., 1999). As reviewed by Dellaire and Bazett-Jones (2004) and Matunis *et al* (2006), PML bodies are involved in many cellular functions, including apoptosis, DNA repair and cellular senescence. They also function by providing access to sites of DNA damage (Matunis et al., 2006). BLM is seen to localise with various proteins in these PML bodies, including Rad51, RPA, p53, Mre11/Rad50/Nbs1 and TopoIIIα (Dellaire and Bazett-Jones, 2004, Yankiwski et al., 2000, Zhong et al., 1999). The importance of the localisation of BLM to these PML bodies was highlighted by studies by Hu *et al* (2001) and Zhong *et al* (1999). It was shown that cells that lack PML bodies exhibit a 2-fold increase in SCEs. The same increase in the frequency of SCEs was reported for cells in which BLM cannot localise to PML bodies. Based on these observations, Hu *et al* (2001) that the localisation of BLM to PML bodies in important in BLM being able to carry out its functions within the cell.

In addition to localising to PML bodies, BLM has also been shown to localise to Rad51 nuclear foci in response to DNA damage (Bischof et al., 2001, Raderschall et al., 1999, Wu et al., 2001). Rad51 facilitates strand invasion during homologous recombination (Raderschall et al., 1999). BLM functions by displacing the Rad51 nucleofilament from single-stranded DNA, therefore preventing the formation of the D-loop (Bugreev et al., 2007). This provides evidence of an anti-recombination role for BLM, which is also seen for Sgs1. The actions of BLM in unwinding Rad51-dependent D-loops facilitates the SDSA pathway and results in the formation of non-crossover products (Adams et al., 2003, Bugreev et al., 2007, McVey et al., 2004). Further evidence of a role for BLM in facilitating the SDSA pathway comes from evidence that it is able to promote the annealing of homologous strands of DNA (Cheok et al., 2005). Therefore, like Sgs1, BLM appears to function in suppressing the formation of an excessive number of crossovers (Raynard et al., 2006, Wu et al., 2006) (discussed further in **Section 1.7.5**).

BLM is seen to associate with several other proteins that respond to DNA damage, including Mlh1 (Pedrazzi et al., 2001), Msh6 (Pedrazzi et al., 2003), Mus81 (Zhang et al., 2005a), BRCA1 (Wang et al., 2000b), ATM (Beamish et al., 2002, Davalos et al., 2004) and RPA (Brosh et al., 2000, Wu et al., 2001). This collection of proteins is referred to as the BASC (BRCA1-Associated Genome Surveillance Complex) complex (Wang et al., 2000b). The foci formed by these BASC proteins appear during late G2/S in response to damage induced by exposure to HU or ionizing radiation (Wang et al., 2000b). Mutations in the BASC proteins lead to sensitivity to ionizing radiation, cell cycle checkpoint defects and chromosomal instability (Wang et al., 2000b). The association of BLM and RPA is not limited to mitosis. RPA, which has been shown to stimulate the helicase activity of BLM (Brosh et al., 2000), is found to co-localise with BLM on meiotic chromosomes (Walpita et al., 1999). This was seen using mouse spermatocytes where both proteins were seen together along the SC during meiotic prophase (Walpita et al., 1999). Therefore, like Sgs1, BLM appears to function during both mitosis and meiosis.

1.6.3.2 WRN

WRN, which maps to chromosome 8p-12 in humans, encodes a 162 kDa, 1432 amino acid protein (Yu et al., 1996). It possesses the characteristic seven helicase motifs that are conserved amongst all RecQ helicases (Mohaghegh et al., 2001) (**Figure 1.9**). It also contains the RQC, HRDC and AR domains that are seen in most RecQ helicases (Liu et al., 1999, Morozov et al., 1997). Like BLM, WRN has an NLS in the C-terminal, which directs the protein to the nucleus. However, unlike other

RecQ helicases, WRN possesses a 3'-to-5' exonuclease activity which maps to a domain in the N-terminus (Huang et al., 1998, Kamath-Loeb et al., 1998, Shen et al., 1998).

Mutation in the WRN gene leads to the cancer predisposition disorder Werner's syndrome (WS) (Epstein et al., 1966, Goto et al., 1997, Oshima, 2000, Shen and Loeb, 2000a, Shen and Loeb, 2000b, Yu et al., 1996). Individuals who suffer from this disease are phenotypically normal until they reach adolescence. On reaching puberty, they suffer from growth retardation as they fail to exhibit a growth spurt. However, they exhibit various phenotypes indicative of accelerated aging. Individuals suffer from various age related disorders including greying of the hair and alopecia, atherosclerosis, osteoporosis, cataracts, type II diabetes as well as a predisposition to cancer. This phenotype is not as severe as that seen in BS. Most of the cancers seen in WS patients are mesenchymal cancers (Goto et al., 1996). Patients usually die before the age of 50. Death is mainly attributed to cancer or disorders of the heart.

The majority of affected individuals are of Japanese origin attributed to a founder mutation. This founder mutation is a deletion of exon 26 resulting from a splice site mutation (Moser et al., 1999). However, over 50 different mutations have been reported in WS individuals (Huang et al., 2006). The majority of these lead to a truncation of the protein. Due to the presence of an NLS in the C-terminal of the WRN protein (**Figure 1.9**), it has been suggested that WS is caused by inability of these truncated WRN proteins to reach the nucleus (Goto et al., 1997, Matsumoto et al., 1997, Moser et al., 1999). Werner's syndrome patients also have mutations within the helicase domain (Goto *et al*., 1997, Moser *et al*., 1999). A dominant-negative mutation of WRN, *K1577M*, which abolishes both the helicase and ATPase activities, has been expressed in mice and has been shown to result in a down-regulation of the levels of the WRN protein (Wang *et al*., 2000a). WS individuals are also reported to exhibit increased translocations (Grigorova et al., 2000, Hoehn et al., 1975), increased spontaneous mutations, rearrangements (Salk et al., 1985) and deletions that lead to defects in DNA replication (Fukuchi et al., 1989, Yamamoto et al., 2008).

Studies by Opresko *et al* (2001) and Machwe *et al* (2007) suggest that the helicase activity and the exonuclease activity of WRN act in concert to act on recombination substrates. For example, Machwe *et al* (2007) showed that the exonuclease activity of WRN is able to degrade the leading strand of recombination substrates that resemble replication forks. This activity then promotes fork regression which is carried out by the helicase activity of WRN (Machwe et al., 2006, 2007). Therefore, the data support a role for WRN in restarting stalled replication forks (Saintigny et al.,

2002) (further reviewed in Ouyang *et al* (2008) Singh *et al* (2008)). In agreement with this, cells from Werner's syndrome (WS) patients exhibit a retarded progression through S-phase, resulting in decreased DNA synthesis and a decreased G1 DNA content (Rodriguez-Lopez et al., 2002). Using bromodeoxyuridine (BrdU) labelling, Rodriguez-Lopez *et al* (2002) and Sidorova *et al* (2008) showed that the progression of replication forks is impaired in the absence of WRN, leading to bidirectional forks that display asymmetry. In addition to this, WS cells are hypersensitive to several agents, including clastrogens (which induce replication fork blockage), DNA inter-strand cross linking agents and HU (Bohr et al., 2001, Okada et al., 1998, Pichierri et al., 2001, Poot et al., 2001) indicative of a role for WRN in mitosis. Further support of this mitotic role comes from evidence that shows that Rad52, which is seen at stalled replication forks, stimulates the helicase activity of WRN (Baynton et al., 2003). In addition to this, the *Xenopus* homolog of WRN, FFA-1, has been shown to be essential for replication foci formation (Yan et al., 1998).

WRN has also been implicated in promoting the resolution of Holliday junctions (Constantinou et al., 2000, Machwe et al., 2007, Rodriguez-Lopez et al., 2007, Saintigny et al., 2002). Rodriguez-Lopez *et al* (2007) and Saintigny *et al* (2002) added the bacterial Holliday junction resolvase RusA to WS cells. They noted that this led to an increase in the proliferation of these WS cells and also was able to restore their rates of DNA replication. These data suggest the inability to resolve Holliday junctions in WS leads to their accumulation during homologous recombination. Further support for this role comes from studies that have shown that WRN is able to promote the branch migration of recombination substrates that resemble Holliday junctions in an ATP-dependent manner (Constantinou et al., 2000, Shen and Loeb, 2000a). Using synthetic X-junctions, Yang *et al* (2002) showed that the role of WRN in processing Holliday junctions is regulated by p53, which is seen to interact with the C-terminal of WRN (Blander et al., 1999, Brosh et al., 2001a, Spillare et al., 1999). Further to this, p53-dependent apoptosis is carried out in WS cells (Spillare et al., 1999). However, this can be rescued upon addition of WRN cDNA (Spillare et al., 1999). These data led Oakley and Hickson (2002) to propose that p53 may act to target WRN to replication forks that have stalled due to the presence of lesions. Further evidence of the importance of this interaction comes from studies that have shown that the majority of missense mutations in WS are found in the C-terminus and may therefore disrupt interactions with p53 (Shen and Loeb, 2000b).

The exonuclease and helicase activities of WRN have also been implicated in the maintenance of telomeres. Telomeres, which are present as a 5 to 20 kilo base TTAGGG repeat, serve to protect the ends of chromosomes. Chromosomal DNA ends have a 100 to 200 base pair single-stranded

overhang. This single-stranded overhang can invade the telomeric DNA in a G-rich sequence forming a G-quadruplex structure (discussed in **Section 1.3.1**). The structure formed by this invasion resembles a D-loop, however, due to its association with telomeric DNA, it is referred to as a t-loop (Griffith et al., 1999). These structures are suggested to be acted upon by WRN (Fry and Loeb, 1999). This is consistent with other RecQ helicases that have been shown to act on G-quadruplex DNA structures (Sun et al., 1998, 1999). The exonuclease activity of WRN has also been shown to promote the telomere overhang in t-loops. This then facilitates the dissolution of the t-loop in a helicase-dependent manner (Opresko et al., 2004). In the absence of WRN, an increase in SCE events is seen at telomeres, suggestive of a hyper-recombination phenotype (Laud et al., 2005).

The specific role of WRN at telomeres may be as part of the ALT (Alternative Lengthening of Telomeres) pathway. This pathway is used for telomere maintenance in the absence of telomerase, an enzyme which extends the length of telomeres. Evidence for this potential role of WRN comes from studies that show it co-localises with foci in human cells that maintain their telomeres using the ALT pathway (Johnson et al., 2001, Opresko et al., 2004). These foci are known as ALT-associated PML bodies and are made up of Rad51, Rad52, RPA, TRF1 and TRF2 (Yeager et al., 1999). BLM is also seen to localise to these ALT-associated PML bodies (Yankiwski et al., 2000). In fact, WRN has been found to interact with BLM (von Kobbe et al., 2002). This telomere maintenance role may be a conserved role of the RecQ helicases, as BLM has also been implication in acting at telomeres, seen by telomere-defects in BS cells (Lillard-Wetherell et al., 2004). Like WRN, BLM has been shown to associate with the proteins TRF1, TRF2 and POT1 which act to regulate telomere lengthening (Crabbe et al., 2004, Lillard-Wetherell et al., 2004, Opresko et al., 2005). In addition to this, Du *et al* (2004) saw that the addition of mutations in BLM or WRN in telomerase-negative mice leads to an acceleration of the phenotypes associated with telomere loss. In addition to this, Sgs1 is also seen to act in the resolution of recombination intermediates that are formed in the absence of telomerase (Johnson et al., 2001, Cohen and Sinclair, 2001, Huang et al., 2001, Lee et al., 2007). These structures are proposed to arise from recombination events that occur to extend the length of the telomere in the absence of telomerase. Due to the telomere defects seen in WS cells (Crabbe et al., 2007, Epstein et al., 1965, Tahara et al., 1997), as well as BS cells (Du et al., 2004, Lillard-Wetherell et al., 2004), one can postulate that RecQ helicases may be involved in the destabilisation of Hoogsteen base pairing which facilitate the formation of G-quadruplex structures at telomeres. The role of WRN in the maintenance of telomeres suggests that telomere defects, which lead to cellular senescence, may be the underlying cause of the premature ageing phenotype seen in WS.

1.6.3.3 RECQL4

RECQL4 was originally identified by Kitao *et al* (1998). It maps to chromosome 8q-24.3 in humans and encodes a 133 kDa 1208 amino acid protein (Kitao et al., 1999b). Mutation of RECQL4 can lead to Rothmund-Thomson Syndrome (RTS) (Kitao et al., 1999a, 1999b). Various studies have been carried out to characterise this disorder (Vennos et al., 1992, Vennos and James, 1995, Wang et al., 2001). RTS is an early-onset disease. Individuals who suffer from RTS are seen to suffer from premature aging phenotypes including an early greying or loss of hair and juvenile cataracts. Other symptoms include alopecia, photosensitivity, retarded growth and a predisposition to cancer. The onset of cancer is usually before the age of 25 and the most common cancer associated with RTS is the bone cancer osteosarcoma (Anbari et al., 2000).

Most RTS individuals suffer from mutations in the helicase domain of RECQL4, which lead to the truncation of the protein (Kitao et al., 1999b, Lindor et al., 2000). Most of these mutations are nonsense or frameshift mutations which lead to the destabilisation of the RECQL4 mRNA (Kitao et al., 1999a). RTS cells are characterised by chromosomal instability (Kitao et al., 1999b, Lindor et al., 1996, Orstavik et al., 1994, Vennos and James, 1995) as well as an increase in the rate of chromosomal breaks and rearrangements (Miozzo et al., 1998). Trisomies and aneuploidy are also seen in RTS cells (Der Kaloustian et al., 1990, Mann et al., 2005). This aneuploidy may be the result of premature centromere separation that has been reported in *RECQL4*$^{-/-}$ mice (Mann et al., 2005). Therefore, the data suggest that RECQL4 may have a role in sister chromatid cohesion.

RECQL4 has also been implicated in DNA replication, as RTS cells show sensitivity to ionizing radiation and HU (Jin et al., 2008, Vennos and James, 1995, Werner et al., 2006). These damage-inducing agents lead to a decrease in DNA synthesis and a decrease in the number of cells that enter the S-phase of the cell cycle when compared to cells from normal individuals (Sangrithi et al., 2005, Werner et al., 2006). The specific role of RECQL4 in DNA replication has been suggested to include aiding in its initiation, as the N-terminus of RECQL4 has been shown to recruit DNA polymerase α (Matsuno et al., 2006, Sangrithi et al., 2005). Further support for this comes from observations that RECQL4 also interacts with Cut5, which is essential for the loading of DNA polymerases onto chromatin (Hashimoto and Takisawa, 2003). Its roles in DNA replication may not be limited to initiation, as RECQL4 has also been shown to interact with Rad51, which is indicative of a potential role in DSB repair (Petkovic et al., 2005).

The helicase activity of RECQL4 has come under recent scrutiny. Studies by Macris *et al* (2006) have suggested that RECQL4 does not possess any helicase activity (also reviewed in Ouyang *et al* (2008)). Macris *et al* (2006) showed that RECQL4 could not unwind various substrates including blunt-ended DNA molecules, duplex substrates with 5' or 3' overhangs, synthetic D-loops, four-way junctions, G-quadruplex and forked DNA substrates. These substrates are readily unwound by other RecQ helicases. This finding was surprising as RECQL4 has been shown to possess the characteristic helicase motifs that are conserved amongst all RecQ helicases (Kitao et al., 1998). However, RECQL4 is seen to lack the conserved RQC and HRDC motifs that reside C-terminal to the helicase domain (**Figure 1.9**). These motifs have been shown to be important for the helicase activity of both BLM and *E. coli* RecQ (Wu et al., 2005). However, as RECQL4 does exhibit single-stranded DNA binding, DNA dependent ATPase and single-stranded DNA annealing activities, Macris *et al* (2006) concluded that RECQL4 can still participate in homologous recombination. They suggest that the single-stranded DNA annealing activity of RECQL4 may facilitate the bringing together of DNA strands that have been unwound by other RecQ helicases (Macris et al., 2006). This single-strand annealing activity may also be important in the potential role of RECQL4 in DSB repair (Petkovic et al., 2005) where it may act in the non-homologous end joining pathway (Macris et al., 2006).

Recently, it has been shown that mutations in RECQL4 can lead to two other disorders. The first is RAPADILINO (RAdial hypoplasia, Patella hypoplasia and cleft of Arched palate, DIarrhea and dislocated joints, LIttle size and limb malformation, Nose slender and nOrmal intelligence) syndrome (Siitonen et al., 2003). Most of the mutations in RAPADILINO syndrome are in-frame deletions, which do not affect the structure of the helicase domain (Siitonen et al., 2003). This shows that sequences additional to those within the helicase domain are important for the functions of RECQL4. The second is Baller Gerold syndrome, which is characterised by radial hypoplasia (disorders of the limbs) and craniosyntostosis (problems with normal brain and skull growth) (Van Maldergem et al., 2006). Only two mutations have been reported in Baller Gerold syndrome. The first is a *RECQL4-R1021W* missense mutation in exon 18 located in the C-terminal of *RECQL4*. The second is a deletion of a threonine residue at position 2886 in exon 9 which maps to the helicase domain of *RECQL4* (Van Maldergem et al., 2006).

1.6.3.4 RECQL1 and RECQ5

Of all five human homologs, RECQL1 was found to be the most abundant in resting B cells (Kawabe et al., 2000). At 649 amino acids long, it is the smallest RecQ helicase and has not been linked to any human disorders (Puranam and Blackshear, 1994, Seki et al., 1994). Its helicase activity was

characterised by Cui *et al* (2003) who showed that RECQL1 is capable of unwinding short duplex DNA substrates. Cui *et al* (2004) went onto show that this unwinding activity is enhanced by upto 500 base pairs in the presence of RPA. RECQL1 has been shown to promote the annealing of homologous single-stranded DNA *in vitro* (Sharma et al., 2005). RECQL1 has recently shown to be able to promote the branch migration of three- and four-way structures (Bugreev et al., 2008). This branch migration activity is dependent on the presence of ATP and, unlike other RecQ helicases, is able to carry out this reaction with a 3'-to-5' polarity (Bugreev et al., 2008). This means that RECQL1 can disassemble D-loops that have been formed by the invasion of a 5' single-stranded overhang, as opposed to a 3' single-stranded overhang which is seen in homologous recombination. As the D-loops that are formed by invasion of a 5' single-stranded overhang cannot be extended by the actions of DNA polymerase, they are suggested to represent an unproductive recombination intermediate that could potentially be toxic to the cell (Bugreev et al., 2008). Therefore, Bugreev *et al* (2008) proposed that the role of RECQL1 is to prevent the accumulation of D-loops in order to maintain the fidelity of recombination. Consistent with a role in homologous recombination, Sharma and Brosh (2007) have shown that RECQL1 is able to interact with Rad51. This hypothesised role may also be linked to the Exo1, as RECQ1 has been shown to co-immunoprecipitate with Exo1 and enhances its incision activity (Doherty et al., 2005). In addition to this, they also showed that deletion of *RECQL1*$^{-/-}$ leads to an increase in the frequency of SCEs, as well as sensitivity of the cell to ionizing radiation (Sharma et al., 2005). As discussed below, this increase in SCEs may be related to the ability of RECQL1 to interact with TopoIIIα (Johnson et al., 2000). The helicase activity of RECQL1 has also been shown to be stimulated by the mismatch repair proteins Msh2 and Msh6 (Doherty et al., 2005).

The other human homolog, RECQ5, has not been linked to any human disorders. In both humans and *Drosophila*, RECQ5 exists as different isoforms that are generated by alterative splicing (Sekelsky et al., 1999). Humans possess three isoforms – RECQ5α, RECQ5ß and RECQ5γ. Both RECQ5α and RECQ5γ are found in the cytoplasm, whereas RECQ5ß resembles other RecQ helicases in that it localises to the nucleus and resides within the nucleoplasm (Shimamoto et al., 2000). RECQ5ß has been shown to also participate in the conserved interaction with topoisomerases, as it is seen to interact with both TopoIIIα and TopoIIIß (Shimamoto et al., 2000). Like RECQL1, RECQ5ß also has been shown to promote the annealing of homologous single-stranded DNA *in vitro* (Garcia et al., 2004). It has also been shown to interact with Rad51, suggesting that it also plays a role in homologous recombination like the other members of the RecQ helicase family (Hu et al., 2007). Similarly to other RecQ helicases, this is suggested to reflect an anti-recombination role. This is

because RECQ5 has been shown to inhibit Rad51-dependent D-loop formation (Hu et al., 2007). Hu *et al* (2007) suggested that it carries out this role by displacing Rad51 from single-stranded DNA molecules. They propose that this role, which facilitates the processing of DSBs, decreases the likelihood of gross chromosomal rearrangements (GCRs) from occurring (Hu et al., 2007). This is based on observations that mouse models, in which RECQ5 has been deleted, show an increased frequency of DSBs and a hyper-recombination phenotype as well as an increased susceptibility to cancer (Hu et al., 2007). In addition to this, RECQ5 has also been suggested to aid in the suppression of SCEs, as the rate of SCEs is increased in RECQ5$^{-/-}$/BLM$^{-/-}$ cells when compared to BLM$^{-/-}$ cells (Hu et al., 2005, Otsuki et al., 2008, Wang et al., 2003b). Therefore, RECQ5 is also proposed to aid in maintaining the fidelity of recombination.

Both RECQL1 and RECQ5 have been shown to be required for cell growth in the absence of BLM (Otsuki et al., 2008, Wang et al., 2003b). This is based on observations by both Otsuki *et al* (2008) and Wang *et al* (2003b) who saw that both *RECQ1*$^{-/-}$*/BLM*$^{-/-}$ and *RECQ5*$^{-/-}$*/BLM*$^{-/-}$ chicken DT40 cells grew more slowly than BLM$^{-/-}$ cells. Taken together with the observation that deletion of *recq1* leads to an increased frequency of SCEs (Sharma et al., 2005) and that both interact with TopoIIIα (Johnson et al., 2000, Shimamoto et al., 2000), Otsuki *et al* (2008) propose that both RECQL1 and RECQ5 interact with TopoIIIα to partially substitute for the functions of BLM in its absence. This suggests that the RecQ helicase family in humans have overlapping functions to maintain the fidelity of recombination (Singh et al., 2008).

1.6.4 Other RecQ helicases

The *S. pombe* RecQ helicase homolog is known as *rqh1+*. When exposed to HU, mutation in *rqh1* leads to a loss of cell viability (Murray et al., 1997, Stewart et al., 1997). In addition to this, these *rqh1* mutants suffer from an increase in recombination and an increase in chromosomal loss (Stewart et al., 1997). This has been attributed to an inability to resolve lesions or aberrant structures that are present on replication forks (Stewart et al., 1997) (**Figure 1.1**). These mutants are also seen to suffer from a 'cut' (cell untimely torn) phenotype (Stewart et al., 1997) which involves the cleavage of a nucleus that has not undergone division (reviewed by Yanagida (1998)).

Killoran and Keck (2008) also investigated the HRDC domain of the RecQ helicase homolog from the bacterium *Deinococcus radiodurans*. The RecQ helicase from this species, termed DrRecQ, possesses three HRDC domains which are suggested to aid in the orientation of DrRecQ on DNA binding (Killoran and Keck, 2008). In their analysis of the C-terminal most HRDC domain, Killoran and Keck (2008) saw that this HRDC domain contains a highly acidic patch that is responsible for the binding of

DNA. Mutation of an aspartic acid residue at position 816 of this HRDC domain leads to defects in the ability of DrRecQ to bind a variety of DNA substrates (Killoran and Keck, 2008). Another species of bacteria whose RecQ homolog contains three HRDC domains is the obligate human bacterial pathogen *Neisseria gonorrhoeae* (also known as *Gonococci*). Killoran *et al* (2009) showed that deletion of the two C-terminal most HRDC domains decreases the affinity of GcRecQ for single-stranded DNA.

Recently, a zebrafish RecQ helicase homolog has been identified that is said to resemble the human homologs BLM and RECQL5 in its ability to suppress mitotic recombination (Xie et al., 2007). This provides more evidence that the RecQ helicase family carry out anti-recombination functions within the cell.

1.7 The role of the RecQ helicases during mitotic growth

1.7.1 RecQ helicases and the intra-S checkpoint

SGS1, along with the human homologs BLM and WRN, play a role in maintaining the stability of the genome (Khakhar et al., 2003, Myung et al., 2001b, Watt et al., 1996) as well as in chromosome segregation (Watt et al., 1995). The expression of Sgs1, and the human homolog BLM, peaks during the S-phase of the cell cycle (Cho et al., 1998, Frei and Gasser, 2000) and is hardly detectable during the M- or G1-phase of the cell cycle (Frei and Gasser, 2000). Its expression has been shown to be controlled via two SCB (Swi4-Swi6 cell cycle box) elements that are usually found in the promoter regions of genes that are involved in the progression of late G1 or S phase of the cell cycle (Cho et al., 1998). *SGS1* has been shown to localise to the nucleus (Frei and Gasser, 2000) and is involved in the activation of the intra S-phase checkpoint in the presence of DNA damaging agents. Sgs1 has been shown to co-localise with Rad53 in S-phase specific foci in response to hydroxyurea treatment (Frei and Gasser, 2000). In the absence of *sgs1*, phosphorylation of Rad53 is decreased (Frei and Gasser, 2000). These data suggest that Sgs1 acts upstream of the effector kinase Rad53 (Cartagena-Lirola et al., 2008). Sgs1 has been shown to act alongside DNA polε and Mec1 (Cobb et al., 2003) to signal cell cycle arrest in response to DNA damage, therefore acting as part of the intra-S phase checkpoint response to DNA damage. This and other cell cycle checkpoints that act in *S. cerevisiae* are depicted in **Figure 1.10**.

As reviewed by Oakley and Hickson (2002) and also shown in **Figure 1.10**, DNA damage is sensed by various checkpoint proteins, including genes belonging to the *RAD9* or *RAD24* epistasis group that act during damage in G1 or G2 (de la Torre-Ruiz et al., 1998). As reviewed in Kolodner *et al* (2002), two branches make up the genes involved in the S-phase response. One branch consists mainly of *RAD9*, *RAD17* and *RAD24* (Paulovich et al., 1997), whereas the other branch mainly consists of *SGS1* (Frei and Gasser, 2000), *TOF1* (Foss, 2001), *TOP3* (Chakraverty et al., 2001) and *SRS2* (Liberi et al., 2000). Studies that further confirmed this S-phase response were carried out by Myung and Kolodner (2001c, 2002) who, using MMS sensitivity assays, showed that inactivating only one of the branches of this checkpoint pathway has little effect on the stability of the genome. However, they showed that impairment of both pathways led to a synergistic increase in the rate of genome rearrangements (Myung et al., 2001c, Myung and Kolodner, 2002). These results suggested that both pathways act in a redundant manner in the maintenance of genome stability. In addition to this, Myung and Kolodner (2001c, 2002) also showed that inactivating both of these branches of the intra-S checkpoint, along with the inactivation of the downstream kinase Mec1 (as shown in **Figure 1.10**) leads to a 12000-fold increase in genome rearrangements. Another group of genes, including

Rfc4-5 (Sugimoto et al., 1996, Sugimoto et al., 1997), Pol2 (Araki et al., 1992, Navas et al., 1995, Navas et al., 1996) and Dpb11 (Wang and Elledge, 1999), make up the S/M checkpoint, which is involved in the response to the depletion of dNTPs (i.e. by hydroxyurea (HU)) and functions by stalling mitosis until DNA synthesis is completed (Lydall and Weinert, 1996).

The above mentioned sensor checkpoint proteins lead to the activation of Rad53 and phosphorylation of Rad9 in a Mec1-dependent manner (Sanchez et al., 1996, Sun et al., 1996). *MEC1* and *RAD53* have been proposed to prevent aberrant origin firing, as late origin firing is seen in mutations of these genes (Neecke et al., 1999, Tercero and Diffley, 2001). Both also have a role in the stabilisation of the replication fork in response to MMS, as mutants that are exposed to MMS result in irreversible replication fork collapse, whereas in a wild-type cell, normal replication is restored after exposure to MMS (Tercero and Diffley, 2001). Defects in the human homolog of Mec1, ATM, have been shown to lead to the disorder ataxia telangiectasia, which is associated with increased chromosomal instability as well as a predisposition to cancer (Kastan and Lim, 2000, Rotman and Shiloh, 1999).

Further evidence for a role of the RecQ helicases in the intra-S checkpoint comes from various studies that show that their deletion leads to the hyper-sensitivity of cells to various inhibitors of DNA replication. For example, deletion of *sgs1* leads to sensitivity to MMS (Frei and Gasser, 2000, Miyajima et al., 2000a, Miyajima et al., 2000b, Mullen et al., 2000, Onoda et al., 2000, Saffi et al., 2000) which induces lesions in the DNA leading to the stalling of replication forks. *sgs1* cells are also sensitive to the ribonucleotide reductase inhibitor HU (Miyajima et al., 2000b, Onoda et al., 2000, Yamagata et al., 1998) which affects DNA replication by depleting the pool of dNTPs. Similar sensitivities to HU and MMS are seen for the human homologs BLM (Wang et al., 2000b) and WRN (Pichierri et al., 2001, Sidorova et al., 2008) as well as the *S. pombe* RecQ homolog *rqh1+* (Murray et al., 1997, Stewart et al., 1997).

Cell cultures from Werner's syndrome (WS) patients exhibit an impaired rate of S-phase progression, along with a decrease in DNA synthesis activity and a decrease in G1 content (Poot et al., 1992, Rodriguez-Lopez et al., 2002). Support for a role of WRN at replication forks comes from studies that show WRN is required for activation of the ATM kinase and also the phosphorylation of the downstream substrates of ATM in response to inter-strand crosslink induced DSBs (Cheng et al., 2008). Cell cultures from Blooms Syndrome (BS) patients also have a decreased rate of DNA synthesis (Hand and German, 1975) as well as an abnormal profile of DNA replication intermediates

(Lonn et al., 1990), which indicate defects during S-phase. In addition to this, BLM has been shown to relocalise to Rad51 foci, which mark the sites of repair, when cells are exposed to DNA damaging agents in late G2/S (Bischof et al., 2001, Wu et al., 2001). This movement of BLM is said to be dependent on the ATM and ATR kinases (Beamish et al., 2002, Davalos et al., 2004). As mentioned in **Section 1.6.3.1**, these foci also contain other DNA repair proteins, such as Mus81 (Zhang et al., 2005a), Rad51D (Braybrooke et al., 2003), RPA (Bischof et al., 2001), BRCA1 (Wang et al., 2000b) and the MMR proteins Mlh1 (Pedrazzi et al., 2001) and Msh6 (Pedrazzi et al., 2003) and form a complex collectively known as BASC (BRCA1-associated genome surveillance complex) (Wang et al., 2000b).

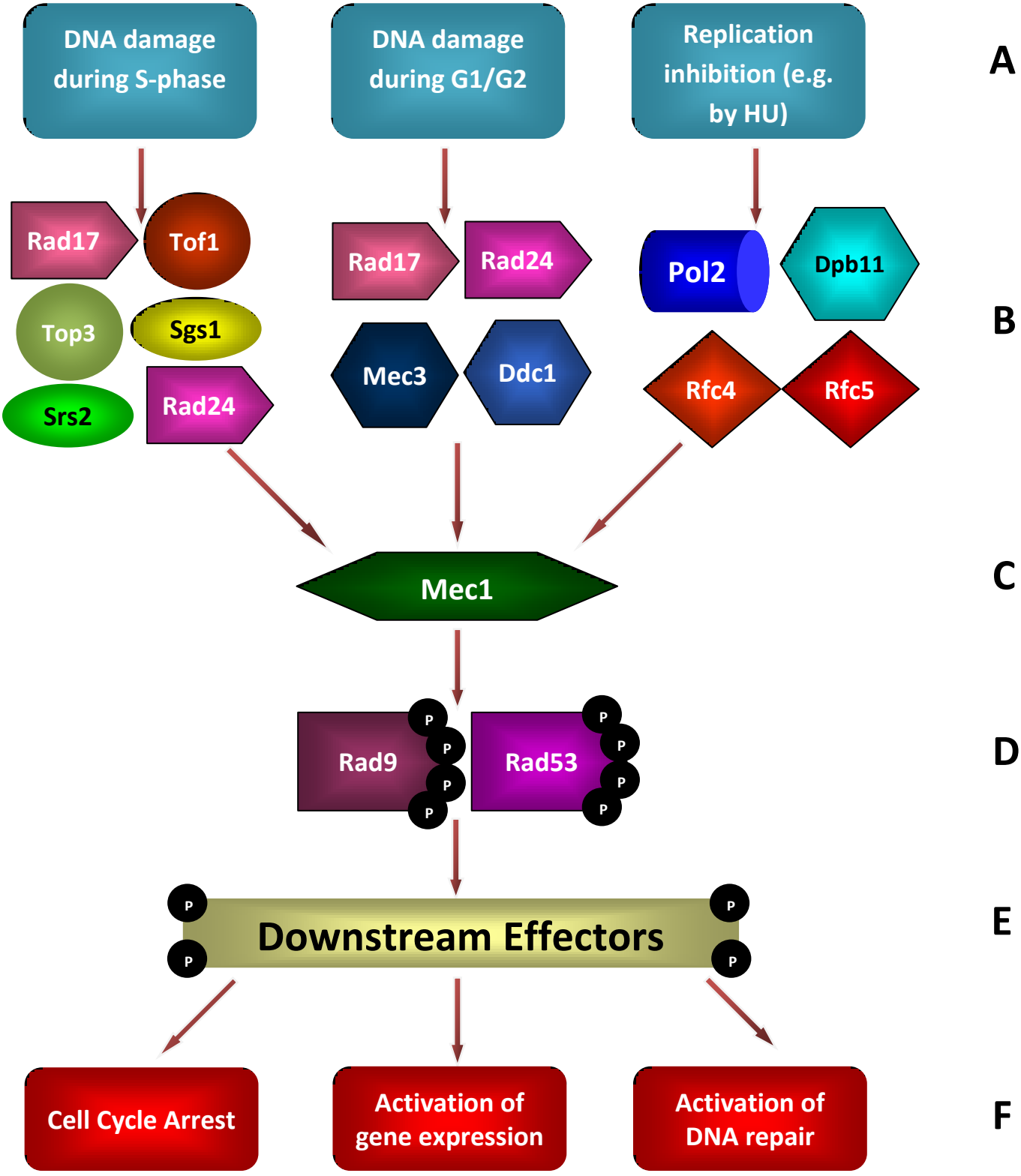


Figure 1.10: Cell cycle checkpoint cascade in *S. cerevisiae*
Modified from Oakley and Hickson (2002)
DNA damage (**A**) is sensed and processed by various 'sensor' checkpoint proteins (**B**) which initiate a signal transduction pathway (**C-F**). Rad9 phosphorylation and Rad53 activation (in a Mec1-dependent manner) (**C-D**) leads to the phosphorylation of downstream effectors (**E**) which leads to the processing of damage (**F**).

1.7.2 RecQ helicases and mitotic hyper-recombination

When Sgs1 is deleted, cells have been shown to suffer from a mitotic hyper-recombination phenotype (Gangloff et al., 1994, Miyajima et al., 2000b, Mullen et al., 2000, Watt et al., 1996). Watt *et al* (1996) specifically saw increases in inter-chromosomal homologous recombination that is partially dependent on Rad52, increases in intrachromosomal excision recombination, increases in the frequency of ectopic recombination and an increase in subtelomeric Y' instability. In addition to these defects, Yamagata *et al* (1998) also saw that *sgs1* cells displayed an increase in illegitimate recombination and this defect was suppressed by the addition of the Sgs1 human homologs BLM or WRN. Onoda *et al* (2000) reported that *sgs1* cells display an increase in unequal sister chromatid exchange (USCE) events that was later shown to be dependent on the activity of *MSH2* (Onoda et al., 2004), as defects in *msh2* significantly decreases the elevated USCE frequency seen in *sgs1* cells.

BS cells also display an elevated level of SCE events (Chaganti et al., 1974) indicative of a mitotic hyper-recombination phenotype, and this feature is actually used in the diagnosis of the syndrome. Analysis of metaphase spreads shows that BS individuals exhibit a 10-fold increase SCE events when compared to normal individuals (Chaganti et al., 1974). *BLM*$^{-/-}$ mice have also been shown to display increased rates of mitotic recombination (Luo et al., 2000) and increased chromosomal instability (Chester et al., 2006). Prince *et al* (2001) showed that WRN plays a role in mitotic recombination. As the initiation of recombination was similar in control cell lines and WS cell lines, Prince *et al* (2001) suggested that these WS cell lines were unable to resolve recombination intermediates. Further support for a role of WRN in mitotic recombination came from studies by Yamamoto *et al* (2008). They used transgenic mice that overexpressed a point mutation, K577M, that was identified in humans by Wang *et al* (2000a) and leads to the ablation of the helicase activity of WRN. They assessed the frequency of deletions caused by intrachromosomal homologous recombination events across a 70 kilo base duplication on chromosome 7 in mice. These deletions caused a colour change in the retinal pigment epithelium of mice. They saw that WRN mice had a significantly greater number of deletions when compared to control mice, which they suggested might be due to the inability to process abnormal structures in the absence of WRN (Yamamoto et al., 2008).

Sinclair *et al* (1997) showed that *sgs1* cells have an accumulation of extrachromosomal rDNA circles (ERCs) that are seen earlier than normal. This correlates with an increase in the instability of repetitive DNA sequences. This instability was most notable at the tandemly duplicated rDNA locus, and these observations led Sinclair *et al* (1997) to suggest that Sgs1 may have a role in the suppression of ageing in yeast, much like its human homolog WRN. This suggestion was made based

on the observations that ageing in *S. cerevisiae* is associated with the accumulation of ERCs. The presence of these ERCs may 'titrate out' necessary replication factors that carry out replication and/or transcription, and this depletion which would ultimately lead to senescence (Sinclair and Guarente, 1997). Support for this suggestion comes from the observations that *sgs1* cells exhibit a fragmented nucleolus, as other mutations in yeast that lead to a premature aging phenotype also show this phenotype (Frei and Gasser, 2000, Sinclair and Guarente, 1997). Taking these studies into account, it is reasonable to suggest that one of the many roles of Sgs1 might be to suppress the formation of ERCs and the resulting instability caused by deletion of *sgs1* leads to a decrease in the lifespan of yeast (Sinclair et al., 1997).

1.7.3 RecQ helicases and strand resection

Recent work has implicated Sgs1 and BLM in the resection of mitotic DSBs (Gravel et al., 2008, Mimitou and Symington, 2008, Zhu et al., 2008). In mitosis, homologous recombination is used to repair breaks in DNA that have been caused by damage, such as ionizing radiation or even by chemotherapeutic agents. This differs from homologous recombination in meiosis (**Figure 1.2**) where the strand breaks are generated enzymatically at specific sites along the DNA (Keeney et al., 1997). Mitotic homologous recombination is described as a 'high fidelity' mechanism (Singh et al., 2008) as repair is carried out mainly using the sister chromatid (Johnson and Jasin, 2000, Liang et al., 1998). If these DSBs are not repaired, they broken ends will become unprotected, rendering them unstable. This may result in their degradation and could lead to the deletion of genes that are close to the break (Ouyang et al., 2008). In mitosis, the generation of single stranded DNA by strand resection causes the activation of a Mec1-dependent DNA damage checkpoint, which will then lead to the activation of Rad53 (Ira et al., 2004, Zou and Elledge, 2003) (**Figure 1.10**). It has been previously shown that ExoI is involved in the resection of DSBs in *S. cerevisiae* (Cotta-Ramusino et al., 2005). Gravel *et al* (2008) showed that Sgs1 comprises a distinct mechanism for the resection of DSBs. They showed that mating type switching, which is initiated by a DSB created by the HO endonuclease, was delayed to in an *exoI sgs1* double mutants but not in the single mutants (Gravel et al., 2008). It was also shown that the rate of degradation was markedly decreased in the *exoI sgs1* double mutant (Gravel et al., 2008) and that this double mutation leads to the accumulation of partially resected intermediates (Mimitou and Symington, 2008). It was also shown that in the *exoI sgs1* double mutant Rad53 is not phosphorylated. It is also not phosphorylated in a helicase-defective mutation of *sgs1* or a nuclease-defective mutation of *exoI* (Gravel et al., 2008). However, it was shown that Rad53 did become phosphorylated by the addition of ExoI or Sgs1 to the *exoI sgs1*

double mutant (Gravel et al., 2008). Using siRNA, Gravel *et al* (2008) showed that BLM is also involved in strand resection in a similar ExoI-independent fashion to Sgs1 (Gravel et al., 2008).

The MRX (Mre11-Rad50-Xrs2) complex has also been implicated in strand resection (Lee et al., 1998, Lisby and Rothstein, 2004), and was shown to act independently to ExoI and Sgs1, as *exoI mre11* double mutations cause a resection defect that is more severe than either single mutant (Moreau et al., 2001, Nakada et al., 2004) and the triple *exoI mre11 sgs1* mutant was shown to be lethal (Gravel et al., 2008). Sae2, which is also implicated in strand resection, has been shown to interact with the MRX complex and its deletion leads to a phenotype that is similar to deletion of the components of the MRX complex (Clerici et al., 2005). Clerici *et al* (2006) observed a marked decrease in 5'-to-3' resection in a *exoI sae2* double mutation, but were still able to detect some levels of resection and homologous recombination. To further elucidate the roles of ExoI, Sae2 and Sgs1, Mimitou and Symington (2008) and Zhu *et al* (2008) used an assay where they probed the region adjacent to the HO restriction site to measure the rates of strand resection. They were able to monitor product formation using Southern blots and saw a delay caused by an *exoI* mutation when compared to a *sae2* mutation (Mimitou and Symington, 2008, Zhu et al., 2008). This observation led them to suggest that Sae2, as well as the MRX complex, is involved in the initial processing of the DSB. This processing leads to the production of an intermediate that is then subsequently acted on by ExoI. In the absence of ExoI, they suggest that Sgs1 is required, as the detectable products that accumulate in an *exoI* mutation are eliminated in an *exoI sgs1* double mutant (Mimitou and Symington, 2008, Zhu et al., 2008).

The interacting partners of Sgs1, Top3 and Rmi1 (discussed further in **Section 1.7.5**) are also involved in strand resection (Zhu et al., 2008). Zhu *et al* (2008) showed that *sgs1*, *top3* and *rmi1* mutations were defective in the processing of resection, but resembled the wild-type in terms of their ability to initiate resection. Also, using ChIP analysis, it was shown that Sgs1, along with the gene Dna2 which possesses both helicase and nuclease activity, are both recruited to DSB ends, but then move away from the breaks (Zhu et al., 2008). This is in contrast to the MRX complex whose recruitment was shown to be limited to the sequences that are immediately adjacent to the break (Shroff et al., 2004). It was also shown that DSBs are still processed in the absence of both *sae2* and *mre11*, suggesting that the Sgs1-Top3-Rmi1 complex and ExoI or Dna2 can still act on these DSB ends, but at a much lower efficiency (Mimitou and Symington, 2008, Zhu et al., 2008). These data support the model proposed by Mimitou and Symington (2008) that Sae2 and the MRX complex are involved in the initial processing of the strand, whereas ExoI and the Sgs1-Top3-Rmi1 complex are

independently involved at a later stage to process an intermediate created by Sae2 and the MRX complex (Zhu et al., 2008) (**Figure 1.11**). After this strand resection, the DSB can be repaired by homologous recombination (**Figure 1.12**). An alternative method for the repair of DSBs is the Break Induced Repair (BIR) pathway. The BIR pathway is an example of a 'high-fidelity' mechanism of repair, much like the repair of DSBs by homologous recombination shown in **Figure 1.11**, as it preferably uses the sister chromatid strand for repair of the DSB. The BIR pathway is depicted in **Figure 1.13**.

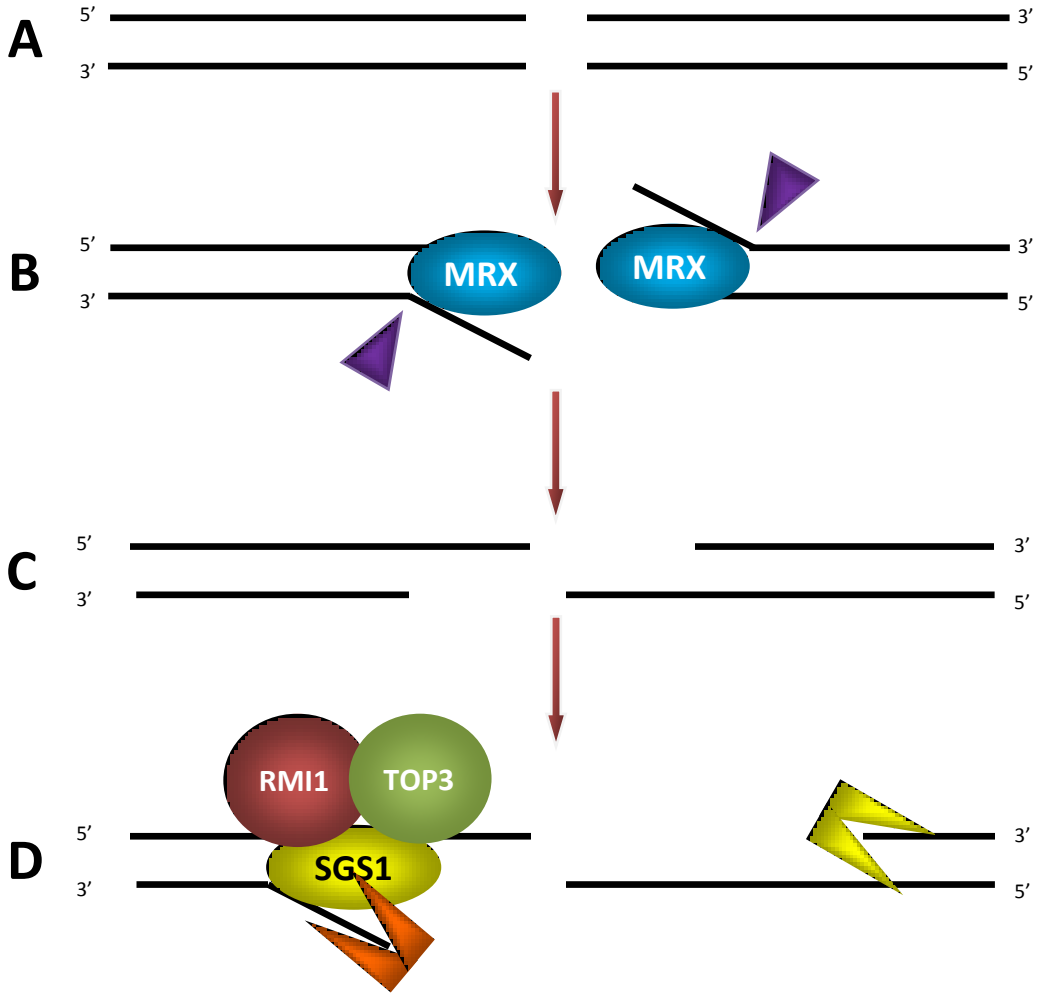
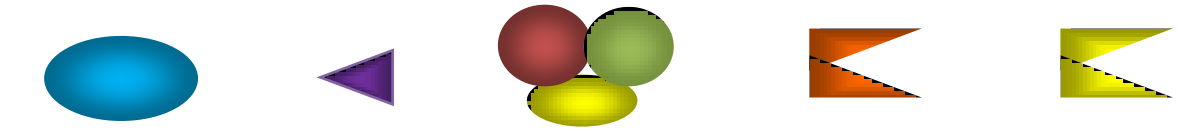


Figure 1.11: Model to show the processing of mitotic DSBs

Modified from Mimitou and Symington (2008) and Zhu *et al* (2008)

After a DSB is formed (**A**), Sae2 and the MRX complex initiate resection (**B**) leading to 3' single stranded overhangs (**C**). These 3' single stranded overhangs are acted on by ExoI (*right*) or Dna2 and the Sgs1-Top3-Rmi1 complex (*left*) (**D**).



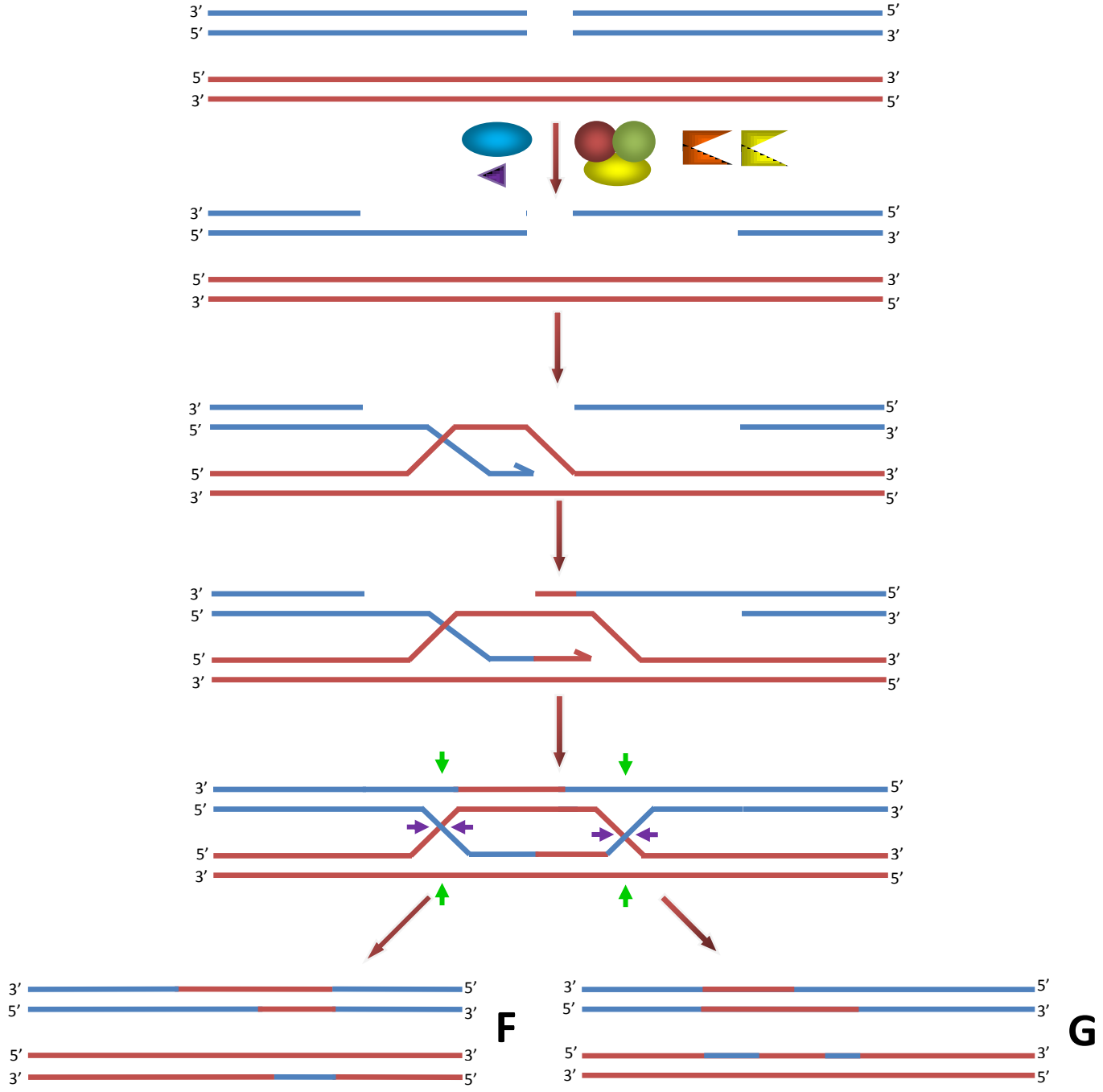
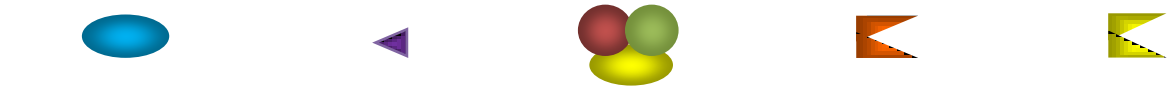


Figure 1.12: Model showing the repair of DSBs by homologous recombination

Modified from Paques and Haber (1999)

After the formation of the DSB (**A**), the MRX complex, Sae2, Exo1, Dna2 and the Sgs1-Top3-Rmi1 complex (as shown in **Figure 1.11**) resect the 5′ ends leading to the formation of 3′ single stranded overhangs (**B**). The 3′ single stranded overhangs invade the homologous sister chromatid strand (**C**) which primes leading strand synthesis, D-loop displacement and strand capture (**D**), leading to the formation of a double Holliday junction (**E**). The resolution of the dHJ can give rise to a crossover product (where the dHJ is resolved by cutting the crossed strands – *purple arrows*) (**F**) or a non-crossover product (where the dHJ is resolved by cutting the uncrossed strands – *green arrows*) (**G**). This model is based on the DSBR model as originally proposed by Szostak *et al* (1983).



The MRX Complex | Sae2 | Sgs1-Top3-Rmi1 | Dna2 | Exo1

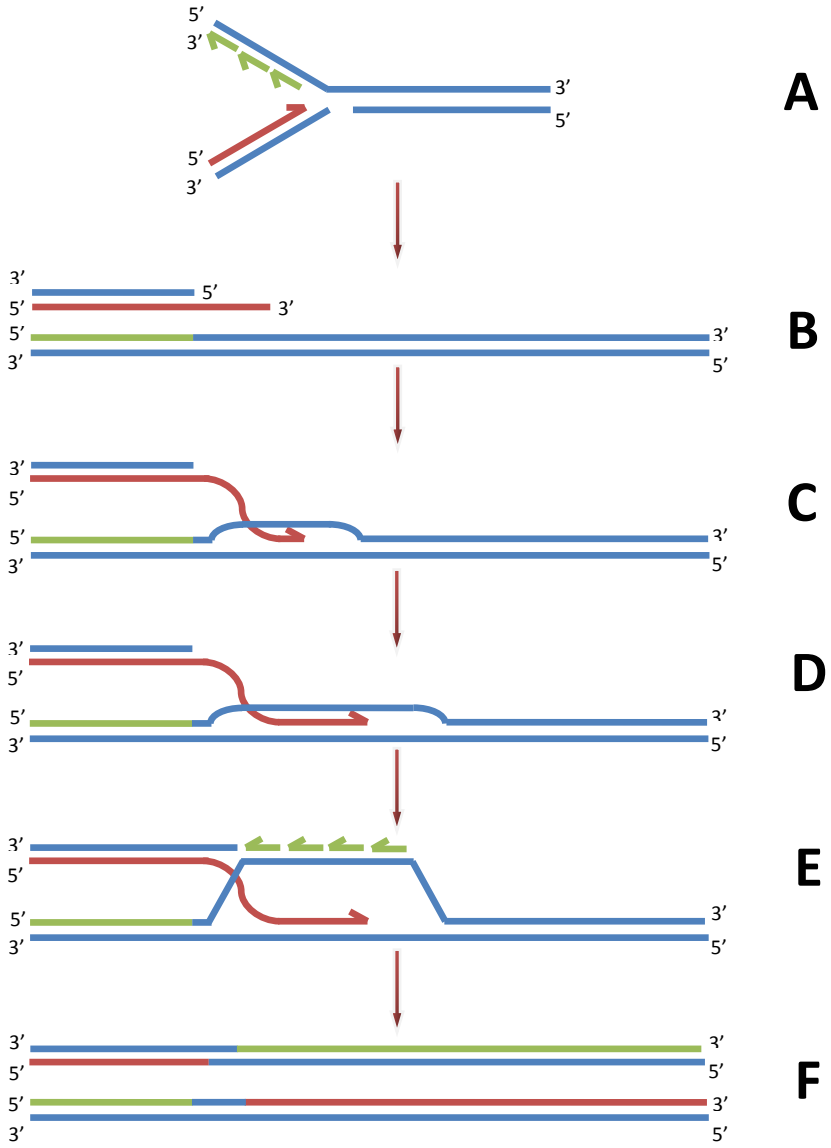


Figure 1.13: The Break Induced Repair Pathway

Modified from Oakley and Hickson (2002)

This pathway is used to repair DSBs that arise from either ionizing radiation or by the collapse of the replication fork. The collapse of a replication fork that has occurred by the presence of a single stranded gap ahead of the fork (**A**). Fork collapse leads to the formation of a DSB, which is resected by the action of nucleases, leading to the formation of a 3′ single stranded overhang (**B**). This 3′ single stranded overhang invades the homologous strand, which, in most cases for this type of repair, will be the sister chromatid. Invasion by the 3′ single stranded overhang leads to the formation of a single end invasion (**C**), which primes leading strand synthesis and D-loop displacement (**D**). Following this, lagging strand synthesis is initiated while the synthesis of the leading strand continues (**E**). This allows for DNA replication to continue throughout the entire length of the molecule (**F**).

1.7.4 Sgs1 acts together with Top3 to restart stalled replication forks

Sgs1 is involved in the processing of DSBs and in restarting replication forks that have stalled due to the presence of damage (**Figure 1.1**). Replication forks may stall due to damage on either the leading or lagging strand. One way in which Sgs1 can restart replication forks that have stalled due to damage on the leading strand is by facilitating its regression to form a structure known as the 'chicken foot' (McGlynn and Lloyd, 2000) (**Figure 1.14**). This structure leads to the switching of the template so that the replication fork can be re-established by reverse branch migration. This means that the lagging strand can be used as the template and the leading strand can be extended so that the lesion is bypassed and the fork does not need to be broken. The lesion is processed at a later stage during homologous recombination. In *E. coli*, the formation of this 'chicken foot' structure requires several proteins, including the RecG helicase (McGlynn and Lloyd, 2000), RuvAB (Seigneur et al., 1998) and RecA (Seigneur et al., 2000). The human RecQ helicases BLM and WRN have also been shown to aid in the regression of stalled replication forks, leading to the formation of a 'chicken foot' structure (Machwe et al., 2006, Ralf et al., 2006). The structure of the 'chicken foot' was physically confirmed by scanning force microscopy and by restriction enzymes that were able to cut the 'middle toe' (Postow et al., 2001a, Postow et al., 2001b).

Another way in which Sgs1 can restart replication forks that have stalled due to damage on the leading strand is through its association with Top3. Top3 is a topoisomerase that is involved in the decatenation of inter-linked DNA molecules (as discussed in **Section 1.5**). Sgs1 is proposed to act on Holliday junction-type structures, forming an intermediate that is acted upon by Top3 (**Figure 1.15**). *sgs1Δ* or *top3Δ* cells are seen to accumulate Rad51-dependent X-shaped molecules, referred to as rec-X molecules, in response to MMS (Liberi et al., 2005). These structures are said to be either Holliday junctions or ternary and quaternary joint molecules in *sgs1Δ* cells (Oh et al., 2007) and hemicatenates in *top3Δ* cells (Liberi et al., 2005). These structures are also present in wild-type cells, but are eventually resolved (Liberi et al., 2005). Sgs1 and Top3 also act together to restart replication forks stalled due to the presence of damage on the lagging strand (**Figure 1.16**). This is achieved by a 'dissolution' activity that dissolves Holliday junction-type structures via branch migration to form non-crossover products (Ira et al., 2003, Karow et al., 2000a, Mankouri and Hickson, 2006, Seki et al., 2006). This branch migration activity is also seen for the human homologs BLM (Karow et al., 2000a) and WRN (Constantinou et al., 2000).

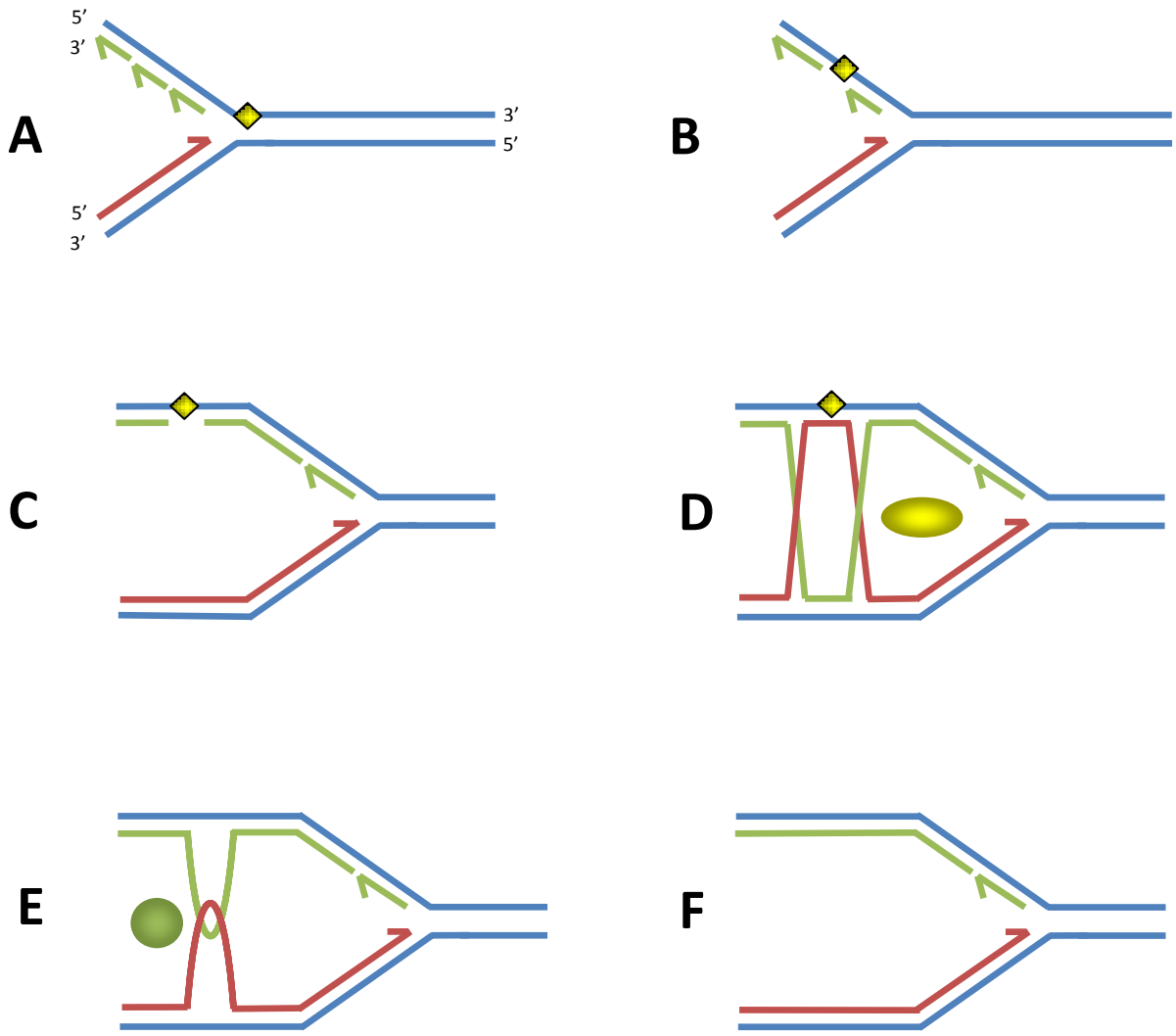


Figure 1.14: Sgs1 and Top3 act when lesions are on the lagging strand.
Modified from Oakley and Hickson (2002)
Due to the discontinuous nature of lagging strand synthesis, the presence of a lesion (**A**) does not block replication (**B**). Bypass of the lesion is achieved by the formation of a single-stranded gap (**C**) which is repaired by homologous recombination. This repair is achieved by the formation of a double Holliday junction so that the leading strand can be used as a template (**D**). Sgs1 is involved in the branch migration of the double Holliday junction, which leads to the formation of a hemicatenate (**E**). This hemicatenate is dissolved by Top3, which restores the replication fork (**F**).

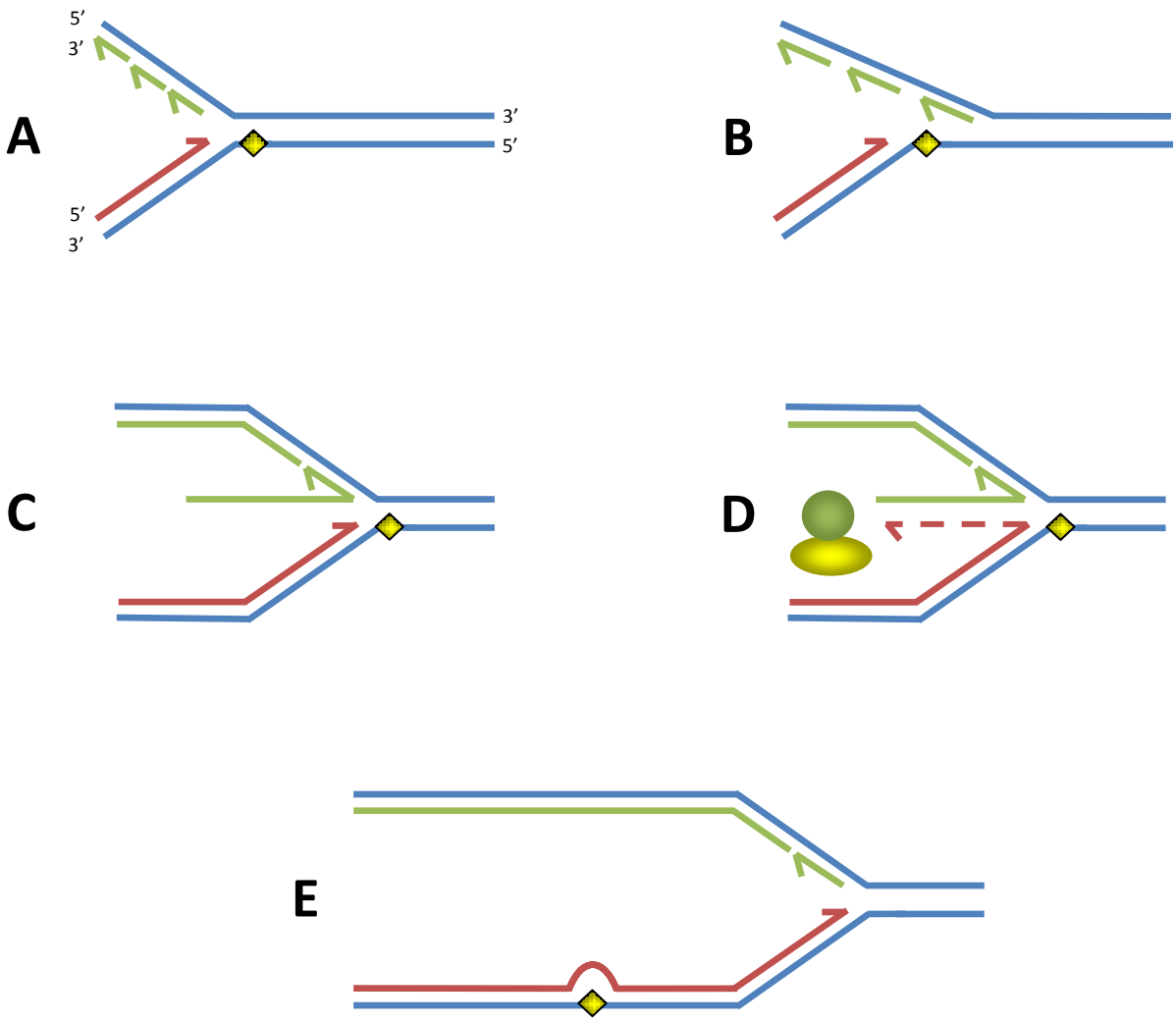


Figure 1.15: The 'chicken foot' structure allows restoration of the replication fork.
Modified from Oakley and Hickson (2002)
The presence of a lesion on the leading strand (**A**) leads to the stalling of replication on the leading strand while replication continues on the lagging strand (**B**). This can lead to regression of the replication fork (**C**) where the leading strand uses the nascent lagging strand as a template (**D**). The regressed fork is unwound by Sgs1 and Top3, which bypasses the lesion and restores the replication fork (**E**).

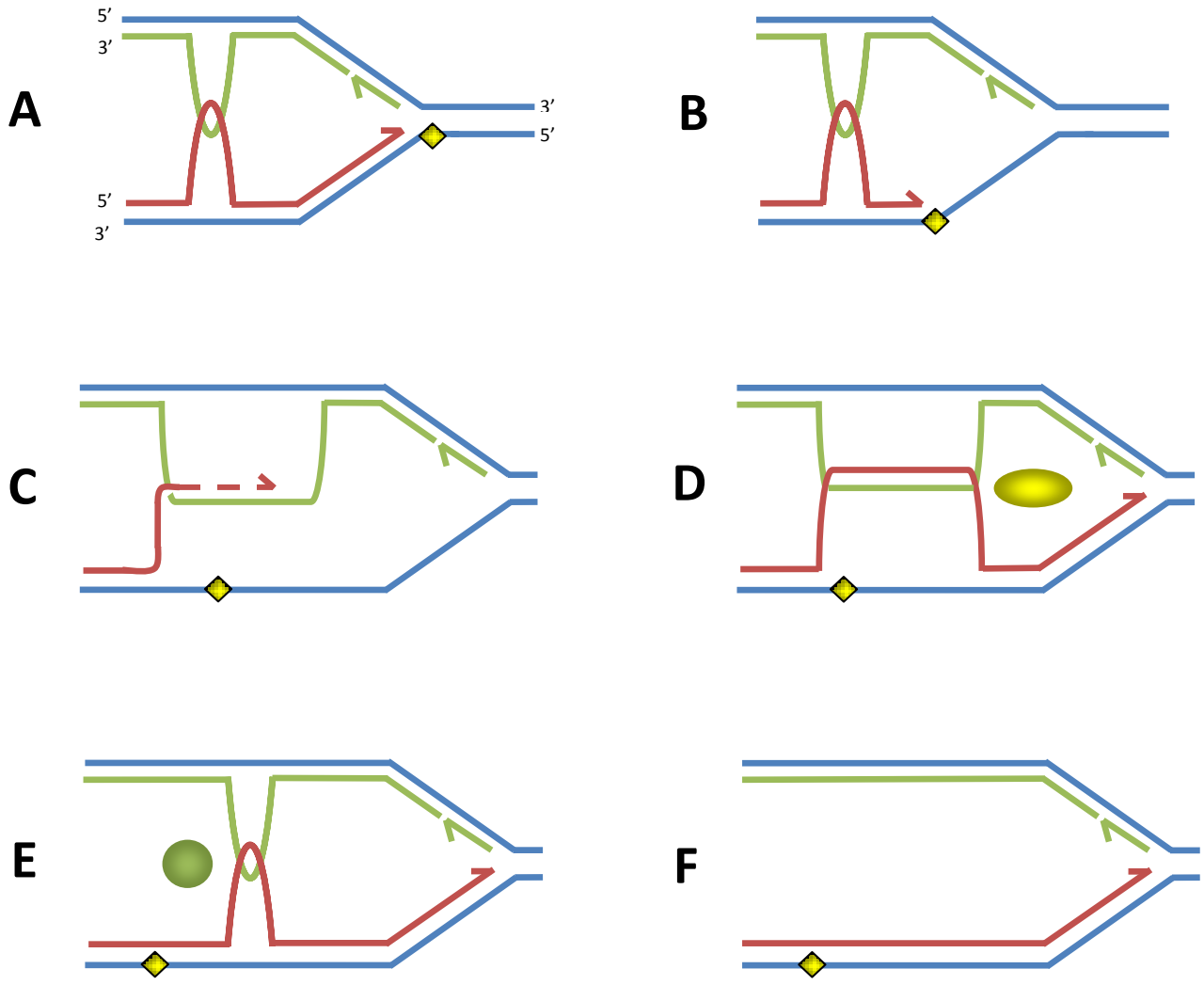


Figure 1.16: Sgs1 and Top3 act to decatenate when lesions are on the leading strand.
Modified from Oakley and Hickson (2002)
Despite the presence of a lesion on the leading strand, hemicatenates are still able to form behind the replication fork (Lopes et al., 2003) (**A**). However, the presence of a lesion on the leading strand leads to the stalling of replication on the leading strand while replication continues on the lagging strand (**B**). The hemicatenates, referred to as sister chromatid junctions (Lopes et al., 2003), are precursors for Rad51-dependent strand invasion that facilitate template switching (**C**). The continuation of replication from the lagging strand leads to the formation of a rec-X structure (**D**). Sgs1 can act on this Holliday junction-type structure to form a hemicatenate (**E**). This hemicatenate is dissolved by Top3, which bypasses the lesion and restores the replication fork (**F**).

1.7.5 Rmi1 comprises a third component to the Sgs1-Top3 complex

The interaction between Sgs1 and Top3 is evolutionarily conserved. The *E. coli* topoisomerase III protein interacts with RecQ and has been shown to be able to decatenate two covalently-closed double-stranded DNA molecules (Harmon *et al*., 1999). This activity of *E. coli* Top3 can be substituted by the activity of *S. cerevisiae* Top3 (Harmon *et al*., 1999). In addition to this, the defects caused by deletion of *top3* in *S. pombe* cells are suppressed by mutating the RecQ helicase *rqh1* (Goodwin *et al*., 1999), which is suggestive of their interaction in *S. pombe*. The observed decatenation function may explain why deletion of these proteins leads to defects in chromosome segregation (Goodwin *et al*., 1999, Murray *et al*., 1997, Watt *et al*., 1995). The observation that mutation in RecQ helicases can suppress defects in type IA topoisomerases extends to *A. thaliana*. Hartung *et al* (2007) observed that the phenotypes displayed by a mutation in Top3 (*top3α-1*), which includes severe developmental defects and the inability to germinate, can be partially suppressed by the *recq4A-4* mutation. In addition to this, Hartung *et al* (2008) demonstrated that the *A. thaliana* homologues RECQ4A and TOP3α also act together to suppress crossover formation during homologous recombination.

BLM and TopoIIIα also interact (Johnson *et al*., 2000, Wu *et al*., 2000). Wu *et al* (2000) determined, by co-immunoprecipitation studies, that TopoIIIα is able to interact with both the N-terminus and C-terminus of BLM. Their interaction is essential in the recruitment of TopoIIIα to PML bodies (Johnson *et al*., 2000, Wu *et al*., 2000), as TopoIIIα does not localise to PML bodies in BS cells (Bischof *et al*., 2001, Wu *et al*., 2001). BLM and TopoIIIα have also been shown to act together to dissolve double Holliday junctions (Hu *et al*., 2001, Plank *et al*., 2006, Wu and Hickson, 2003). This activity is not possible when either protein acts alone (Wu and Hickson, 2003). Also, the mRNA of BLM and TopoIIIα, as well as TopoIIIß, is expressed at high levels in the testis of mice (Seki *et al*., 1998a, Seki *et al*., 1998b, Seki *et al*., 1998c) indicative of a role for all three proteins during meiosis. TopoIIIα and TopoIIIß have also been shown to interact with an alternative splice variant of the RecQ helicase RecQ5 (Shimamoto *et al*., 2000) which is further evidence that various combinations of RecQ helicases and topoisomerases can exist in human cells (Oakley and Hickson, 2002). However, the purpose of these interactions are suggested to differ, as studies by Wu *et al* (2005) showed that neither WRN, RECQ1 nor RECQ5ß could substitute BLM to carry out the dissolution of Holliday junction-type structures with TopoIIIα.

Recently, it has been shown that there is a third component to the Sgs1-Top3 complex. This protein, named Rmi1 (RecQ Mediated Genome Instability 1), was originally identified in humans as a

component of the BLM-TopoIIIα complex (Meetei *et al*., 2003, Yin *et al*., 2005). RMI1 was also called BLAP75 (BLM-Associated Polypeptide, 75 kDa) before amino acid sequence homology revealed that they are the same protein. It has been shown to contain an oligonucleotide/oligosaccharide-binding (OB)-fold domain that is predicted to facilitate the binding of DNA (Chang *et al*., 2005, Mullen *et al*., 2005, Yin *et al*., 2005). Using immunofluorescence, it was also identified as part of a BLM-containing complex known as BRAFT (or BLM complex I), which is made up of BLM, TopoIIIα, at least five Fanconi anemia proteins and several novel polypeptides known as BLAPs (Yin *et al*., 2005). RMI1 was identified as one of these BLAPs and it is also seen to associate with BLM in other BLM-containing complexes (Bloom complexes II and III), as shown by Yin *et al* (2005) using SDS-PAGE analysis. Bloom complex II is similar to BLM complex I but lacks the Fanconi anemia proteins, whereas BLM complex III is made up of BLM, TopoIIIα, RMI1 and MLH1 (Yin *et al*., 2005). Interactions between RMI1 and TopoIIIα were confirmed by pull-down assays and Western blotting by Wu *et al* (2006). In *S. cerevisiae*, interactions between Sgs1, Top3 and Rmi1 were confirmed by co-immunoprecipitation studies by Mullen *et al* (2005).

Rmi1 is seen to colocalise with BLM in subnuclear foci that arise in response to DNA damage (Yin *et al*., 2005). Using RNA interference, Yin *et al* (2005) showed that decreasing the levels of RMI1 in the cell results in the destabilisation of BLM and TopoIIIα, as both exhibit a decrease in their protein levels. On the other hand, depletion of BLM does not affect the protein levels of either TopoIIIα or RMI1, as their protein levels appear wild-type in BS cells (Meetei *et al*., 2003, Yin *et al*., 2005). These results indicate that RMI1 is involved in stabilising TopoIIIα, and BLM has no role in this stability (Yin *et al*., 2005). Under wild-type conditions, both BLM and RMI1 exhibit a diffuse nuclear staining pattern in the cell. However, when exposed to DNA damaging agents, such as HU, both appear as distinct foci (Yin *et al*., 2005). Immunofluorescence data show that the BLM and RMI1 foci overlap in response to DNA damage (Yin *et al*., 2005). However, when the levels of RMI1 are depleted, BLM exhibits the same diffuse staining pattern that is seen when no damaging agents are added (Yin *et al*., 2005). In addition to this, depletion of RMI1 leads to an increase in the levels of SCE that is comparable to the levels seen in BS cells (Yin *et al*., 2005) which further indicates that the importance of the interactions between BLM, TopoIIIα and RMI1.

In humans, RMI1 aids in the recruitment of BLM and TopoIIIα to Holliday junctions and enhances their dissolution activities to specifically yield non-crossover products (Bussen *et al*., 2007, Mullen *et al*., 2005, Raynard *et al*., 2006, Wu *et al*., 2006). This dissolution activity is said to inhibit the formation of crossovers, and therefore actively suppress SCE events from occurring (Wu and

Hickson, 2003). The dissolution assay used to investigate this was comprised of two inter-linked radiolabelled oligonucleotides that resemble a Holliday junction-type structure (Wu and Hickson, 2003). The dissolution of this structure in a non-crossover dependent manner yields two intact circular oligonucleotides (Wu and Hickson, 2003). Using this assay, it was shown that the addition of RMI1 stimulated the dissolution activities of BLM and TopoIIIα in a concentration-dependent manner (Wu *et al*., 2006). When RMI1 was added to either BLM or TopoIIIα independently, no dissolution activity was observed (Wu *et al*., 2006). However, when RMI1 was added to reactions where the concentrations of TopoIIIα were limiting, a strong stimulatory effect on dissolution was observed (Wu *et al*., 2006). As this was not seen when the concentrations of BLM were limiting, it suggests that RMI1 may act to only stimulate the dissolution activities of TopoIIIα (Wu *et al*., 2006). This result indicates that both RMI1 and TopoIIIα act downstream of BLM in the dissolution of these recombination intermediates. Raynard *et al* (2008) saw that disruption of the lysine residue at position 166 resulted in the inability of RMI1 to interact with TopoIIIα. The interaction between RMI1, TopoIIIα and BLM has been shown to be highly specific, as the RMI1-TopoIIIα complex has no effect on the activities of WRN, RECQ1, RECQ5ß or *E. coli* RecQ (Bussen *et al*., 2007, Wu *et al*., 2005). In addition to this, Bussen *et al* (2007) and Wu *et al* (2006) also showed that substituting *E. coli* Top3 or Top1 for the human TopoIIIα does not enhance the activities of BLM.

Similar observations can be seen in yeast and in plants, as the phenotype of cells deleted for *rmi1* resemble cells deleted for *top3* in *S. cerevisiae* (Chang *et al*., 2005, Mullen *et al*., 2005) and *A. thaliana* (Hartung *et al*., 2007, 2008). Hartung *et al* (2008) showed that both Rmi1 and TOP3α are involved in the suppression of crossovers and in the resolution of meiotic recombination intermediates in *A. thaliana*. Deletion of either of these proteins leads to sterility in *A. thaliana* (Hartung *et al*., 2008). *A. thaliana* cells that have been deleted for *rmi1* display a hyper-recombination phenotype as well as sensitivity to genotoxic agents such as MMS and cisplatin indicative of defects during mitosis as well as meiosis (Hartung *et al*., 2008). Mutation of *rmi1* and *top3α* lead to an increase in the entanglement of chromosomes, which are subsequently torn apart during anaphase I which shows that both play an integral role in the resolution of recombination intermediates (Hartung *et al*., 2008). These findings are consistent with the role of BLM in human cells in promoting the decatenation of sister chromatids during anaphase (Chan *et al*., 2007) (described in **Section 1.6.3.1**).

Mullen *et al* (2005) saw that *rmi1Δ* yeast cells display a hyper-recombination phenotype with an increase in the rate of gross chromosomal rearrangements, are slow growing, are sensitive to DNA

damaging agents and exhibit a decreased efficiency of sporulation. As well as these phenotypes, Chang *et al* (2005) reported that *rmi1Δ* yeast cells were shown to accumulate as large budded cells containing only one nucleus, indicative of a delay at the G2/M phase of the cell cycle. This is similar to what is seen in *top3Δ* cells (Gangloff *et al*., 1994) which suggests that the absence of *rmi1* cells leads to a checkpoint induced mitotic delay. This was confirmed using a mobility shift assay that showed that *rmi1Δ* cells lead to the phosphorylation of Rad53, whereas wild-type cells do not (Chang *et al*., 2005). As with *top3*, deletion of *sgs1* can suppress several phenotypes exhibited by *rmi1Δ* cells, which suggests that both Top3 and Rmi1 function downstream of Sgs1 (Chang *et al*., 2005, Mullen *et al*., 2005). In addition to this, Chang *et al* (2005) and Mullen *et al* (2005) saw that *rmi1Δ* mutants exhibit the same spectrum of synthetic lethality as *sgs1Δ* cells. *rmi1Δ* cells, like *sgs1Δ* cells, were shown to be synthetically lethal when combined with *mms4Δ*, *mus81Δ*, *slx1Δ* and *slx4Δ* (Chang *et al*., 2005, Mullen *et al*., 2001, Mullen *et al*., 2005). Chang *et al* (2005) also saw that the slow growth phenotype of *rmi1Δ* cells, like *top3Δ* cells, is suppressed by mutations in the homologous recombination genes Rad51, Rad52, Rad54 and a checkpoint-defective allele of Rad53 (*rad53-11*) (Oakley *et al*., 2002, Weinert *et al*., 1994). This further supports the suggestion that Sgs1 is involved in the processing of recombination intermediates during homologous recombination, the products of which are toxic to the cell in the absence of Rmi1 (or Top3) (Chang *et al*., 2005).

1.7.6 RecQ helicases and mitotic homeologous recombination

As discussed in **Section 1.4.1**, studies by Chen and Jinks-Robertson (1998) and Zahrt and Maloy (1997) proposed that the impeding actions of the MMR proteins on the heteroduplex leads to a 'helicase-catalysed reversal of heteroduplex formation'. The involvement of a helicase, along with MMR proteins, in heteroduplex rejection was further supported by a study by Spell and Jinks-Robertson (2004) who carried out a genetic screen and a candidate gene approach to elucidate the proteins involved in the suppression of homeologous recombination. The inverted-repeat assay used was similar to that developed by Chen and Jinks-Robertson (1998) which has been described ain **Section 1.4.1.2**. For this assay, prototrophy to both histidine and lysine were assessed. After performing UV mutagenesis, Spell and Jinks-Robertson (2004) then used fluctuation analysis to elucidate the frequency of recombination by counting the numbers of HIS or LYS papillations. Candidate strains that had increases in *HIS* and *LYS* papillations were then crossed to several tester strains, each containing a deletion of a gene suspected to be involved in the suppression of homeologous recombination – *msh2*, *msh3*, *msh6*, *mlh1*, *pms1*, *rad1* and *sgs1*. If the resulting cross did not lead to a decrease in the rate of recombination, it is an indication of allelism with the corresponding mutant gene, showing that they act in the same pathway (Spell and Jinks-Robertson,

2004). The results from this screen showed that Sgs1 is involved in the suppression of homeologous recombination (Spell and Jinks-Robertson, 2004).

To further investigate how Sgs1 acts in this suppression of homeologous recombination, Spell and Jinks-Robertson (2004) looked at the effects of a C-terminal truncation of Sgs1 (*sgs1-ΔC200*) and the helicase-defective mutation of *sgs1* (*sgs1-K706A*). *sgs1-K706A* was indistinguishable from the deletion phenotype of *sgs1*, therefore showing that the helicase domain is important in the ability of Sgs1 to maintain genome integrity (Spell and Jinks-Robertson, 2004). *sgs1-ΔC200* led to a 3.2-fold increase in homeologous recombination without an associated increase in homologous recombination, suggesting that this mutation directly affects the fidelity of recombination but does not lead to a general hyper-recombination phenotype (Spell and Jinks-Robertson, 2004). This region may be important as it allows interactions between Sgs1 and Mlh1 (Gellon *et al*., 2002), and between the human homologs BLM and human Mlh1 (Langland *et al*., 2001, Pedrazzi *et al*., 2001).

In addition to this, Spell and Jinks-Robertson (2004) investigated the interactions of MMR proteins and Sgs1 in the suppression of homeologous recombination by testing the effects of deleting *sgs1* in strains deleted for *mlh1* or *msh2*. Comparisons between the single *sgs1* deletion to the double deletions revealed similar increases on the homologous/homeologous ratio, suggesting that both the MMR proteins and Sgs1 work in the same pathway to suppress this type of homeologous recombination with Sgs1 acting downstream of the MMR proteins to potentially unwind the heteroduplex DNA (Spell and Jinks-Robertson, 2004).

This is in contrast to what was reported by Myung *et al* (2001b), who showed a synergistic increase in the rate of accumulating gross chromosomal rearrangements (GCRs) when both *sgs1* and *msh2* were deleted. Myung *et al* (2001b) investigated the effects of *sgs1* and *msh2* mutations on the rate of accumulating various mutations. They noted that the frequency of frameshift mutations and base substitution mutations were not altered in *sgs1* mutations (confirming the observations by Watt *et al* (1996)), however they saw a 20-fold increase in the rate of accumulating gross chromosomal rearrangements (GCRs), including translocations and deletions (Myung et al., 2001b). Myung *et al* (2001b) used an inverted-repeat assay developed by Datta *et al* (1996) where recombination results in the formation of full length *HIS3*, using substrates that were either 100% or 91% identical. Using this assay, they noted that a deletion of *sgs1* led to a 6-fold increase in the rate of homeologous recombination that was further increased by deletion of *msh2* (Myung et al., 2001b). This indicates that Msh2 and Sgs1 may act in separate pathways in the suppression of GCRs. The discrepancy

between whether Sgs1 acts together with or independently to Msh2 in the suppression of homeologous recombination may be due to the different assays used. Whereas Spell and Jinks-Robertson (2004) assayed for the effects of mismatches, Myung *et al* (2001b) assayed for more types of events including deletions and translocations.

Deletion of *top3* was also shown to lead to an increase in the rate of gross chromosomal rearrangements (GCRs) amongst homeologous substrates during mitosis (Myung *et al*., 2001b, Putnam *et al*., 2009). These studies assayed for GCRs carried out by the *HXT13-DSF1* interval on chromosome V with homeologous regions on chromosomes IV, X and XIV. It was shown that the rate of GCRs for the *sgs1 top3* double mutant was not different from the *sgs1* single mutant, suggesting that both Sgs1 and Top3 act in the suppression of GCRs during mitosis (Myung *et al*., 2001b, Putnam *et al*., 2009).

Further support for a 'helicase-catalysed reversal of heteroduplex formation' (Chen and Jinks-Robertson, 1998) came from studies by Sugawara *et al* (2004) and Goldfarb and Alani (2005) and their investigations involving heteroduplex rejection during SSA. In addition to showing that the mismatch binding and ATP hydrolysis activities of Msh2-Msh6 were required for heteroduplex rejection, Goldfarb and Alani (2005) also showed that Sgs1 was involved in the unwinding of mismatch-containing recombination intermediates formed during single-strand annealing (SSA) (**Figure 1.7**). The SSA assay (Sugawara et al., 2004) involved two *URA3* sequences that are either 100% or 97% identical and a galactose-induced HO cut site in between. DSBs were induced via the HO cut site. The repair of the DSB by SSA (**Figure 1.7**), which is followed by non-homologous tail removal, leads to a deletion of the intervening sequences between the two *URA3* repeats. However, heteroduplex rejection, caused by the presence of mismatches in one copy, will prevent the repair of the DSB and will ultimately lead to a decrease in cell viability. It was found that the heteroduplex rejection phenotypes seen for the helicase mutation, *sgs1-K706A* (Lu et al., 1996), *sgs1-ΔC795* (a deletion of the C-terminal 795 amino acids of *SGS1* (Mullen et al., 2000)) and *sgs1-ΔN644* (a deletion of the N-terminal 644 amino acids of *SGS1* (Mullen et al., 2000)) all resemble the heteroduplex rejection phenotype seen for an *sgs1* deletion strain (Goldfarb and Alani, 2005). These data led both groups to suggest that heteroduplex rejection occurs by an unwinding mechanism that involves the helicase activity of Sgs1 and that this activity is recruited to the mismatch-containing sites by the MMR complex (Goldfarb and Alani, 2005, Sugawara et al., 2004). The data from these studies therefore provide evidence that the helicase suggested to act in the reversal of heteroduplex formation suggested by Chen and Jinks-Robertson (1999) is Sgs1 in yeast.

The eukaryotic members of the RecQ helicase family have also been implicated in the suppression of mitotic homeologous recombination. Recent studies in *Drosophila melanogaster* by Kappeler *et al* (2008) saw that the frequency of SSA (**Figure 1.7**) is decreased by the presence of increasing homeology, and this suppression was found to be dependent on *MUS309*, which is the *Drosophila* homologue of the Bloom helicase (Kappeler et al., 2008).

1.8 Sgs1 acts in the suppression of meiotic homeologous recombination

The RecQ helicase family, along with various components of the MMR complex, have been implicated in the suppression of homeologous recombination. The roles of RecQ helicases in the suppression of mitotic homeologous recombination have been discussed above in **Section 1.7.6**. However, work in our laboratory has also implicated the MMR complex in the suppression of meiotic homeologous recombination (Chambers et al., 1996, Hunter et al., 1996) (**Section 1.4**). Further to this, Chambers (1999) carried out a screen to identify genes that, when mutated, lead to an increase in the levels of homeologous recombination. Sgs1 was identified as one of these genes. Based on this work, Chaix (2007) investigated whether Sgs1 was also implicated in the suppression of meiotic homeologous recombination using the partial hybrid strain created by Chambers *et al* (1996). The data obtained by this study implicate the helicase in this suppression. Deletion of *sgs1* led to a marked increase in homeologous recombination along chromosome III. This increase is associated with a decrease in meiosis I non-disjunction events. In addition to this, Chaix (2007) also showed that deletion of *sgs1* leads to an increase in unequal recombination events. Therefore, one of the many roles of Sgs1 appears to be in the suppression of both mitotic and meiotic homeologous recombination.

Due to the above results which implicated both the MMR system and Sgs1 in the suppression of meiotic homeologous recombination, we wanted to characterise their mode of action (Chaix, 2007, Chambers et al., 1996, Hunter et al., 1996). Mitotic data have led to the hypothesis that Sgs1 is involved in heteroduplex rejection in response to mismatches that are sensed by the MMR complex. We propose that this heteroduplex rejection model is also the mechanism of choice in the suppression of meiotic homeologous recombination (**Figure 1.17**). The model proposes that the MMR complex, consisting of Msh2, Msh6, Mlh1 and Pms1, scans the genome for SEI events that take place between diverged sequences. On finding such events, the MMR complex binds to them and prevents the SEI from progressing. The MMR complex then recruits Sgs1, whose helicase activity unwinds the heteroduplex DNA. This allows the invading strand to continue its homology search.

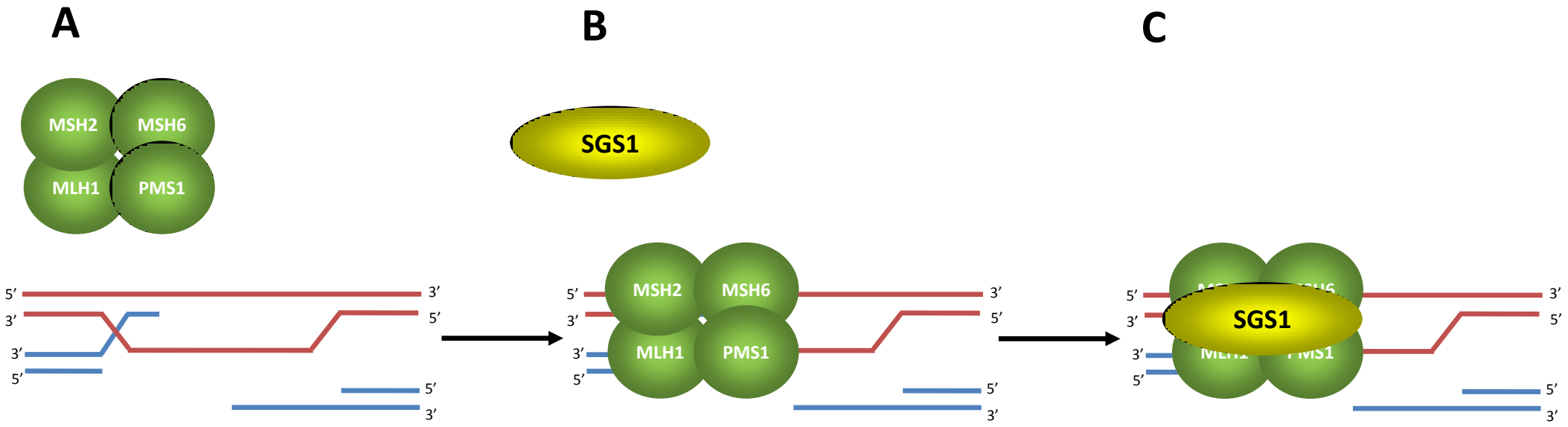


Figure 1.17: Model proposed to account for the suppression of homeologous recombination

We propose that the mismatch repair complex, which is involved in the recognition of mismatches, scans the genome looking for SEI events occurring between diverged sequences (**A**). On finding such events, they bind to them and block the progression of the SEI (**B**). The MMR complex then recruits Sgs1, whose helicase activity unwinds the heteroduplex DNA, therefore releasing the invading strand and allowing it to continue its homology search (**C**).

In addition to this Chambers *et al* (1996) saw an increase in the number of three viable spores in the partial hybrid strain, and the dead spore was inferred to be recombinant. Rockmill *et al* (1994) suggested that 3 viable spores can be caused by either precocious separation of sister chromatids or meiosis II non-disjunction (**Figure 1.4**). These possibilities were ruled out by CHEF gel analysis as both defects would lead to two copies of chromosome III resulting in a doubly intense band for chromosome III on the CHEF gels. This was not seen (Chambers *et al*., 1996). After analysing the rates of recombination across chromosome III, it was shown that the majority of the dead spores in the three viable spore class of tetrads were recombinant in the partial hybrid strain. If death was random, the number of recombinants would be seen at equal frequencies to the number of non-recombinants. Indeed, this was seen for the wild-type homologous cross (Chambers *et al*., 1996). However, when homeology was introduced, recombinants were seen six-times more frequently in the three viable spore class of tetrads (Chambers *et al*., 1996). This suggests that attempted recombination, leading to the formation of heteroduplex DNA, but a failure to complete a reciprocal crossover, is the underlying cause of spore death in these tetrads (Chambers *et al*., 1996).

To further characterise this attempted crossover defect, Chambers *et al* (1996, 1999) investigated the effects of the MMR proteins Msh2, Pms1 and Msh6. They noted that deletion of *msh2* or *pms1* resulted in a restoration of the recombinant : non-recombinant ratio to equal frequencies (Chambers *et al*., 1996). The data suggest that Msh2 and Pms1 play a role in the rejection of this attempted crossover due to the presence of mismatches. By deleting *msh2* or *pms1*, this intermediate is not rejected, and the reciprocal crossover is successfully completed. In contrast to this, deletion of *msh6* did not restore the recombinant : non-recombinant ratio to equal frequencies, and the dead spore was still seen to be recombinant (Chambers, 1999). This therefore suggests that Msh6 plays no role in this process. The data obtained from these studies led to the proposal of a model (**Figure 1.18**) in which Msh2 and Pms1 act in the rejection of the reciprocal crossover, leading to the rejection of the formation of a dHJ, in response to mismatches. This model is an extension of the model described in **Figure 1.17**, which suggests a role for Msh2, Mlh1, Msh6 and Pms1 in the rejection of a SEI in response to mismatches. However, the data suggest that Msh2 and Pms1 also play a role in the subsequent step. This suggests that while sequences may be contain sufficient homology for the initiation of recombination, components of the MMR complex continue to play a role in assessing that there is an adequate degree of homology for resolution.

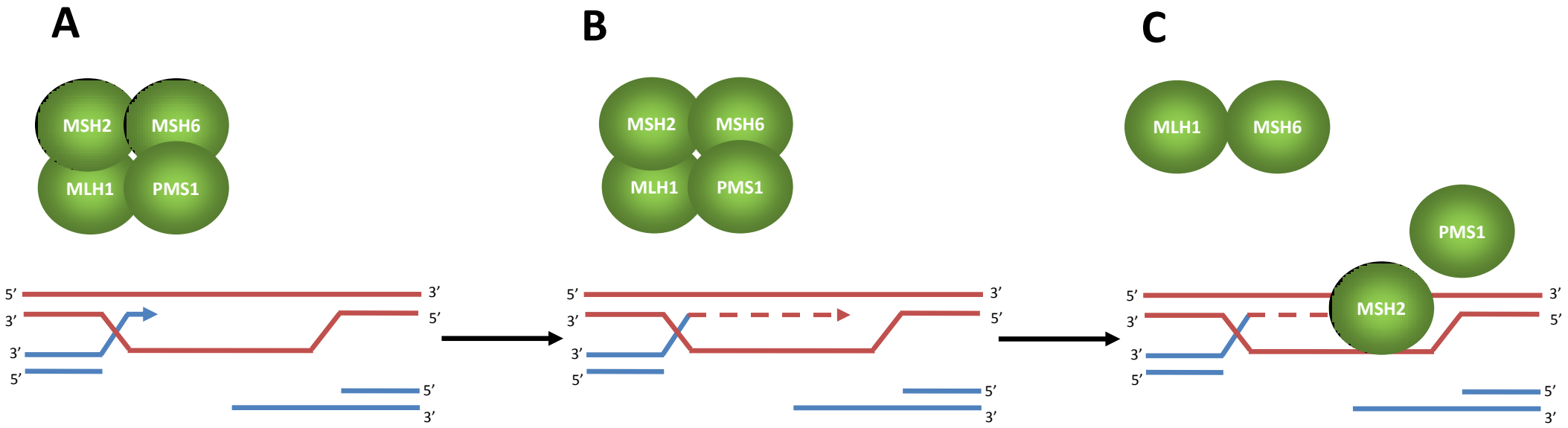


Figure 1.18: The rejection of strand capture leading to spore death is dependent on the activities of Msh2 and Pms1

The mismatch repair complex scans the genome looking for SEI events occurring between diverged sequences (**A**). If there is sufficient homology for the initiation of recombination, the MMR complex plays no role and DNA synthesis begins (**B**). Msh2 is required for the assessment of sequence divergence while Pms1 is required for its processing when the attempt is made to carry out strand capture. If the sequences are too diverged, Msh2 and Pms1 reject that attempted strand capture, leading to death of the spore (**C**).

1.9 Aim

The aim of this investigation was characterise the role of the *Saccharomyces cerevisiae* RecQ helicase Sgs1 in the suppression of meiotic homeologous recombination (**Figure 1.17**) by analysing its functional domains (**Figure 1.19**). In order to analyse the meiosis-specific effects of Sgs1, we first aimed to create a meiotic null mutant of Sgs1 by cloning the promoter of the *CLB2* gene upstream of *SGS1* (**Figure 1.19A**). The *CLB2* promoter leads to expression during mitosis but not meiosis (Lee and Amon, 2003) (**Chapter 3**).

We also aimed to characterise whether the mismatch repair (MMR) complex was responsible for the recruitment of Sgs1 to the sites of homeologous recombination at both the single end invasion (SEI) stage (**Figure 1.17**) and the strand capture stage (**Figure 1.18**) by mutating the Mlh1-interacting domain of Sgs1 (**Figure 1.19B**) (Gellon et al., 2002) (**Chapter 4**).

As mitotic data have implicated that Sgs1 and Top3 act in the same pathway to suppress gross chromosomal rearrangements (GCRs), we aimed to assess whether their interaction was important in the suppression of meiotic homeologous recombination. Therefore, we assessed the ability of a mutation which abolished the interactions between Top3 and Sgs1 to suppress homeologous recombination (**Figure 1.19B**) (**Chapter 5**).

Our model (**Figure 1.17**) proposes that the helicase activity of Sgs1 is responsible for heteroduplex rejection in meiosis. Therefore, we aimed to assess the ability of a helicase-defective mutant of Sgs1 in suppressing homeologous recombination (**Figure 1.19B**) (**Chapter 5**).

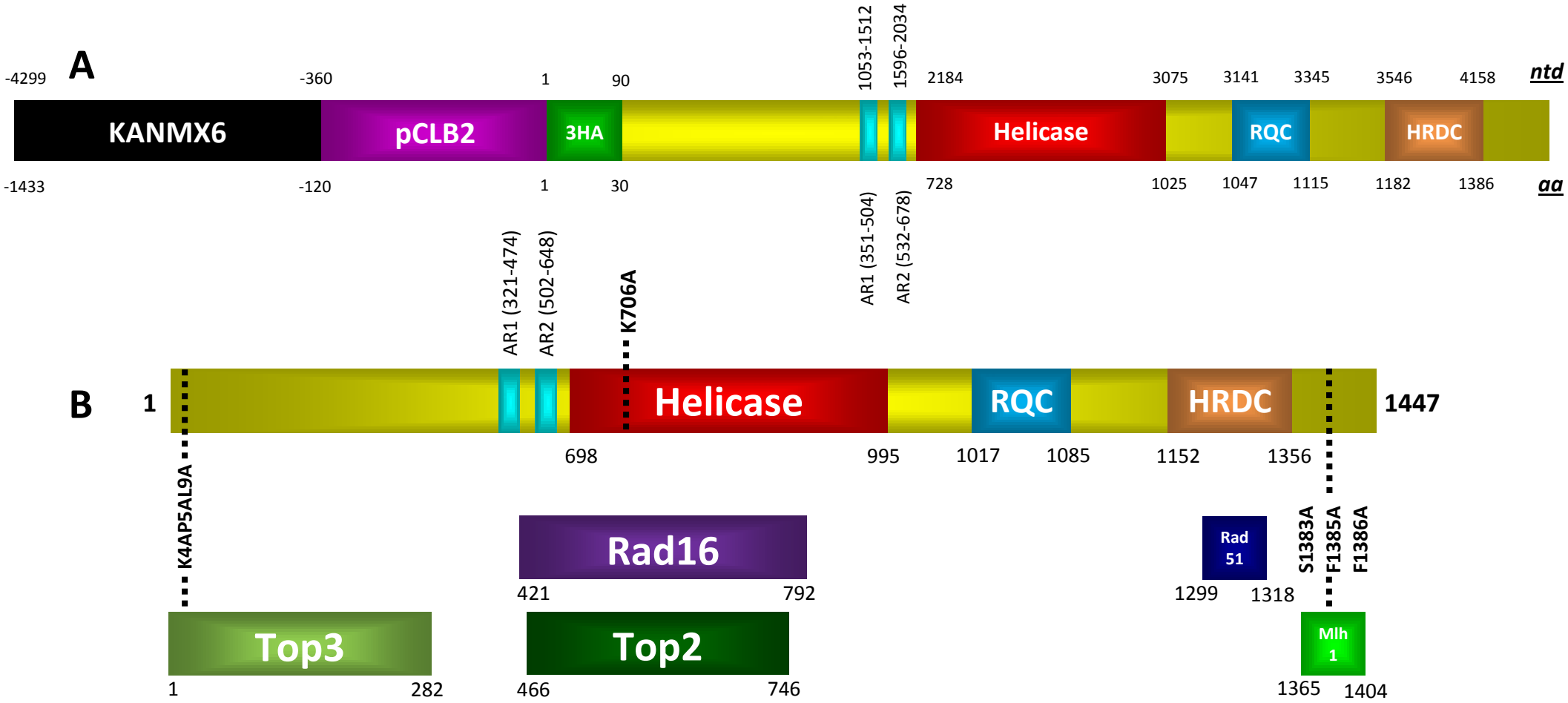


Figure 1.19: Different Sgs1 constructs and mutations used in this investigation

A: The *pCLB2-SGS1* strain in which the promoter of the *CLB2* gene was inserted upstream of *SGS1* (described in detail in **Section 3.2.1**). Both the nucleotide (ntd) and amino acid (aa) positions of the relevant domains are highlighted with respect to the start codon present immediately upstream of the 3HA protein tag.

B: The interacting domains of Sgs1 highlighting the amino acid changes used to disrupt the Top3-interacting domain of Sgs1 (*sgs1-top3-id*), to disrupt the helicase activity of Sgs1 (*sgs1-K706A*) and to disrupt the Mlh1-interacting domain of Sgs1 (*sgs1-mlh1-id*).

Chapter 2: Materials & Methods

2.1 Materials

2.1.1 *S. cerevisiae* Yeast Strains

All of the strains used in this study are in a Y55 background and are listed in **Table 2.1**.

Table 2.1: Genotypes of haploid strains

Strains	Key Features	Genotype	Source
SCT 14	*S. paradoxus* N17 chromosome III	*ade1-1; α; HOΔ; met13-4; ura3::nco; cyh2-1; kar1Δ13*	Chambers (1996)
ACT 2	*S. paradoxus* N17 *sgs1Δ*	*ade1-1; α; HOΔ; met13-4; ura3::nco; cyh2-1; kar1Δ13; sgs1::KANMX4*	Chaix (2007)
ACT 53	*SGS1*	*ade1-1; α; HOΔ; met13-2; ura3::nco; cyh2-1*	Chaix (2007)
ACT 56	*sgs1Δ*	*ade1-1; α; HOΔ; met13-2; ura3::nco; cyh2-1; sgs1::KANX4*	Chaix (2007)
ACT 57	*SGS1*	*ade1-1; HML::ADE1; his4-r1; leu2-r1; α; HOΔ; lys2-c; met13-2; ura3::nco; trp1:bsu36; cyh2-1*	Chaix (2007)
ACT 65	*HYG-CYH/HYG*	*ade1-1; HML::ADE1; HYG-CYH2-his4-r1; leu2-r1-HYG; α; HOΔ; lys2-c; met13-2; ura3::nco; trp1::bsu36; cyh2-1*	Chaix (2007)
ACT 66	*HYG-CYH/HYG sgs1Δ*	*ade1-1; HML::ADE1; HYG-CYH2-his4-r1; leu2-r1-HYG; α; HOΔ; lys2-c; met13-2; ura3::nco; trp1::bsu36; cyh2-1; sgs1::KANMX4*	Chaix (2007)
ACT 83-1	*sgs1-ΔC795*	*ade1-1; α; HOΔ; met13-2; ura3::nco; cyh2-1; sgs1Δ-C795::NATMX4*	Chaix (2007)
Y55 3540	*sgs1-K706A*	*ade1-1; HML::ADE1; HYG-CYH2-his4-r1; leu2-r1-HYG; α; HOΔ; lys2-c; met13-2; ura3::nco; trp1::bsu36; cyh2-1; sgs1-K706A*	Amin (*unpublished*)
Y55 3541	*sgs1-top3-id*	*ade1-1; HML::ADE1; HYG-CYH2-his4-r1; leu2-r1-HYG; α; HOΔ; lys2-c; met13-2; ura3::nco; trp1::bsu36; cyh2-1; sgs1-K4AP5AL9A*	Chaix (2007)
Y55 3543	*sgs1-mlh1-id 1.6*	*ade1-1; HML::ADE1; HYG-CYH2-his4-r1; leu2-r1-HYG; α; HOΔ; lys2-c; met13-2; ura3::nco; trp1::bsu36; cyh2-1; sgs1-S1383AF1385AF1386A*	Mason (*unpublished*)
Y55 3544	*sgs1-mlh1-id 2.14*		
Y55 3545	*sgs1-mlh1-id 6.10*		
Y55 3565	*S. paradoxus* N17 *pCLB2-SGS1*	*ade1-1; α; HOΔ; met13-4; ura3::nco; cyh2-1; kar1Δ13; KANMX6::pCLB2-SGS1*	This study
Y55 3567	*pCLB2-SGS1*	*ade1-1; α; HOΔ; met13-2; ura3::nco; cyh2-1; KANMX6::pCLB2-SGS1*	This study
Y55 3588	*sgs1-ΔHRDC*	*ade1-1; HML::ADE1; HYG-CYH2-his4-r1; leu2-r1-HYG; α; HOΔ; lys2-c; met13-2; ura3::nco; trp1::bsu36; cyh2-1; sgs1-ΔC360::KANMX4*	This study
Y55 3589	*sgs1-K706AΔHRDC*	*ade1-1; HML::ADE1; HYG-CYH2-his4-r1; leu2-r1-HYG; α; HOΔ; lys2-c; met13-2; ura3::nco; trp1::bsu36; cyh2-1; sgs1-K706AΔC360::KANMX4*	This study
Y55 3590	*msh2::KANMX4*	*ade1-1; HML::ADE1; HYG-CYH2-his4-r1; leu2-r1-HYG; α; HOΔ; lys2-c; met13-2; ura3::nco; trp1::bsu36; cyh2-1; msh2::KANMX4*	Malik (*unpublished*)
QM1	*S. paradoxus* N17 *pCLB2-MSH2*	*ade1-1; α; HOΔ; met13-4; ura3::nco; cyh2-1; kar1Δ13; KANMX6::pCLB2-MSH2*	Malik (*unpublished*)
QM2	*pCLB2-MSH2*	*ade1-1; α; HOΔ; met13-2; ura3::nco; cyh2-1; KANMX6::pCLB2-MSH2*	Malik (*unpublished*)

2.1.2 Creating Diploids

Yeast strains exist in one of two mating types *MAT* $\underline{a}$ or *MAT* α. To create diploids, a *MAT* $\underline{a}$ and *MAT* α strains with complementing auxotrophic markers were crossed on a YEPD plate. This plate was incubated at 30°C for 12 to 16 hours to allow mating to occur. Diploids were selected via complementation by replicating the YEPD plate onto a minimal medium plate. This plate was then incubated at 30°C for 12 to 16 hours. The resulting diploid was then streaked to single colonies on YEPD. The diploid strains used in this study are listed in **Table 2.2**.

Table 2.2: Genotypes of diploid strains

Diploid Strains	Haploid Strains	Genotype	Key Features	Source
ACD 94	SCT 14	*ade1-1 LYS2 HML HIS4 LEU2 α HOΔ TRP1 ura3::nco met13-4 cyh2-1 kar1Δ13*	*SGS1/SGS1* homeologous	Chaix (2007)
	ACT 65	*ade1-1 lys2-c HML::ADE1 HYG-CYH2-his4-r1 leu2-r1-HYG a HOΔ trp1::bsu36 ura3::nco met13-2 cyh2-1 KAR1*		
ACD 95	ACT 56	*ade1-1 LYS2 HML HIS4 LEU2 α HOΔ TRP1 ura3::nco met13-2 cyh2-1 sgs1::KANMX4*	*sgs1Δ/sgs1Δ* homologous	Chaix (2007)
	ACT 66	*ade1-1 lys2-c HML::ADE1 HYG-CYH2-his4-r1 leu2-r1-HYG a HOΔ trp1::bsu36 ura3::nco met13-2 cyh2-1 sgs1::KANMX4*		
ACD 96	ACT 2	*ade1-1 LYS2 HML HIS4 LEU2 α HOΔ TRP1 ura3::nco met13-4 cyh2-1 sgs1::KANMX4 kar1Δ13*	*sgs1Δ/sgs1Δ* homologous	Chaix (2007)
	ACT 66	*ade1-1 lys2-c HML::ADE1 HYG-CYH2-his4-r1 leu2-r1-HYG a HOΔ trp1::bsu36 ura3::nco met13-2 cyh2-1 sgs1::KANMX4 KAR1*		
ACD 97	ACT 53	*ade1-1 LYS2 HML HIS4 LEU2 α HOΔ TRP1 ura3::nco met13-2 cyh2-1*	*SGS1/SGS1* homologous	Chaix (2007)
	ACT 65	*ade1-1 lys2-c HML::ADE1 HYG-CYH2-his4-r1 leu2-r1-HYG a HOΔ trp1::bsu36 ura3::nco met13-2 cyh2-1*		
ACD 116	ACT 56	*ade1-1 LYS2 HML HIS4 LEU2 α HOΔ TRP1 ura3::nco met13-2 cyh2-1 sgs1::KANMX4*	*sgs1Δ/SGS1* homologous	Chaix (2007)
	ACT 65	*ade1-1 lys2-c HML::ADE1 HYG-CYH2-his4-r1 leu2-r1-HYG a HOΔ trp1::bsu36 ura3::nco met13-2 cyh2-1 SGS1*		
ACD 117	ACT 2	*ade1-1 LYS2 HML HIS4 LEU2 α HOΔ TRP1 ura3::nco met13-4 cyh2-1 sgs1::KANMX4 kar1Δ13*	*sgs1Δ/SGS1* homologous	Chaix (2007)
	ACT 65	*ade1-1 lys2-c HML::ADE1 HYG-CYH2-his4-r1 leu2-r1-HYG a HOΔ trp1::bsu36 ura3::nco met13-2 cyh2-1 SGS1 KAR1*		
ADA 1	Y55 3567	*ade1-1 LYS2 HML HIS4 LEU2 α HOΔ TRP1 ura3::nco met13-2 cyh2-1 KANMX6::pCLB2-SGS1*	*pCLB2-SGS1/sgs1Δ* homologous	This study
	ACT 66	*ade1-1 lys2-c HML::ADE1 HYG-CYH2-his4-r1 leu2-r1-HYG a HOΔ trp1::bsu36 ura3::nco met13-2 cyh2-1 sgs1::KANMX4*		
ADA 2	Y55 3565	*ade1-1 LYS2 HML HIS4 LEU2 α HOΔ TRP1 ura3::nco met13-4 cyh2-1 KANMX6::pCLB2-SGS1 kar1Δ13*	*pCLB2-SGS1/sgs1Δ* homologous	This study
	ACT 66	*ade1-1 lys2-c HML::ADE1 HYG-CYH2-his4-r1 leu2-r1-HYG a HOΔ trp1::bsu36 ura3::nco met13-2 cyh2-1 sgs1::KANMX4 KAR1*		
ADA 3	ACT 83-1	*ade1-1 LYS2 HML HIS4 LEU2 α HOΔ TRP1 ura3::nco met13-2 cyh2-1 sgs1-ΔC795*	*sgs1-ΔC795/sgs1Δ* homologous	This study
	ACT 66	*ade1-1 lys2-c HML::ADE1 HYG-CYH2-his4-r1 leu2-r1-HYG a HOΔ trp1::bsu36 ura3::nco met13-2 cyh2-1 sgs1::KANMX4*		
ADA 4	ACT 56	*ade1-1 LYS2 HML HIS4 LEU2 α HOΔ TRP1 ura3::nco met13-2 cyh2-1 sgs1::KANMX4*	*sgs1-mlh1-id /sgs1Δ* homologous	This study
	Y55 3543	*ade1-1 lys2-c HML::ADE1 HYG-CYH2-his4-r1 leu2-r1-HYG a HOΔ trp1::bsu36 ura3::nco met13-2 cyh2-1 sgs1- S1383AF1385AF1386A*		
ADA 5	ACT 2	*ade1-1 LYS2 HML HIS4 LEU2 α HOΔ TRP1 ura3::nco met13-4 cyh2-1 sgs1::KANMX4 kar1Δ13*	*sgs1-mlh1-id/sgs1Δ* homologous	This study
	Y55 3543	*ade1-1 lys2-c HML::ADE1 HYG-CYH2-his4-r1 leu2-r1-HYG a HOΔ trp1::bsu36 ura3::nco met13-2 cyh2-1 sgs1- S1383AF1385AF1386A KAR1*		
ADA 6	ACT 56	*ade1-1 LYS2 HML HIS4 LEU2 α HOΔ TRP1 ura3::nco met13-2 cyh2-1 sgs1::KANMX4*	*sgs1-mlh1-id/sgs1Δ* homologous	This study
	Y55 3544	*ade1-1 lys2-c HML::ADE1 HYG-CYH2-his4-r1 leu2-r1-HYG a HOΔ trp1::bsu36 ura3::nco met13-2 cyh2-1 sgs1- S1383AF1385AF1386A*		
ADA 7	ACT 2	*ade1-1 LYS2 HML HIS4 LEU2 α HOΔ TRP1 ura3::nco met13-4 cyh2-1 sgs1::KANMX4 kar1Δ13*	*sgs1-mlh1-id/sgs1Δ* homologous	This study
	Y55 3544	*ade1-1 lys2-c HML::ADE1 HYG-CYH2-his4-r1 leu2-r1-HYG a HOΔ trp1::bsu36 ura3::nco met13-2 cyh2-1 sgs1- S1383AF1385AF1386A KAR1*		
ADA 8	ACT 56	*ade1-1 LYS2 HML HIS4 LEU2 α HOΔ TRP1 ura3::nco met13-2 cyh2-1 sgs1::KANMX4*	*sgs1-mlh1-id/sgs1Δ* homologous	This study
	Y55 3545	*ade1-1 lys2-c HML::ADE1 HYG-CYH2-his4-r1 leu2-r1-HYG a HOΔ trp1::bsu36 ura3::nco met13-2 cyh2-1 sgs1- S1383AF1385AF1386A*		
ADA 9	ACT 2	*ade1-1 LYS2 HML HIS4 LEU2 α HOΔ TRP1 ura3::nco met13-4 cyh2-1 sgs1::KANMX4 kar1Δ13*	*sgs1-mlh1-id/sgs1Δ* homologous	This study
	Y55 3545	*ade1-1 lys2-c HML::ADE1 HYG-CYH2-his4-r1 leu2-r1-HYG a HOΔ trp1::bsu36 ura3::nco met13-2 cyh2-1 sgs1- S1383AF1385AF1386A KAR1*		
ADA 12	ACT 56	*ade1-1 LYS2 HML HIS4 LEU2 α HOΔ TRP1 ura3::nco met13-2 cyh2-1 sgs1::KANMX4*	*sgs1-top3-id/sgs1Δ* homologous	This study
	Y55 3541	*ade1-1 lys2-c HML::ADE1 HYG-CYH2-his4-r1 leu2-r1-HYG a HOΔ trp1::bsu36 ura3::nco met13-2 cyh2-1 sgs1-K4A, P5A, L9A*		

Diploid Strains	Haploid Strains	Genotype	Key Features	Source
ADA 13	Y55 3567	*ade1-1 LYS2 HML HIS4 LEU2 α HOΔ TRP1 ura3::nco met13-2 cyh2-1 KANMX6::pCLB2-SGS1*	*sgs1-top3-id/pCLB2-SGS1* homologous	This study
	Y55 3541	*ade1-1 lys2-c HML::ADE1 HYG-CYH2-his4-r1 leu2-r1-HYG a HOΔ trp1::bsu36 ura3::nco met13-2 cyh2-1 sgs1-K4A, P5A, L9A*		
ADA 14	Y55 3565	*ade1-1 LYS2 HML HIS4 LEU2 α HOΔ TRP1 ura3::nco met13-4 cyh2-1 KANMX6::pCLB2-SGS1 kar1Δ13*	*sgs1-top3-id/pCLB2-SGS1* homeologous	This study
	Y55 3541	*ade1-1 lys2-c HML::ADE1 HYG-CYH2-his4-r1 leu2-r1-HYG a HOΔ trp1::bsu36 ura3::nco met13-2 cyh2-1 sgs1-K4A, P5A, L9A KAR1*		
ADA 15	Y55 3567	*ade1-1 LYS2 HML HIS4 LEU2 α HOΔ TRP1 ura3::nco met13-2 cyh2-1 KANMX6::pCLB2-SGS1*	*sgs1-K706A/pCLB2-SGS1* homologous	This study
	Y55 3540	*ade1-1 lys2-c HML::ADE1 HYG-CYH2-his4-r1 leu2-r1-HYG a HOΔ trp1::bsu36 ura3::nco met13-2 cyh2-1 sgs1-K706A*		
ADA 16	Y55 3565	*ade1-1 LYS2 HML HIS4 LEU2 α HOΔ TRP1 ura3::nco met13-4 cyh2-1 KANMX6::pCLB2-SGS1 kar1Δ13*	*sgs1-K706A/pCLB2-SGS1* homeologous	This study
	Y55 3540	*ade1-1 lys2-c HML::ADE1 HYG-CYH2-his4-r1 leu2-r1-HYG a HOΔ trp1::bsu36 ura3::nco met13-2 cyh2-1 sgs1-K706A KAR1*		
ADA 17	Y55 3567	*ade1-1 LYS2 HML HIS4 LEU2 α HOΔ TRP1 ura3::nco met13-2 cyh2-1 KANMX6::pCLB2-SGS1*	*sgs1-ΔHRDC/pCLB2-SGS1* homologous	This study
	Y55 3588	*ade1-1 lys2-c HML::ADE1 HYG-CYH2-his4-r1 leu2-r1-HYG a HOΔ trp1::bsu36 ura3::nco met13-2 cyh2-1 sgs1-ΔHRDC::KANMX4*		
ADA 18	Y55 3565	*ade1-1 LYS2 HML HIS4 LEU2 α HOΔ TRP1 ura3::nco met13-4 cyh2-1 KANMX6::pCLB2-SGS1 kar1Δ13*	*sgs1-ΔHRDC/pCLB2-SGS1* homeologous	This study
	Y55 3588	*ade1-1 lys2-c HML::ADE1 HYG-CYH2-his4-r1 leu2-r1-HYG a HOΔ trp1::bsu36 ura3::nco met13-2 cyh2-1 sgs1-ΔHRDC::KANMX4 KAR1*		
ADA 19	Y55 3567	*ade1-1 LYS2 HML HIS4 LEU2 α HOΔ TRP1 ura3::nco met13-2 cyh2-1 KANMX6::pCLB2-SGS1*	*sgs1-K706AΔHRDC/pCLB2-SGS1* homologous	This study
	Y55 3589	*ade1-1 lys2-c HML::ADE1 HYG-CYH2-his4-r1 leu2-r1-HYG a HOΔ trp1::bsu36 ura3::nco met13-2 cyh2-1 sgs1-K706AΔHRDC::KANMX4*		
ADA 20	Y55 3565	*ade1-1 LYS2 HML HIS4 LEU2 α HOΔ TRP1 ura3::nco met13-4 cyh2-1 KANMX6::pCLB2-SGS1 kar1Δ13*	*sgs1-K706AΔHRDC/pCLB2-SGS1* homologous	This study
	Y55 3589	*ade1-1 lys2-c HML::ADE1 HYG-CYH2-his4-r1 leu2-r1-HYG a HOΔ trp1::bsu36 ura3::nco met13-2 cyh2-1 sgs1-K706AΔHRDC::KANMX4 KAR1*		
ADA 21	ACT53	*ade1-1 LYS2 HML HIS4 LEU2 α HOΔ TRP1 ura3::nco met13-2 cyh2-1 SGS1*	*sgs1-K706A/SGS1* homologous	This study
	Y55 3540	*ade1-1 lys2-c HML::ADE1 HYG-CYH2-his4-r1 leu2-r1-HYG a HOΔ trp1::bsu36 ura3::nco met13-2 cyh2-1 sgs1-K706A*		
ADA 22	SCT14	*ade1-1 LYS2 HML HIS4 LEU2 α HOΔ TRP1 ura3::nco met13-4 cyh2-1 SGS1 kar1Δ13*	*sgs1-K706A/SGS1* homeologous	This study
	Y55 3540	*ade1-1 lys2-c HML::ADE1 HYG-CYH2-his4-r1 leu2-r1-HYG a HOΔ trp1::bsu36 ura3::nco met13-2 cyh2-1 sgs1-K706A KAR1*		
ADA 23	ACT 53	*ade1-1 LYS2 HML HIS4 LEU2 α HOΔ TRP1 ura3::nco met13-2 cyh2-1 SGS1*	*SGS1/sgs1Δ* homologous	This study
	ACT 66	*ade1-1 lys2-c HML::ADE1 HYG-CYH2-his4-r1 leu2-r1-HYG a HOΔ trp1::bsu36 ura3::nco met13-2 cyh2-1 sgs1::KANMX4*		
ADA 24	SCT 14	*ade1-1 LYS2 HML HIS4 LEU2 α HOΔ TRP1 ura3::nco met13-4 cyh2-1 SGS1 kar1Δ13*	*SGS1/sgs1Δ* homeologous	This study
	ACT 66	*ade1-1 lys2-c HML::ADE1 HYG-CYH2-his4-r1 leu2-r1-HYG a HOΔ trp1::bsu36 ura3::nco met13-2 cyh2-1 sgs1::KANMX4 KAR1*		
QMD1	QM1	*ade1-1 LYS2 HML HIS4 LEU2 α HOΔ TRP1 ura3::nco met13-2 cyh2-1 KANMX6::pCLB2-MSH2*	*pCLB2-MSH2/msh2Δ* homologous	Malik (*unpublished*)
	Y55 3590	*ade1-1 lys2-c HML::ADE1 HYG-CYH2-his4-r1 leu2-r1-HYG a HOΔ trp1::bsu36 ura3::nco met13-2 cyh2-1 msh2::KANMX4*		
QMD2	QM2	*ade1-1 LYS2 HML HIS4 LEU2 α HOΔ TRP1 ura3::nco met13-4 cyh2-1 kar1Δ13 KANMX6::pCLB2-MSH2*	*pCLB2-MSH2/msh2Δ* homeologous	Malik (*unpublished*)
	Y55 3590	*ade1-1 lys2-c HML::ADE1 HYG-CYH2-his4-r1 leu2-r1-HYG a HOΔ trp1::bsu36 ura3::nco met13-2 cyh2-1 KAR1 msh2::KANMX4*		

2.1.3 Yeast Media

The yeast media used in this study were made using ingredients purchased from *Sigma Aldrich*.

The complete medium for yeast, YEPD, was made up of yeast extract (1% w/v), Bactopeptone (2% w/v), dextrose (D-glucose) (2% w/v) and 10ml/L of 0.5% adenine. The pH was adjusted to between 6-6.5 by adding 1M HCl. 2.5% solid agar was added to the liquid media in order to make plates. The media was then autoclaved for one hour.

Synthetic media were used for the purpose of studying the segregation of auxotrophic markers following tetrad dissection (**Section 2.2.11.1**). These media were made by supplementing minimal medium with the necessary 'drop-out' mixture. The 'drop-out' mixture consists of all of the amino acids, adenine and uracil (listed in **Table 2.3**) except the required supplement. Minimal media were made up of yeast nitrogen base (2.7% w/v) and dextrose (D-glucose) (2% w/v). The pH was adjusted to between 6-6.5 by adding 2.5M NaOH. 870mg/L of the 'drop-out' mixture (without the required amino acid) was added to this. 2.5% solid agar was added in order to make solid media. Media were then autoclaved for 20 minutes at 115°C at a pressure of 10 psi.

Table 2.3: List of amino acid powders used to make the 'drop-out' powder

Amino Acid	**Amount (mg)**
Adenine	800
Arginine	800
Aspartic Acid	4000
Histidine	800
Leucine	800
Lysine	1200
Methionine	800
Phenylalanine	2000
Threonine	800
Tryptophan	800
Tyrosine	1200
Uracil	800

Antibiotic drugs, 5-fluororotic acid (5-FOA) or methyl methane sulfonate (MMS) were added to the specific media after autoclaving. **Table 2.4** lists the drugs used in this study and the amounts required.

Table 2.4: List of drugs used and the concentration required per plate

Drugs	**Medium**	**Concentration**	**Supplier**
Ampicillin	Luria Broth	0.1%	*Sigma*
Canavanine	Arginine drop out	2%	*Sigma*
Cycloheximide	YEPD	1%	*Sigma*
Geneticin (G418)	YEPD	1.6%	*Invitrogen*
Hygromycin B	YEPD	0.6%	*Invitrogen*
Methyl Methane Sulfonate (MMS)	YEPD	0.02%	*Sigma*
5-FOA	Uracil drop out + 50mg uracil	0.1%	*Sigma*

2.1.4 Bacterial Media

Luria Broth (LB) medium, used to grow *Escherichia coli*, was made up of 1% w/v Bactopeptone, 0.5% w/v yeast extract, 1% w/v NaCl and was adjusted to pH7. The LB medium was supplemented with ampicillin (**Table 2.4**) to allow for plasmid selection.

2.1.5 Chemically Competent Bacterial Cells

Chemically competent *E. coli* cells were purchased from *Amersham* (for use with the *pMOS-Blue Transformation Kit*) or *Fermentas* (for use with the *pJET Cloning Kit*). The cells were transformed as per the manufacturer's instructions.

2.1.6 Oligonucleotides

Oligonucleotides were purchased from *Invitrogen*. **Table 2.5** lists the oligonucleotides used in this study.

Table 2.5: Oligonucleotides used in this study

Oligonucleotide	Sequence (5' to 3')	Purpose
pCLB2-SGS1 Fwd	GGAAAAAATACAGATTATTGTTGTATATATTTAAAAAATCATACACGTACACACAAGGCGGTAGAATTCGAGCTCGTTTAAAC	Insertion of the *CLB2* promoter
pCLB2-SGS1 Rev	GTAAAGTCGCCGTTTCCTTTAACCATTTGTGCTCCCTTCTTAAGTTATGTGACGGCTTCGTCACGCACTGAGCAGCGTAATCTG	
pCLB2-SGS1 A1	AATTCCCATGGCTCAAACTG	Checking *pCLB2-SGS1* transformants
pCLB2-SGS1 A4	TTGAAGGCGGATCACCTCTA	
pCLB2-SGS1 Seq F1	TAAGGTGCCTTAGGGGGACT	Sequencing *CLB2* promoter
pCLB2-SGS1 Seq F2	GAATCTTTCTGGTATTAATTTTGTCC	
sgs1-top3-id Fwd	ATAGTTCAGCCGTGCGTTTC	Sequencing for *K4AP5AL9A* mutation
sgs1-top3-id Rev	TTGAAGGCGGATCACCTCTA	
sgs1K706A Fwd	ATGAACACCCACCACCATCT	Sequencing for *K706A* mutation
sgs1K706A Rev	CTGATGTTTGCTCGCATGAT	
sgs1ΔHRDC trunc Fwd	GATGGCAGCAATATTACCAATGAATGATTCGGCTTTTGCAACTTTAGGCACAGTGGAGGACCGTACGCTGCAGGTCGAC	Deleting the C-terminus of *SGS1* from the HRDC domain
sgs1ΔHRDC trunc Rev	CCCAAAAAGAATGCTTGGCGAATGGTGTCGTAGTTATAAGTAACACTATTTATTTTTCTACTCTATCGATGAATTCGAGCTCG	
sgs1ΔHRDC trunc A1	GCGCTTTATAAGTGCGAAGG	Checking *sgs1ΔHRDC* transformants
sgs1ΔHRDC trunc A4	TGCGAACGAAACTGAATGAG	
K2	TTCAGAAACAACTCTGGCGCA	Internal primers to check for integration of *KANMX4*
K3	CATCCTATGGAACTGCCTCGG	

Blue underlined sequences, as described by Longtine *et al* (1998), are homologous to *pA6a-KANMX6-pCLB2-3HA* plasmid (described in **Table 2.6**)
Green underlined sequences are homologous to *pFA6kanMX4* plasmid (described in **Table 2.6**)

2.1.7 Plasmids

Table 2.6 lists the plasmids used in this study.

Table 2.6: Plasmids used in this study

Plasmid	Description	Reference
pFA6KANMX4	*KANMX4*	Wach *et al* (1994)
pFA6a-KANMX6-pCLB2-3HA	*CLB2* promoter	Lee and Amon (2003)
pRED 56	*SacI-SacII* fragment of *CYH2* ORF in pBluescript marked with *URA3*	Szent-Giorgy (*unpublished*)

2.1.8 Enzymes

The DNA modifying enzymes and their concentrations are listed in **Table 2.7**.

Table 2.7: Enzymes used in this study

Enzyme	Concentration	Purchased From
Phusion High-Fidelity DNA polymerase	2 units/µl	*New England BioLabs*
Proteinase K	10mg/ml	*Roche*
Restriction Enzymes	1-5 units/reaction	*New England BioLabs*
RNase A	1mg/ml	*Sigma*
Taq DNA polymerase	0.1-1 unit/reaction	*ABgene*
T4 DNA ligase	20 units/reaction	*Roche*

2.1.9 DNA Molecular Weight Markers

The *λBstEII* DNA marker was purchased from *New England BioLabs* and was used at a concentration of 25ng/µl. The *GeneRuler™ 1kb Ladder* from *Fermentas* was also used at a concentration of 0.1µg/µl.

2.1.10 Microscopes

Tetrad dissection was carried out using a *Zeiss* phase contrast microscope which had been fitted with a micromanipulator needle. The dissecting needles were purchased from *Singer Instruments*. A *Zeiss Axioskop 2* microscope was used for analysis of the DAPI staining.

2.2 Methods

2.2.1 Agarose Gel Electrophoresis

LE agarose powder was purchased from *Roche*. The appropriate amount of powder (depending on the desired gel concentration) was dissolved in 1x Tris-Borate EDTA (TBE) solution, which is made up of 90mM Tris base, 90mM boric acid and 2mM EDTA and is then adjusted to pH 8.3. The 1x TBE solution is also used as the running buffer for the agarose gel electrophoresis (Sambrook et al., 1989). 10mg/ml of ethidium bromide (*Sigma*) was added to stain the DNA. The gels were visualised under ultraviolet light at a wavelength of 302nm. Gel images were taken using the *Kodak 200L* dark chamber and camera and the images were processed using the *Kodak 1D Image* analysis software.

2.2.2 Clamped Homogenous Electric Field (CHEF) Analysis

CHEF Gels are agarose gels that allow the separation of chromosomes by the variation of the time and direction of the electric field. To carry this out, DNA was extracted in agarose plugs as described by Louis and Haber (1990). Yeast strains were cultured in 3ml liquid YEPD for 12-16 hours at 30°C. The cells were harvested by centrifugation at 3000 rpm for 5 minutes. The cells were then resuspended in 1ml 50mM EDTA. The tubes were centrifuged at 13000 rpm for 30 seconds and the supernatant was discarded. The cells were then resuspended in 200µl 50mM EDTA. 100µl SCE Solution (1M sorbitol, 0.1M sodium citrate, 10mM EDTA, 5-ß-mercaptoethanol and 10mg/ml zymolyase) was added along with 0.5ml low melting point (LMP) agarose (1% SeaKem LMP agarose [*Cambrex Bio Science*] was melted in 0.125M EDTA and then cooled to approximately 45°C). After pipetting to mix thoroughly, the solution was transferred to a 75µl plug former (*Bio-Rad*) on ice and was left to set. Once set, the polymerised plugs were transferred to clean Eppendorf tubes that contained 0.5ml EDTA Solution (0.5M EDTA, 0.1M Tris-HCl pH7.5 and 5% w/v 5-ß-mercaptoethanol). These tubes were incubated for 6 hours at 37°C. The EDTA Solution was then removed using a pipette and was replaced by Proteinase K/RNase Solution (0.5M EDTA, 1% w/v sodium sarkosyl, 10mg/ml Proteinase K and 0.1mg/ml RNase). The tubes were incubated for 12 hours at 37°C so that the proteins and RNA are degraded. The Proteinase K/RNase Solution was removed using a pipette and the plugs were stored in 1ml Tris/EDTA Solution (0.5M EDTA and 0.1M Tris-HCl at pH 7.5) at 4°C.

For the CHEF Gel, the one-third of the plug was cut and loaded into the well of a 1% agarose gel (LE agarose powder dissolved in 0.5x TBE). The CHEF Gels were run for 24 hours in 0.5x TBE running buffer. The programme that was used to separate the chromosomes is given below. The separation of chromosomes is carried out using two switching times, where the current that is running though the electrode alternates at a 120° angle. The program used is as follows:

Stage 1:	Initial Switch Time:	60 seconds
	Final Switch Time:	60 seconds
	Run Time:	15 hours
	Voltage:	6 volts/cm
	Angle:	120°
Stage 2:	Initial Switch Time:	90 seconds
	Final Switch Time:	90 seconds
	Run Time:	9 hours
	Voltage:	6 volts/cm
	Angle:	120°
Pump Speed:	80	

2.2.3 DNA Extraction

Genomic DNA was extracted using a modified version of the phenol chloroform method as described by Borts *et al* (1986). Yeast strains were cultured in liquid YEPD for 12-16 hours at 30°C. The cells were harvested by centrifugation at 3000 rpm for 5 minutes. They were then resuspended in 0.5ml of Solution A (1M sorbitol, 0.2M tris-HCl pH8.5, 0.02M EDTA, 0.1% 5-ß-mercaptoethanol and distilled water). 50µl of 10mg/ml T20 zymolyase was added to this and the cells were incubated at 37°C until they had become spheroblasted. Once spheroblasted, the cells were centrifuged at 13000 rpm for 3 minutes and the supernatant was discarded. The spheroblasted cells were *gently* resuspended in 50µl 1M sorbitol and 0.5ml of Solution B (50mM Tris-HCl pH7.5, 100mM NaCl, 100mM EDTA, 0/5% w/v SDS and distilled water). 0.2mg proteinase K and 50µl 1mg/ml RNase was then added and the cells were incubated at 65°C for 12 hours. After this, the cells were chilled on ice for 5 minutes. 0.5ml phenol chloroform was added and the cells were centrifuged at 13000 rpm for 10 minutes. The top, aqueous layer was taken and placed in a new tube. The phenol chloroform extraction was carried out two more times. 1ml 100% ethanol was then added and the tube was centrifuged at 13000 rpm for 10 minutes. The ethanol was removed and the DNA was washed with 1ml 70% ethanol. After centrifugation at 13000 rpm for 1 minute, the pellet was air dried and then dissolved in 400µl 1x TE (0.1M Tris-HCl pH8 and 1mM EDTA).

2.2.4 Precipitation of PCR products

One tenth total volume of 3M sodium acetate (pH5.2) and twice the volume of cold 100% ethanol were added to the DNA. The resulting mixture was centrifuged for 30 minutes at 13000 rpm. The ethanol was discarded and 1ml 70% ethanol was added to the pellet. The tubes were centrifuged for 1 minute at 13000 rpm. The ethanol was removed and the pellet as air dried. The pellet was then dissolved in 34µl 1x TE (0.1M Tris-HCl pH8 and 1mM EDTA).

2.2.5 Lithium Acetate Transformation

Lithium Acetate Transformation procedures were used to either disrupt an endogenous gene in the genome (as described by Wach *et al* (1994) and illustrated in **Figure 2.1**) or to tag the N- or C-terminus of a particular gene. The method used was carried out as described by Geitz *et al* (1992) using a slightly modified procedure. Yeast strains were cultured in liquid YEPD for 12-16 hours at 30°C. After this, the cells were subcultured by making a 1 in 10 dilution in fresh YEPD liquid media, and were incubated at 30°C for a further 3 hours. Cells were harvested at 3000 rpm for 5 minutes and were washed with 1ml distilled water three times. The cells were then resuspended in 1ml 100mM lithium acetate and were centrifuged at 13000 rpm for 15 seconds. The supernatant was discarded and the cells were resuspended in 1ml 100mM lithium acetate. The cells were split into two aliquots, so that one set could be used as a control. These tubes were centrifuged for 15 seconds at 13000 rpm and the supernatant was discarded. The transformation mix was then added to the cells. For the experimental samples, the transformation mix is made up of 240µl 50% w/v PolyEthylene Glycol (PEG) 3350, 36µl 1M lithium acetate, 50µl 2mg/ml salmon sperm DNA, and 34µl precipitated PCR products (described in **Section 2.2.4**). The tubes were vortexed until the cell pellet had completely dissolved. The cells were heat shocked at 42°C for 40 minutes. For the expression of an antibiotic marker, the tubes were then centrifuged at 7000 rpm for 30 seconds and were resuspended in 1ml fresh YEPD liquid medium. The cells were incubated at 30°C for a further three hours to allow expression of the antibiotic resistant marker. This step was eliminated if the selective marker confers for prototrophy. The cells were then washed twice with distilled water, plated on selective media, and incubated at 30°C for 2-3 days.

Transformants were then streaked for single cells on the selective medium and incubated at 30°C. The single colonies were taken and then streaked for singles on non-selective media (YEPD). This is necessary as although the DNA, which goes to the nucleus, may be expressed, it may not have integrated into the genome. By using non-selective medium, we decrease the pressure of selection. As a result, only cells in which DNA has become integrated into the genome will grow. The colonies from the non-selective medium were then replica plated back onto the selective medium.

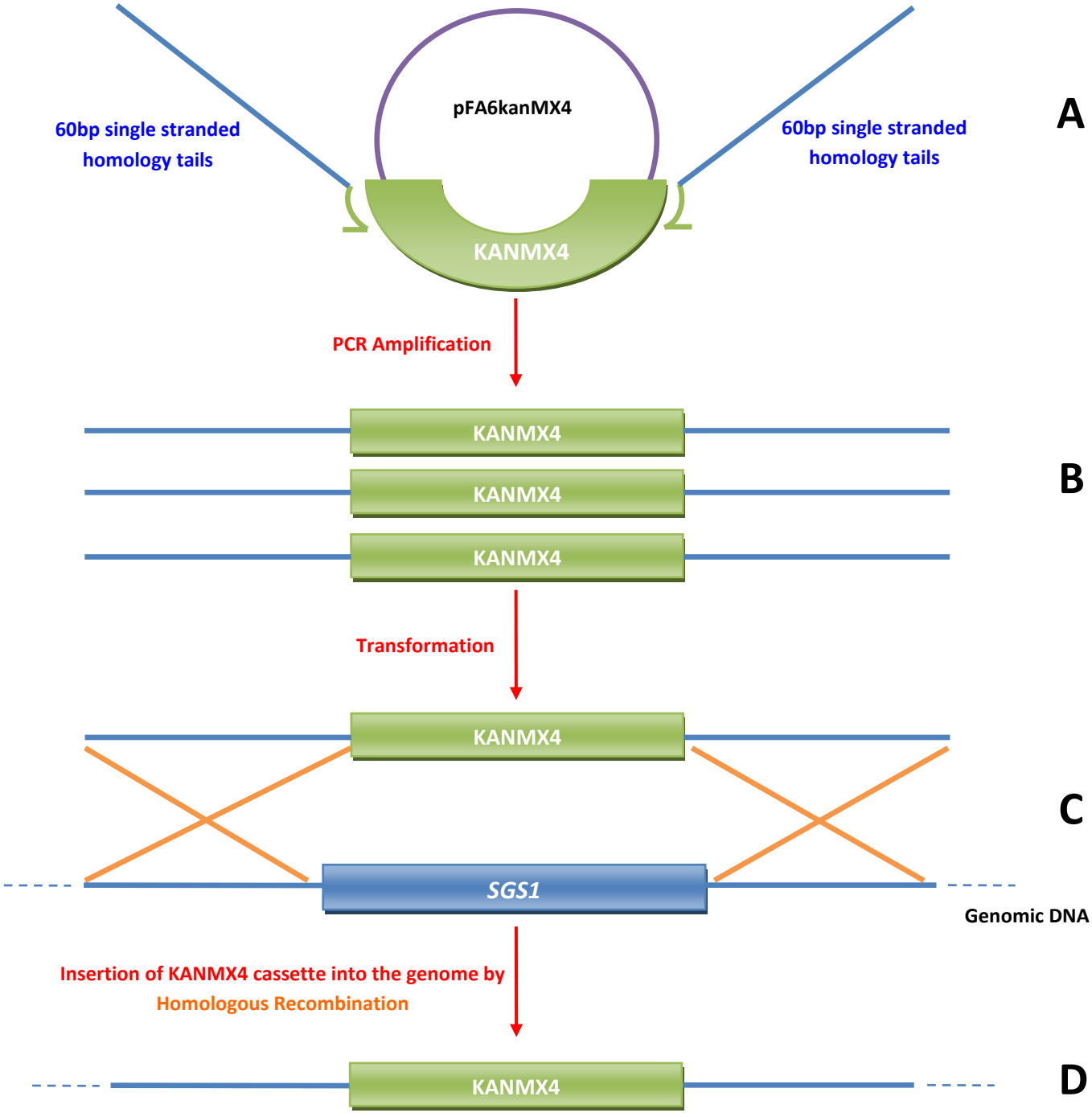


Figure 2.1: An example of PCR-mediated gene deletion (Wach et al., 1994)
In this case, the KANMX4 cassette was amplified from the pFA6kanMX4 plasmid using 80bp primers (**A**). 20bp are designed around the KANMX4 cassette (shown as green arrows) and the remaining 60bp are tails that are homologous to the region to be disrupted in the genome (shown as blue arrows). The PCR products (**B**) are then transformed into the genome using the Lithium Acetate Transformation protocol detailed in **Section 2.2.5**) (**C**). The 60bp tails will direct the construct to the *SGS1* gene where it is integrated into the genome via homologous recombination (**D**).

The correct integration of the transformants was confirmed by PCR amplification of purified genomic DNA (**Section 2.2.3**). This PCR reaction used primers that were designed approximately 100bp upstream and downstream of the desired insertion site along with primers that were constructed within the integrated cassette (**Table 2.6**).

2.2.6 Polymerase Chain Reaction (PCR)

2.2.6.1 Conventional PCR

PCR was originally described by Mullis *et al* (1986). The 11.1x PCR buffer that was used, developed by Jeffreys *et al* (1990), consisted of 45mM Tris-HCl pH8.8, 11mM ammonium sulphate, 4.5mM magnesium chloride, 6.7mM 5-β-mercaptoethanol, 4.4µM EDTA pH8, 1mM dATP, 1mM dCTP, 1mM dGTP, 1mM dTTP and 113µg/ml Bovine Serum Albumin. Primers, ordered from *Invitrogen*, were used at a concentration of 0.2µM per reaction. For cloning experiments, *Phusion* High-Fidelity polymerase, from *New England BioLabs*, was used to decrease the likelihood of nucleotide misincorporation during DNA synthesis. For all other PCR amplification reactions, *Taq* polymerase, from *ABgene*, was used.

The PCR conditions used were as follows - a pre-denaturation step was carried out at 97°C for 3 minutes. This was followed by a cycle of denaturation at 97°C for 30 seconds, an annealing step which varied for each reaction and was determined by gradient PCR (**Section 2.2.6.2**) for 1 minute, and an elongation step at 72°C that varied according to the size of the PCR product. For *Taq* polymerase, the elongation time was 1 minute per kilobase. For *Phusion*, an elongation time of 15 seconds per kilobase was used for the amplification of plasmid DNA. This denaturation-annealing-elongation cycle was repeated 34 more times. This was followed by a final elongation step at 72°C for 10 minutes. All PCR reactions were carried out using *MJ Research Thermal Tetrad Cycler* PCR machines. After completion, the reaction products were run on an agarose gel to confirm amplification (**Section 2.2.1**).

2.2.6.2 Gradient PCR

Gradient PCR was carried out to optimise the annealing temperature for specific primer pairs. The reactions were carried out using the conditions described in **Section 2.2.6** but the annealing temperature was varied from 51°C to 62°C.

2.2.6.3 Sequencing PCR

Sequencing reactions were carried out using either the forward or reverse primer. The reaction consisted of 3µl 5x Sequencing Buffer (supplied by the *PNACL* (Protein Nucleic Acid Chemistry Laboratory) service at the University of Leicester), 1µl Big Dye Terminator v3.1 (supplied by *PNACL*), 2µl 0.02µM forward or reverse primer, 4µl PCR purified DNA (**Section 2.2.7.2**) and double distilled water.

The Sequencing Reaction conditions were as follows - a pre-denaturation step was carried out at 96°C for 5 minutes. This was followed by a cycle of denaturation at 96°C for 10 seconds and then 60°C for 4 minutes to allow synthesis. The last two steps were finally repeated 29 more times. The samples were purified using the *Performa DTR Gel Filtration* cartridges (according to the manufacturer's, *EdgeBio*, instructions) before they were sent to the *PNACL* for sequencing.

2.2.7 Commercial Kits

2.2.7.1 Plasmid miniprep extraction

The QIAgen Miniprep plasmid extraction kit was used to extract plasmid DNA, as described in the manufacturer's instructions, for restriction digestion or PCR amplification (**Section 2.2.6.1**) for Lithium Acetate Transformation reactions (**Section 2.2.5**).

2.2.7.2 - PCR Product Purification

The QIAgen PCR Purification kit was used, as described in the manufacturer's instructions, to purify PCR products before sending them for sequencing (**Section 2.2.6.3**).

2.2.8 Southern Blot Analysis

Southern Blotting (Southern, 1975) was carried out as described by Sambrook *et al* (1989). Hybond-N^+ nitrocellulose membranes (purchased from *Amersham Pharmacia Biotech*) were used to blot fragments of DNA on agarose gels for approximately 12 hours. After the DNA had been transferred, it was cross-linked to the membrane by exposing it to U.V. light at 100µJ/cm^2 for 20 seconds.

2.2.8.1 Probe labelling

The DNA probe was prepared by digesting *pRED 56* with the restriction enzyme *AatII* (*New England BioLabs*). The probe was labelled using DIG High Prime (*Roche*). 16µl of 10ng-3µg of template DNA was heated in a boiling heat block for 5 minutes, following by quickly chilling it on ice. 4µl of DIG-High Prime was added to the denatured DNA sample and the mixture was incubated at 37°C for 1 to

20 hours, depending on the initial concentration of the DNA sample. The reaction was stopped by either the addition of 2µl 0.2M EDTA (pH 8) and/or heating the sample at 65°C for 10 minutes.

2.2.8.2 Pre-hybridisation and Hybridisation of the membrane

After the DNA was cross-linked to the membrane, the membrane was placed DNA side-up in a glass tube. 10ml of pre-heated DIG Easy Hyb (*Roche*) (at 42°C) was added to the tube, which was then placed in a rotating hybridisation oven at 42°C for 1 hour.

During this time, 3.5ml of DNA Easy Hyb was heated at 42°C. To prepare the probe, the stopped probe reaction mixture was added to 50µl of distilled water and the mixture was heated in a boiling heat block for 5 minutes, following by chilling on ice. This mixture was then added to the 3.5ml pre-heated DIG Easy Hyb. After removing the 10ml of DIG Easy Hyb from the glass tube containing the membrane, the probe-DIG Easy Hyb mixture was added to the tube and was incubated in the rotating hybridisation cupboard at 42°C for 12-16 hours.

2.2.8.3 Developing the Blot

After incubating the membrane at 42°C for 12-16 hours, the membrane was washed twice with 200ml of Low Stringency Buffer (2x SSC containing 0.1% SDS) for 5 minutes at room temperature. After this, the membrane was washed twice with 200ml of High Stringency Buffer (0.5x SSC containing 0.1% SDS) at 65°C for 15 minutes. The membrane was then washed with 100ml Washing Buffer (0.1M Maleic acid, 0.15M NaCl pH7.5, 0.3% w/v Tween 20) for 2 minutes at room temperature. This was followed by incubating the membrane with 100ml of 1x Blocking Solution (1g Blocking Reagent [*Roche*] dissolved in 100ml Maleic Acid Buffer [0.1M Maleic acid, 0.15M NaCl pH7.5]) at room temperature for 30 minutes to 3 hours. After this, the membrane was washed with 20ml Antibody Solution (75mU/ml Anti-Digoxigenin 1:10,000 dissolved in Blocking Solution) for 30 minutes at room temperature. The membrane was then washed twice with 100ml Washing Buffer for 15 minutes at room temperature. The membrane was then equilibrated for 3 minutes in 20ml of Detection Buffer (0.1M Tris-HCl, 0.1M NaCl pH9.5). The membrane was then covered with 10µl 0.25mM CSPD (*Roche*) resuspended in 990µl of Detection Buffer and incubated at room temperature for 5 minutes. After this step, the membrane was incubated at 37°C for 10 minutes. The membrane was then placed in an autoradiograph cassette, to which autoradiograph film was added, incubated for 30 minutes and then processed.

2.2.9 Site Directed Mutagenesis

Site directed mutagenesis was used to introduce point mutations at specific locations in *SGS1*. The region of interest was cloned into the *pJET* vector (*Fermentas*) according to the manufacturer's instructions. Inverse primers were designed (as described by the *Stratagene Quick-Change Site Directed Mutagenesis Kit* protocol), which contained the point mutations, such that there were 20 base pairs of homology immediately upstream and downstream from the first and last mutations. After PCR, the products of the reaction were digested with the methylation specific enzyme *DpnI*, and were then transformed into chemically competent cells. *DpnI* specifically cleaves *Dam* methylated sequences that are found in *E. coli* strains but not in PCR products. This ensures that only the PCR products, that will contain the mutated sequence, will be transformed into the chemically competent cells. The DNA was extracted from the colonies (**Section 2.2.7.1**) and was sequenced (**Section 2.2.6.3**) to check for successful construction of the point mutations.

2.2.10 MMS sensitivity

Deletion of Sgs1 during mitosis leads to the cells becoming sensitive to the DNA damaging agents hydroxyurea and methyl methane sulfonate (MMS) (Ui et al., 2001). When placed on agarose plates that have been supplemented with MMS, cells that either lack Sgs1, or have a non-functional Sgs1 protein, will be unable to divide efficiently (Miyajima et al., 2000a, Mullen et al., 2000). Therefore, MMS sensitivity was assessed for *SGS1* and mutant strains by spot assays.

Yeast strains were cultured in liquid YEPD for 12-16 hours at 30°C. The O.D. of each strain was taken at 600nm. Appropriate dilutions were made so that each sample contained the same concentration of cells. Serial dilutions were then made in a total volume of 100µl and 5µl of each dilution was spotted onto the appropriate media – a YEPD plate (used as a control) and a YEPD plate supplemented with 0.02% MMS. The plates were incubated at 20°C for 2 days.

2.2.11 Yeast Techniques

2.2.11.1 Tetrad Dissection and Analysis

Sporulation is achieved by replicating diploid cells onto sporulation media (2% potassium acetate media, 0.22% yeast extract, 0.5% dextrose, 0.0875% complete amino acid and nucleotides mix). The plates were incubated at 23°C for 5 days. After sporulation, the four gametes that arise during meiotic divisions are visible as a four spore tetrad that is surrounded by an ascus. By separating the spores of this tetrad, the products from the same meiotic division can be visualised.

To carry out the dissection, the asci were first resuspended in 100µl Dissecting Buffer (1M sorbitol, 10mM EDTA, 10mM $NaPO_4$ pH7.2) and 5µl 5mg/ml zymolyase (*Sigma*) and were placed at 37°C for 30 minutes. After this incubation, 400µl of Dissecting Buffer was added. The asci were then ready for dissection and were separated using a *Zeiss* phase contrast microscope which had been fitted with a micromanipulator needle. The four spores that make up an individual ascus were spotted at fixed positions along a YEPD plate. The dissected plates were incubated at 30°C for 3 days.

2.2.11.2 Spore Viability

Once the spores had grown, the spore viability was calculated. The following formula was used to calculate the spore viability:

$$\text{Spore Viability} = \frac{([\text{No. 4 spore tetrads} \times 4] + [\text{No. 3 spore tetrads} \times 3] + [\text{No. 2 spore tetrads} \times 2] + [\text{No. 1 spore tetrads} \times 1] + [\text{No. 0 spore tetrads} \times 0])}{[\text{Total No. Tetrads Dissected} \times 4]}$$

2.2.11.3 Replica Plating and Mating Type Testing

After determining the spore viability, the dissection plates were replicated onto different synthetic media plates in order to study the segregation of auxotrophic and drug resistant markers. The mating types of the spores were determined by first replicating the dissecting plate onto a plate covered with *MAT α* or *MAT a* cells. This was incubated at 30°C for 12 hours. The plate, which now contains diploids, was then replicated onto a minimal media plate and was incubated at 30°C for 12 hours. The mating type was determined by assessing the growth on the minimal media plates. The *MAT α* and *MAT a* strains used were *tyr1* and *ura2* which complemented the auxotrophic mutations in the strains used in our laboratory allowing us to score the mating types of the dissected spores. Spores that were *MAT α* would grow when mated to *MAT a* cells but not when mated to *MAT α* cells. Similarly, spores that were *MAT a* would grow when mated to *MAT α* cells but not when mated to M*AT a* cells.

2.2.11.4 DAPI Staining

Diploid cells were cultured in 5ml YEPD for 12-16 hours at 30°C. After this time, 60µl of this overnight culture was added to 50ml of SPS media that was supplemented with amino acids that are required by the diploid. This mixture was incubated at 30°C for 12-16 hours. After this time, the O.D. was measured at a wavelength of 600nm until it reached an O.D. of 1.4. Once this O.D. was reached, the overnight culture was centrifuged at 13000 rpm for 5 minutes. The supernatant was discarded and the pellet was resuspended in potassium acetate medium supplemented with amino

acids as above. This step was repeated once more and the mixture was incubated at 30°C for 24 hours. After this time, 500µl of this mixture was taken and added to 500µl of 100% ethanol. 4µl of this mixture was placed on a glass slide along with 4µl of 1µg/ml DAPI staining solution (*Invitrogen*) and the cells were analysed using a *Zeiss Axioskop 2* microscope. The number of cells with one, two or three/four nuclei was noted.

2.2.11.5 Calculation of the Recombination Rate

After scoring the pattern of segregation of the auxotrophic markers, drug resistance markers and mating types, the data were entered into a *Microsoft Excel* spreadsheet so that it could be analysed using the *MacTetrad 6.9* software. The *MacTetrad 6.9* software calculates the *Parental Ditypes* (PD), *Non-Parental Ditypes* (NPD) and *Tetratypes* (TT) for each interval and also calculates the percentage of recombination in each interval in centiMorgans (cM) according to the *Perkins formula* (Perkins, 1949):

Map Distance = ½(TT + 6NPD) / (PD + NPD + TT)

The PD, NPD, TT and centiMorgan data were calculated for the four viable spore class of tetrads. For the three viable spore class of tetrads, the data were also calculated, but first the genotype of the dead spore was predicted by using the *2nd Law of Mendel* which defines independent assortment.

2.2.11.6 Identification of sister spores

Sister and non-sister spores were classified by the pattern of the centromere marker *TRP1*. In the two viable spore class of tetrads, sisters were identified if both viable spores were auxotrophic or prototrophic for tryptophan. Non-sister spores were identified if one spore was auxotrophic and the other was prototrophic for tryptophan.

2.2.11.7 Identification of Meiosis I Non-Disjunction Events

Meiosis I non-disjunction events for chromosome III were identified by analysing the mating types of the two viable spore class of tetrads (**Figure 1.3D**). Meiosis I non-disjunction leads to two copies of chromosome III from each parent. This means that the spores will contain genetic information from both parents and will therefore be non-maters, which can be assessed by carrying out mating type testing (**Section 2.2.11.3**).

2.2.12 Statistical Analysis

The statistical significance of the data obtained in this study were evaluated using the G-test and the χ^2 test. The type of test used is mentioned where appropriate. Both statistical tests were carried out on two or more sets of data using the null hypothesis, H_0: 'the sets of data are the same' using a 95% confidence level. If the p-value obtained is less than 0.05 ($p < 0.05$), the null hypothesis was rejected and the sets of data are said to be statistically significantly different to each other. If more than two sets of data are compared, the Bonferroni adjustment* was used to minimise the chance of type I errors (i.e. rejecting the null hypothesis even though it is true).

* http://www.quantitativeskills.com/sisa/calculations/bonfer.htm

2.2.13 Analysis of Unequal Sister Chromatid Exchange

Unequal recombination events were investigated using a construct created by Chaix (2007) known as the HYG-CYH/HYG cassette. A hygromycin-cycloheximide cassette has been inserted upstream of *HIS4* and a hygromycin cassette has been inserted downstream of *LEU2* on chromosome III (illustrated in **Figure 2.2A**). The hygromycin cassette confers resistance to hygromycin, whereas the wild type *CYH2* gene confers cycloheximide sensitivity, which is dominant to the endogenous *cyh2* gene located at chromosome VII. When mated to a strain that does not have these cassettes, the rates of unequal recombination can be assessed by analysing the segregation of the hygromycin and cycloheximide markers (**Figure 2.2**). The specific types of unequal events that occurred were determined by CHEF Gel analysis (described in **Section 2.2.2**) and Southern Blottings (described in **Section 2.2.8**). Intra-chromatid events, inter-chromatid events and gene conversion events can be distinguished as illustrated in **Figure 2.2**. This analysis is possible as there are no essential genes present between *HIS4* and *LEU2*.

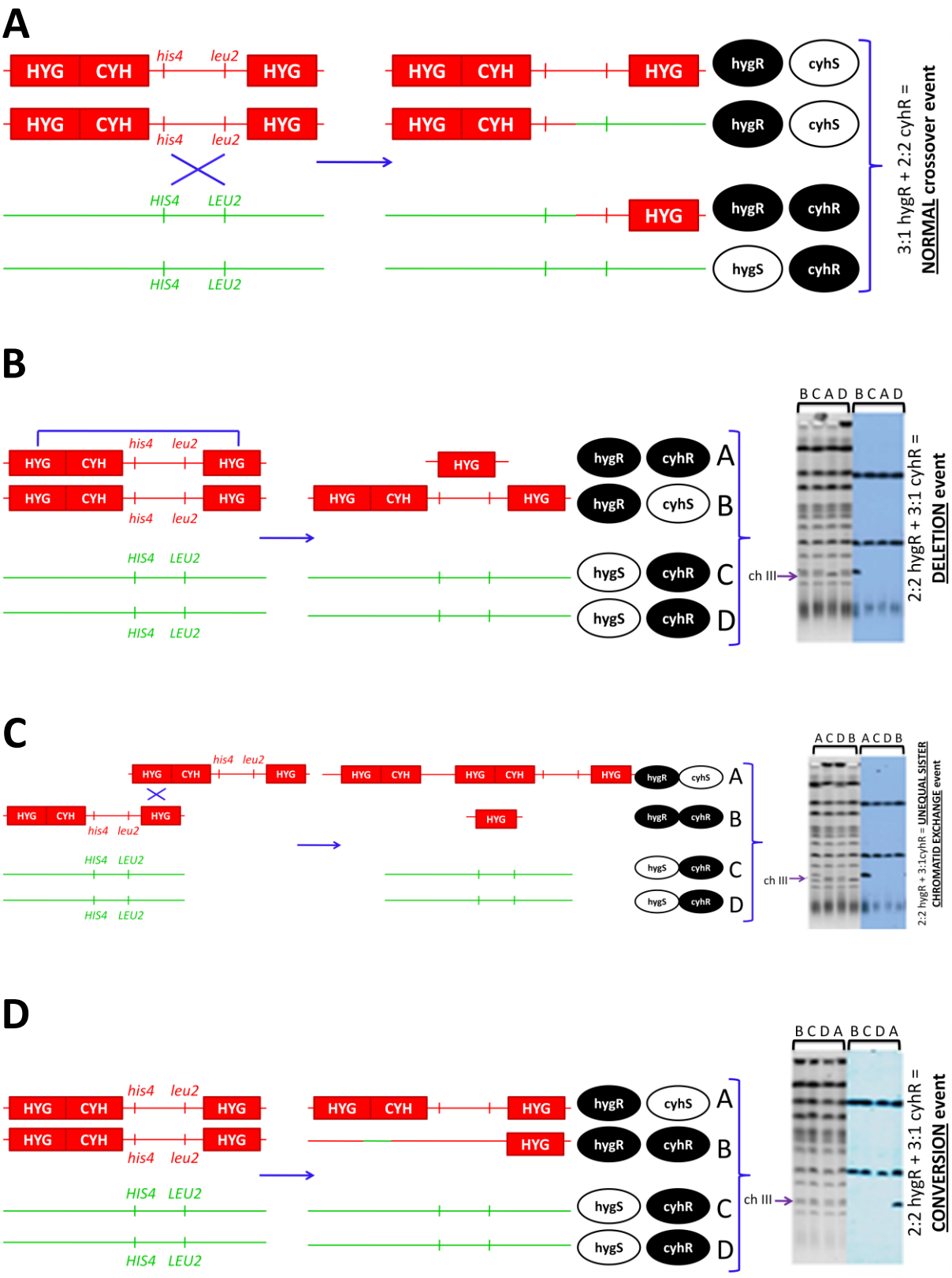

A
HYG
CYH
his4
leu2
HIS4
LEU2
hygR
cyhS
cyhR
hygS
3:1 hygR + 2:2 cyhR =
NORMAL crossover event
B
BCAD BCAD
ch III
2:2 hygR + 3:1 cyhR =
DELETION event
C
ACDB ACDB
2:2 hygR + 3:1cyhR = UNEQUAL SISTER CHROMATID EXCHANGE event
D
BCDA BCDA
2:2 hygR + 3:1 cyhR =
CONVERSION event

Figure 2.2: Unequal Recombination Assay

CHEF Gel and Southern Blot pictures from Chaix (2007)

A: A strain that has the HYG-CYH/HYG cassette (described in **Section 2.2.13** and shown in red) was crossed to a strain that does not (shown in green) in order to assess unequal recombination events. **Figure 2.2A** shows a **normal crossover** event that occurs between *HIS4* and *LEU2*. This event leads to 3:1 hygromycin resistance: sensitivity and 2:2 cycloheximide resistance: sensitivity.

B: Intra-chromatid events (also known as Deletion events). In this event, recombination occurs between the hygromycin cassettes on the same sister strand. This results in a deletion event and is seen as 2:2 hygromycin resistance and 3:1 cycloheximide resistance colonies. Four lanes of a CHEF Gel are shown, each of which represents one spore of a four viable spore tetrad. Chromosome III is indicated with an arrow (→). Due to the deletion event, DNA will have been lost. As a result, the chromosome III band moves down the gel at a faster rate than normal. This results in the absence of a band where expected and a more intense band below, as the band representing chromosome III migrates with the band representing chromosome VI. Southern blot analysis is carried out to confirm the presence of the *CYH2* gene. The probe used is a *URA3-CYH2* probe. The top set of four bands represents the endogenous *URA3* gene on chromosome V, and the second set of four bands represents the endogenous *CYH2* gene on chromosome VII. These serve as controls for the probe. The single band at chromosome III represents the one cycloheximide sensitive spore, confirming the loss of one *CYH2* gene.

C: Inter-chromatid events (also known as Unequal Sister Chromatid Exchange). In this event, a crossover occurs between one hygromycin cassette on one sister strand and another hygromycin cassette on the other sister strand. This event results in a triplication event as well as a reciprocal deletion event and is also seen as 2:2 hygromycin resistance and 3:1 cycloheximide resistance colonies. The triplication event results in an increase in the amount of DNA present. As a result, the band representing chromosome III will migrate more slowly down the gel resulting in a higher band on the CHEF Gel. Once again, Southern blot analysis is used as physical confirmation of the genetic diagnosis, as discussed above (**B**).

D: Gene Conversion events. One other event can arise from 2:2 hygromycin resistance and 3:1 cycloheximide resistance colonies. These are gene conversion events and are not unequal recombination events. It is therefore important to identify these events, so they are not included when analysing the rates of unequal recombination. The gene conversion events do not result in any major increases or decreases in DNA, so the CHEF Gel will look wild type. Therefore Southern Blotting is used to confirm this class of events, as the single band at chromosome III represents the one cycloheximide sensitive spore. This indicates that the event is a 3:1 cycloheximide event, and together with wild-type sized chromosomes, confirms that these are gene conversion events.

Chapter 3: Analysis of the meiotic specific effects of Sgs1

3.1 Introduction

The aim of this investigation was to assess the way in which the Sgs1 helicase suppresses meiotic homeologous recombination during meiosis. However, as discussed in **Section 1.7**, the RecQ helicases, including Sgs1, play a significant role in mitosis. In their absence, DNA damage will persist and will therefore impact meiosis. For instance, in the absence of *sgs1*, mitotic DSBs may not be repaired and the persistence of lesions may lead to replication fork collapse. In humans, BS cells exhibit abnormal replication intermediate formation as well as delayed Okazaki fragment maturation (Davies et al., 2004, Lonn et al., 1990).

In order to assess the meiotic effects of *SGS1*, early studies used a complete deletion. However, defects seen using this complete deletion cannot be specifically attributed to the meiosis-specific defects of *sgs1*, as these defects cannot be differentiated from those caused in the absence of *sgs1* during mitosis. In support of this, Chaix (2007) showed that *sgs1Δ* cells that had undergone approximately 50 mitotic divisions showed a decrease in the number of four viable spore tetrads when compared to *sgs1Δ* cells that had not undergone as many mitotic divisions. This study highlights the impact that mitotic defects can have on spore viability during meiosis. To overcome this issue, many researchers then used a C-terminal truncation of *sgs1*, *sgs1-ΔC795*, that deleted the C-terminal 795 amino acids of the gene, including the helicase domain. This mutation was originally created by Mullen *et al* (2000) who suggested that Sgs1 had a 'bipartite structure'. Studies by Miyajima *et al* (2000a, 2000b) suggested that the N-terminal of *SGS1* is responsible for the mitotic phenotypes of *SGS1*, whereas the C-terminal is mainly responsible for the meiotic roles. This was suggested because the N-terminal 125 amino acids were required for the complementation of the MMS and HU sensitivities and the suppression of the mitotic hyper-recombination. However, cells with only the N-terminal 125 amino acids still exhibited poor sporulation phenotype and a decrease in meiotic recombination, suggesting that the C-terminal is required for normal meiosis (Miyajima et al., 2000b). This was also seen in a study by Rockmill *et al* (2003) who saw that *sgs1-ΔC795* resembles *sgs1Δ* regarding various meiotic defects, including both mutations exhibiting an increase in chromosome 'pseudosynapsis' when combined with a mutation in *zip1* and as well as an increase in crossing over. However, the use of *sgs1-ΔC795* is still an imperfect system, as some of the meiotic functions of *SGS1* may be carried out by the N-terminus and some of the mitotic roles may be dependent on the C-terminus.

The promoter of the *CLB2* gene is increasingly used to overcome this potential problem, as it is expressed only in mitosis and not in meiosis (Grandin and Reed, 1993, Lee and Amon, 2003). Therefore, by cloning this promoter, *pCLB2*, upstream of the start site of *SGS1* will lead to its expression only in mitosis, thereby creating a meiotic null. This *pCLB2-SGS1* construct has already been used in a number of studies (Jessop et al., 2006, Oh et al., 2007, Oh et al., 2008) and has been shown to be a much more suitable method to investigate the meiotic phenotypes of an *sgs1* deletion. Therefore, in order to assess the role of Sgs1 in the suppression of meiotic homeologous recombination, we aimed to clone the *CLB2* promoter upstream of *SGS1* and then cross this meiotic null of *sgs1* to a variety of *sgs1* mutations (**Figure 1.19**).

However, one potential issue with using this approach is the possibility that the Sgs1 helicase may exhibit haploinsufficiency. Haploinsufficiency is seen in diploid organisms where a mutation in one copy of a gene leads to a detectable phenotype. In humans, several genes that cause cancer have been shown to exhibit haploinsufficiency. An example of one of these genes is the breast and ovarian cancer susceptibility gene *BRCA1* (Miki et al., 1994). Staff *et al* (2003) assayed for loss of *BRCA1* in sporadic breast tumours using FISH analysis and RT-PCR. They showed that loss of one copy of the *BRCA1* gene causes a significant decrease in the expression of mRNA, therefore suggesting that *BRCA1* exhibits haploinsufficiency. Another example is the tumour suppressor gene *PTEN* which has been shown to promote prostate cancer (Li et al., 1997). Kwabi-Addo *et al* (2001) showed that the progression of prostate cancer is promoted by the haploinsufficiency of *PTEN*. Mice that retain only one copy of *PTEN* were seen to develop tumours at a significantly greater rate than mice that have both copies of *PTEN* (Kwabi-Addo et al., 2001).

Even the murine homolog of BLM has been shown to exhibit haploinsufficiency (Goss et al., 2002). Goss *et al* (2002) have shown that mice that are heterozygous for *Blm* develop lymphoma much earlier than mice that have both copies of *Blm*. The fact that the murine homolog of BLM is haploinsufficient could affect our experimental design, as we were concerned that Sgs1 may also exhibit haploinsufficiency. Our aim was to cross several mutations of *sgs1* to a meiotic null of *sgs1*. This approach results in only one functional copy of the mutated gene being present. A *Saccharomyces cerevisiae* genome-wide screen has been carried out to determine which *S. cerevisiae* genes showed haploinsufficiency (Deutschbauer et al., 2005). This screen, using heterozygous deletion strains, analysed whether genes exhibited a haploinsufficient growth defect on YPD and minimal media. Although several genes were identified to exhibit this haploinsufficient growth defect, *SGS1* was not one of them. Although this suggests that *SGS1* is not haploinsufficient,

we were concerned that this assay was not sensitive enough to confirm this, as a limitation of this approach was the inability to accurately measure the doubling times of slow growing strains (Deutschbauer et al., 2005). Therefore, we needed to ensure that *SGS1* was not haploinsufficient.

3.1.1 Aim

3.1.1.1 Determining whether *SGS1* exhibits haploinsufficiency

We needed to determine whether *SGS1* exhibited haploinsufficiency in order to use the *pCLB2-SGS1* construct for our investigations, as our experimental approach relies on only one functional copy of the mutated gene being present. To assess whether *SGS1* exhibits haploinsufficieny, we mated an *SGS1* strain to a strain in which *sgs1* had been deleted. After tetrad dissection, we compared the spore viability and recombination data for this heterozygous cross to a *SGS1*/*SGS1* diploid and an *sgs1Δ*/*sgs1Δ* diploid using the G-test, which allows us to compare the distribution of events amongst the different crosses.

3.1.1.2 Analysis of homeologous recombination using a meiotic null of *SGS1*

In order to investigate whether *SGS1* has a role in the suppression of meiotic homeologous recombination, we cloned the *CLB2* promoter upstream of *SGS1*. Using the *pCLB2-SGS1* construct, Jessop *et al* (2006) showed that Sgs1 was degraded rapidly approximately 2 hours after the induction of sporulation, with no detectable amounts of the protein after 4 hours. We utilised two approaches to assess whether our *pCLB2-SGS1* construct caused any mitotic defects. We assessed the sensitivity of our *pCLB2-SGS1* strain to the alkylating agent MMS, which is indicative of an inability to repair lesions leading to replication fork arrest during mitosis. We also compared the overall spore viability of the *pCLB2-SGS1* strain to various other strains, including an *SGS1* and an *sgs1Δ* strain. The ability of Sgs1 to carry out its mitotic functions in the *pCLB2-SGS1* construct is expected to increase the overall spore viability when compared to *sgs1Δ*. On the other hand, the absence of Sgs1 during meiosis is expected to decrease the overall spore viability when compared to *SGS1*.

3.2 Experimental Procedures

3.2.1 Insertion of the *CLB2* promoter upstream of *SGS1*

The *KANMX6-pCLB2-3HA* construct was inserted upstream of *SGS1* in a partial hybrid *S. cerevisiae* strain where one copy of chromosome III has been replaced with chromosome III from the related species *Saccharomyces paradoxus* (SCT14) as well as in a *S. cerevisiae SGS1* strain (ACT 53) (**Figure 1.19A**). This was carried out by amplifying the *KANMX6-pCLB2-3HA* construct using the *pCLB2-SGS1* Fwd and *pCLB2-SGS1* Rev primers (**Table 2.3**) from the *pA6a-KANMX6-pCLB2-3HA* plasmid using *Phusion* High-Fidelity polymerase. The primers were designed so that 20 base pairs were homologous to the *pA6a-KANMX6-pCLB2-3HA* plasmid and 60 base pairs were homologous to the regions immediately upstream and downstream of the ATG of *SGS1*. This ensured that, after transformation (**Chapter 2**) (Wach et al., 1994), the native ATG of *SGS1* was deleted and a 3HA tag was fused in its place. Transformants were plated on YEPD media that was supplemented with Geneticin. After DNA extraction was carried out (**Chapter 2**), the successful insertion of the construct was confirmed by PCR using primers *pCLB-SGS1* A1 and *pCLB2-SGS1* A4 (**Table 2.3**). These primers were designed approximately 500 base pairs upstream and downstream of the *SGS1* start codon. Strains that had successfully incorporated the *KANX6-pCLB2-3HA* construct at the desired location were sequenced (**Chapter 2**) using the *pCLB2-SGS1* Seq F1 and *pCLB2-SGS1* Seq F2 (**Table 2.3**) primers to ensure that no mutations had occurred and also to ensure that the *CLB2* promoter was in-frame with the *SGS1* gene.

3.3 Results

3.3.1 Sgs1 does not exhibit haploinsufficiency

3.3.1.1 Sgs1 is not haploinsufficient with respect to spore viability

In order to test whether *SGS1* exhibits a haploinsufficient phenotype, we made homologous and homeologous chromosome III diploids that were heterozygous for *sgs1Δ*. For the homologous heterozygous diploid (ACD 116), we mated a strain in which *sgs1* had been deleted (*sgs1Δ*) (ACT 56) to an *SGS1* strain (ACT65). For the homeologous heterozygous diploid (ACD 117), we mated the partial hybrid strain, in which *sgs1* had been deleted (ACT2) to an *SGS1* strain (ACT65). After tetrad dissection (**Section 2.2.11.1**), we used the G-test to compare the distribution of viable spores per tetrad from these heterozygous diploids to a homozygous *SGS1* diploid and also to a homozygous *sgs1Δ* diploid (**Figures 3.1** and **3.2**). We used the null hypothesis, H_0: 'the sets of data are the same', using a 95% confidence level. However, by taking into account the Bonferroni adjustment, which minimises the chance of type I errors (as discussed in **Section 2.2.12**), we used a p-value of 0.0125.

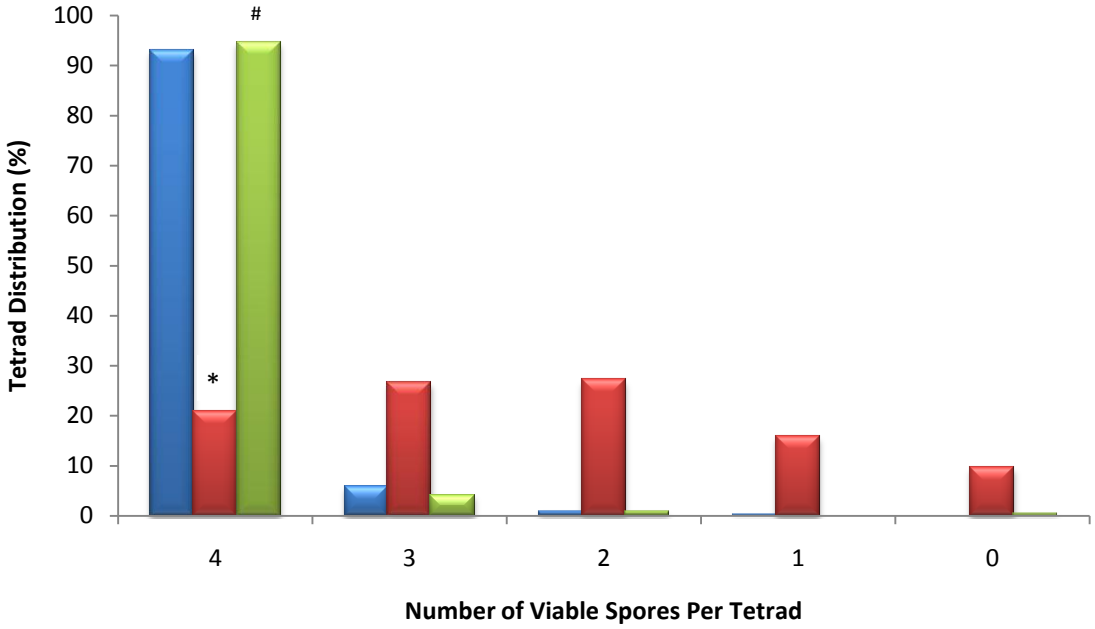


Figure 3.1: Spore viability for homologous chromosome III diploids.
The G-test was used to compare the distribution of four, three, two, one and zero viable spores for the homologous diploids. p-values < 0.0125 were considered significant, allowing us to reject the null hypothesis, H_0: 'the sets of data are the same'. The *SGS1* and *sgs1Δ* data were provided by Chaix (2007).
* =significantly different from *SGS1*/*SGS1*; # = significantly different from *sgs1Δ/sgs1Δ*
SGS1/*SGS1* (ACD 97); *sgs1Δ/sgs1Δ* (ACD 95); *SGS1*/*sgs1Δ* (ACD 116)

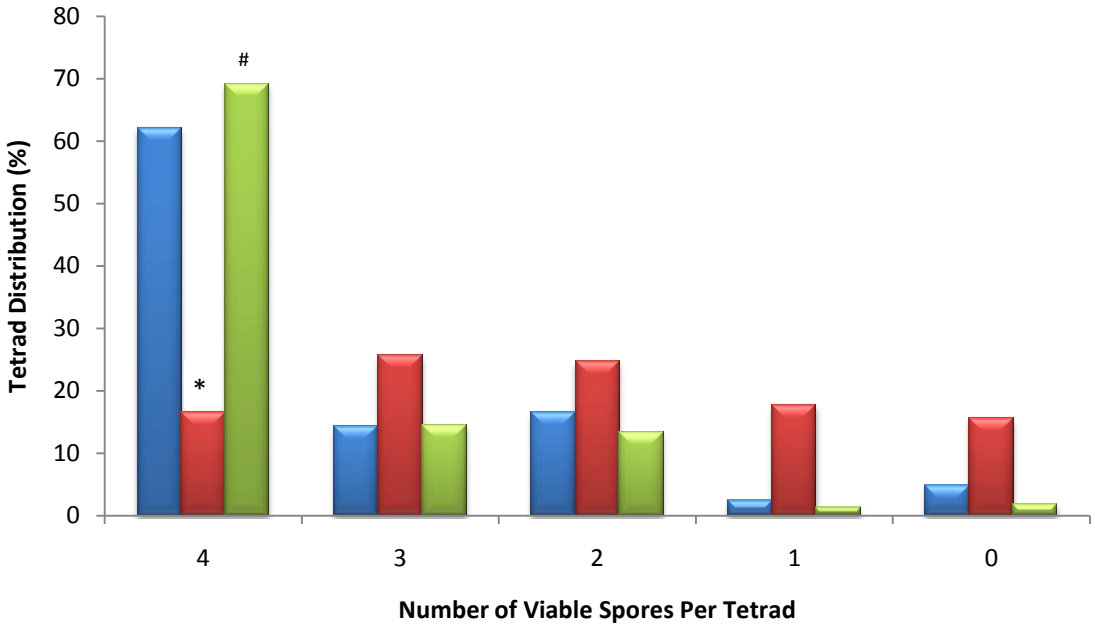


Figure 3.2: Spore viability for homeologous chromosome III diploids.

The G-test was used to compare the distribution of four, three, two, one and zero viable spores for the homeologous diploids. p-values < 0.0125 were considered significant, allowing us to reject the null hypothesis, H_0: 'the sets of data are the same'. The *sgs1Δ* data were provided by Chaix (2007).

* =significantly different from *SGS1*/*SGS1*; # = significantly different from *sgs1Δ/sgs1Δ*

SGS1/*SGS1* (ACD 94); *sgs1Δ/sgs1Δ* (ACD 96); *SGS1*/*sgs1Δ* (ACD 117)

There is no statistical significance between the heterozygous diploids and the *SGS1* homozygous diploids for both the homologous and homeologous data. However, statistical significance is observed when the heterozygous diploids are compared to the *sgs1Δ* homozygous diploids for both the homologous and homeologous data. Therefore, despite only having one copy of *SGS1* the heterozygous diploid exhibits a wild-type phenotype, suggesting that *SGS1* does not display haploinsufficiency with respect to spore viability.

3.3.1.2 Sgs1 does not exhibit haploinsufficiency with respect to crossing over

We assessed the distribution of Parental Ditypes (PD), Non-Parental Ditypes (NPD) and TetraTypes (TT) for the four viable spore tetrads across three intervals along chromosome III – *HML-HIS4*, *HIS4-LEU2* and *LEU2-MAT*. Using the G-test, we compared the distribution of PDs, NPDs and TTs for the heterozygous diploids to the *SGS1* homozygous diploids (**Tables 3.1** and **3.2**). For p-values that were less than 0.05, we rejected the null hypothesis, H_0: 'the sets of data are the same'.

Table 3.1: Map distances on chromosome III in homologous strains.

Interval	Diploid	PD	NPD	TT	Four Viable Spore Tetrads	Map Distance (cM)	p-value
HML-HIS4	*SGS1*/*SGS1* (ACD 97)	196	20	363	579	41.7	n.a.
	SGS1/*sgs1Δ* (ACD 116)	77	7	129	213	40.1	0.834
HIS4-LEU2	*SGS1*/*SGS1* (ACD 97)	404	2	165	571	15.5	n.a.
	SGS1/*sgs1Δ* (ACD 116)	163	1	49	213	12.9	0.246
LEU2-MAT	*SGS1*/*SGS1* (ACD 97)	340	15	250	605	28.1	n.a.
	SGS1/*sgs1Δ* (ACD 116)	133	3	92	228	24.1	0.517

Using the G-test, we compared the distribution of PDs, NPDs and TTs for the heterozygous and *SGS1* homologous diploids. p-values < 0.05 were considered significant allowing us to reject the null hypothesis, H_0: 'the sets of data are the same'. The *SGS1*/*SGS1* data were provided by Chaix (2007).

Table 3.2: Map distances on chromosome III in homeologous strains.

Interval	Diploid	PD	NPD	TT	Four Viable Spore Tetrads	Map Distance (cM)	p-value
HML-HIS4	*SGS1*/*SGS1* (ACD 94)	626	0	4	630	0.317	n.a.
	SGS1/*sgs1Δ* (ACD 117)	221	0	1	222	0.2	0.9506
HIS4-LEU2	*SGS1*/*SGS1* (ACD 94)	630	0	1	631	0.079	n.a.
	SGS1/*sgs1Δ* (ACD 117)	221	0	1	222	0.2	0.7696
LEU2-MAT	*SGS1*/*SGS1* (ACD 94)	611	1	19	631	1.981	n.a.
	SGS1/*sgs1Δ* (ACD 117)	218	1	5	224	2.5	0.6398

Using the G-test, we compared the distribution of PDs, NPDs and TTs for the heterozygous and *SGS1* homeologous diploids. p-values < 0.05 were considered significant allowing us to reject the null hypothesis, H_0: 'the sets of data are the same'.

In all three intervals, for both the homologous and homeologous data, the heterozygous diploid (*SGS1*/*sgs1Δ*) data are not significantly different from *SGS1*/*SGS1*. Therefore, the presence of only one copy of *SGS1* still leads to wild-type rates of crossing over, suggesting that Sgs1 does not exhibit haploinsufficiency with respect to crossing over.

The results suggest that *SGS1* does not exhibit haploinsufficiency. We were thus able to carry out our experimental design where we crossed each of our *sgs1* mutations to either a deletion of *sgs1* or a meiotic null of *sgs1*.

3.3.2 Creation of a meiotic null of *SGS1*

3.3.2.1 MMS sensitivity assay

Various studies have used the *pCLB2-SGS1* construct to assess the meiotic effects of Sgs1 (Jessop et al., 2006, Oh et al., 2007, Oh et al., 2008). Using Western blotting, Jessop *et al* (2006) showed that Sgs1, when under control of the *CLB2* promoter, is degraded approximately 2 hours after sporulation. We cloned the promoter of the *CLB2* gene upstream of *SGS1* in the partial hybrid strain (SCT 14) to create a homeologous meiotic null of *sgs1*, and also in an *SGS1 S. cerevisiae* strain (ACT 53) to use as a homologous control. In order to test whether the insertion of the *CLB2* promoter upstream of *SGS1* led to any mitotic defects we firstly assessed whether our *pCLB2-SGS1* strains were sensitive to the alkylating agent MMS. Exposure to MMS leads to the stalling or collapse of replication forks. If Sgs1 is not present, the forks cannot be restarted, leading to replication fork collapse (Cobb et al., 2003). Therefore, when Sgs1 is deleted in mitosis, the cells become sensitive to MMS (Ui et al., 2001).

Therefore, we assayed our *pCLB2-SGS1* strains (Y55 3565 and Y55 3567) on YEPD plates supplemented with 0.02% MMS. Previous work has shown that this concentration of MMS is suitable for MMS sensitivity assays (Amin, *unpublished*). As a positive control, we used the *SGS1* strain ACT53. As a negative control, we used the *sgs1Δ* strain ACT56. These controls ensured that the MMS had not deteriorated, as previous work has shown that YEPD plates supplemented with MMS are only suitable for use for approximately two weeks (Amin, *unpublished*).

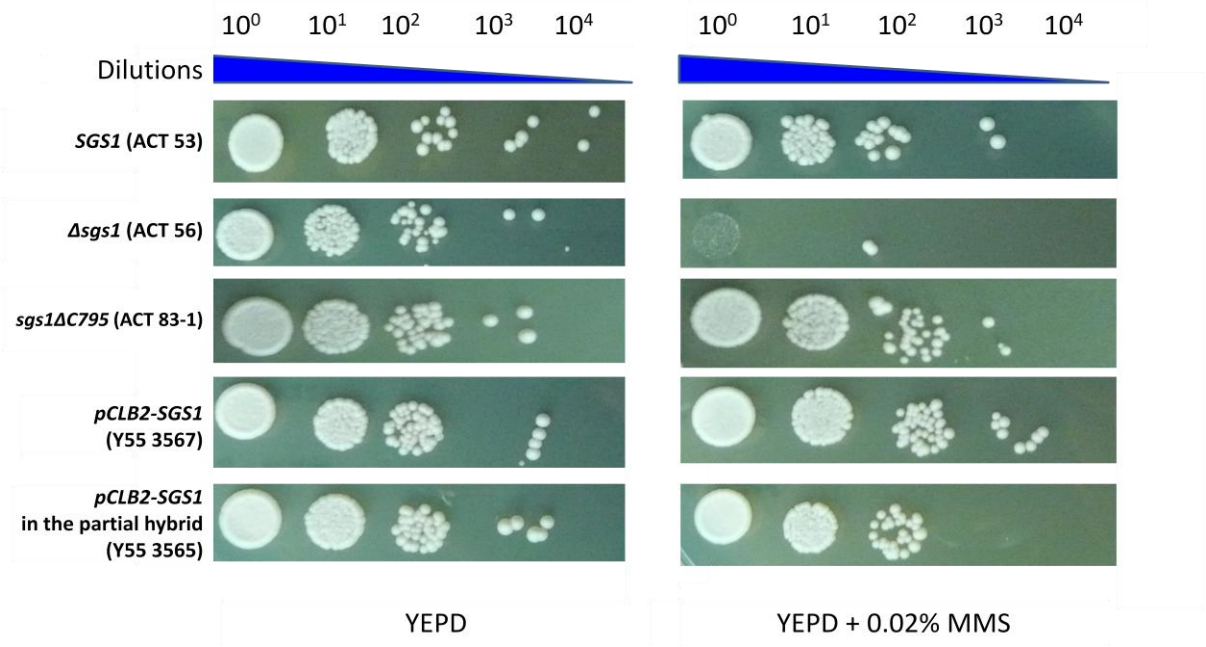


Figure 3.3: Testing the growth of the *pCLB2-SGS1* constructs with respect to MMS resistance by spotting serial dilutions onto YEPD plates (as a control) and YEPD plates supplemented with 0.02% MMS. Failure to grow on YEPD media supplemented with 0.02% MMS is indicative of an inability to repair lesions which lead to the stalling of replication forks during mitosis.

Growth on YEPD plates was used as a control for cell number. As previously shown, deletion of Sgs1 results in sensitivity to MMS (Miyajima et al., 2000a, Mullen et al., 2000, Ui et al., 2001) (**Figure 3.3**).

The purpose of this experiment was to assess whether the introduction of the *KANMX6-pCLB2-3HA* construct in front of *SGS1* affected the ability of Sgs1 to restart replication forks that have stalled due to the presence of lesions induced by exposure to MMS. As seen in **Figure 3.3**, both the homologous and homeologous *pCLB2-SGS1* constructs exhibit resistance to MMS. This suggests that *pCLB2-SGS1* expresses sufficient amounts of Sgs1 protein to confer resistance to MMS. Therefore, sufficient amounts of Sgs1 are being produced to fulfil its mitotic role in restarting stalled replication forks. Interestingly, however, the homeologous *pCLB2-SGS1* strain appears to be slightly sensitive to MMS. This result is discussed further in **Section 3.4**.

3.3.2.2 An improvement in spore viability is seen in the *pCLB2-SGS1* strains when compared to *sgs1Δ*

To further assess whether the *KANMX6-pCLB2-3HA* construct was expressing Sgs1 during mitosis, we utilised the G-test to compare the distribution of viable spores per tetrad for the *pCLB2-SGS1* strain (ADA 1) to an *SGS1* homozygous strain (ACD 97), an *sgs1Δ* homozygous strain (ACD 95) and an *sgs1-ΔC795*/*sgs1Δ* (ADA 3) (**Table 3.3** and **Figure 3.4**). We used the null hypothesis, H_0: 'the sets of data

are the same', using a 95% confidence level. However, as multiple data sets were being compared, we applied the Bonferroni adjustment, which minimises the chance of type I errors (as discussed in **Section 2.2.12**), and therefore used a p-value of 0.01.

Table 3.3: Spore viability of *pCLB2-SGS1* compared to different homologous diploids.

Homologous Chromosome III Diploid	**Spore Viability***	**Percentage of viable spore tetrads (%)**				
		4	**3**	**2**	**1**	**0**
SGS1/SGS1 (ACD 97)	98%	93	6	1	0	0
SGS1/sgs1Δ (ACD 116)	98%	95	4	1	0	0
sgs1Δ/sgs1Δ (ACD 95)	58%	21	27	27	16	10
sgs1-ΔC795/sgs1Δ (ADA 3)	73%	37	31	22	7	3
pCLB2-SGS1/sgs1Δ (ADA 1)	82%	48	32	19	1	0

*$\text{Spore Viability} = \frac{[(\text{No. 4 spore tetrads} \times 4) + (\text{No. 3 spore tetrads} \times 3) + (\text{No. 2 spore tetrads} \times 2) + (\text{No. 1 spore tetrads} \times 1) + (\text{No. 0 spore tetrads} \times 0)]}{[\text{Total No. Tetrads Dissected} \times 4]}$

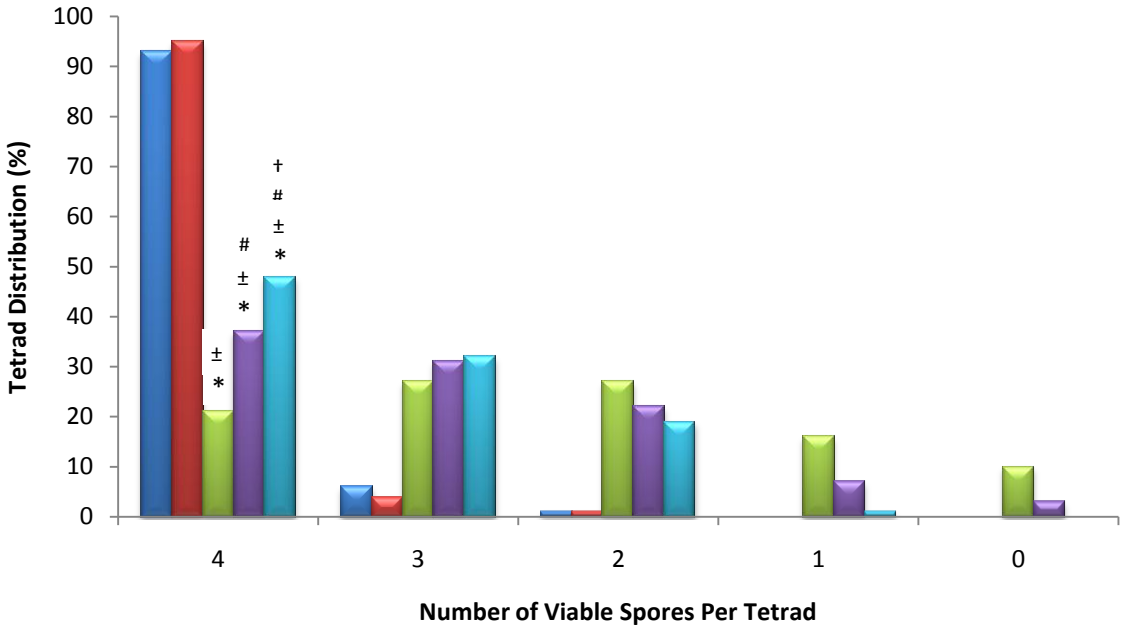


Figure 3.4: Distribution of viable spores for *pCLB2-SGS1* compared to different homologous chromosome III diploids.

The G-test was used to compare the distribution of four, three, two, one and zero viable spores for the homologous diploids. p-values < 0.01 were considered significant, allowing us to reject the null hypothesis, H_0: 'the sets of data are the same'. The *sgs1Δ/sgs1Δ* and *sgs1-ΔC795/sgs1Δ* data were provided by Chaix (2007).

* =significantly different from *SGS1/SGS1*; ± = significantly different from *SGS1/sgs1Δ*;

= significantly different from *sgs1Δ/sgs1Δ*; † = significantly different from *sgs1-ΔC795/sgs1Δ*

SGS1/SGS1 (ACD 97); *SGS1/sgs1Δ* (ACD 116); *sgs1Δ/sgs1Δ* (ACD 95); *sgs1-ΔC795/sgs1Δ* (ADA 3); *pCLB2-SGS1/sgs1Δ* (ADA 1)

The distribution of viable spores shown here is in agreement with the data presented by Jessop *et al* (2006) who also cloned the promoter of the *CLB2* gene upstream of *SGS1*.

The data show that *sgs1Δ/sgs1Δ* exhibits a statistically significant decrease in overall spore viability when compared to the *SGS1*/*SGS1* diploid. This can be attributed to the occurrence of both mitotic and meiotic defects that occur in the absence of *sgs1*, leading to an increase in spore death. The spore viability is significantly improved for the *sgs1-ΔC795/sgs1Δ* diploid when compared to *sgs1Δ/sgs1Δ*. This is expected as the N-terminal region of Sgs1 has been reported to be responsible for the mitotic functions of the gene (Mullen et al., 2000). Therefore, by retaining this part of the gene, Sgs1 is supposedly present during mitosis. Sgs1 is therefore able to fulfil its mitotic roles, and hence we see an improvement in the spore viability when compared to a full deletion. The spore viability for *pCLB2-SGS1/sgs1Δ* is also significantly improved when compared to *sgs1Δ/sgs1Δ*. As the MMS sensitivity assay (**Section 3.3.2.1**) suggests that sufficient amounts of Sgs1 protein are being produced in the *pCLB2-SGS1/sgs1Δ* strain during mitosis, the improvement in spore viability can be attributed to Sgs1 being able to fulfil its mitotic roles. This further implies that cloning the *CLB2* promoter upstream of Sgs1 does not affect the ability of Sgs1 to act during mitosis. Interestingly, the *pCLB2-SGS1/sgs1Δ* data are significantly improved when compared to the *sgs1-ΔC795/sgs1Δ* data. This supports the hypothesis that the *sgs1-ΔC795* mutant does not complement all of the mitotic defects seen when *sgs1* is deleted. Therefore, the *sgs1-ΔC795* mutant may not suitable for analysing the meiotic-specific effects of Sgs1, as we see a greater amount of death when compared to the meiotic null of *sgs1*.

3.4 Discussion

The data presented show that both the homologous and homeologous heterozygous diploids are not statistically significantly different to *SGS1* (**Figures 3.1** and **3.2** and **Tables 3.1** and **3.2**). If haploinsufficiency results in an abnormal phenotype when only one copy of the gene is present, we can say, from this data, that *SGS1* does not exhibit haploinsufficiency. The data further support the observations made by Deutschbauer *et al* (2005) who reported that *SGS1* does not display a haploinsufficient phenotype with respect to its ability to grow on YPD and minimal media. Therefore, we can conclude that having one functional copy of *SGS1* is enough to lead to a wild-type response.

The promoter of the *CLB2* gene was inserted in front of *SGS1* to make a meiotic null of *SGS1*. Previously, the C-terminal truncation *sgs1-ΔC795* was used (Mullen et al., 2000), as it is proposed that the mitotic functions, but not the meiotic functions, of Sgs1 are carried out by the N-terminus. Rockmill *et al* (2003) showed that *sgs1-ΔC795* resembled *sgs1Δ* for various meiotic defects, including an increase in crossing over and increased chromosome synapsis. However, this is predicted to be an imperfect system, as it cannot be certain that the mitotic functions of Sgs1 are exclusively carried out by the N-terminus.

After inserting the *KANMX6-pCLB2-3HA* construct in front of *SGS1*, we needed to ensure that it did not disrupt the mitotic functions of Sgs1. To test this, we carried out MMS sensitivity assays (**Figure 3.3**) on YEPD plates supplemented with 0.02% MMS, as deletion of Sgs1 in mitosis leads to sensitivity to MMS (Ui et al., 2001). This reflects an inability for cells lacking Sgs1 in restarting replication forks that have stalled due to the presence of lesions caused by exposure to MMS. As shown in **Figure 3.3**, the *pCLB2-SGS1* strains are resistant to MMS. However, interestingly, the partial hybrid strain containing the *pCLB2-SGS1* construct (Y55 3565) shows slight sensitivity to MMS. This may reflect an issue with the *pCLB2-SGS1* construct which affects the levels of expression of Sgs1 during mitosis. To ascertain whether cloning the *CLB2* promoter upstream of *SGS1* affects vegetative growth in this strain, growth curves or FACS analysis could be carried out and compared to *SGS1* and *sgs1Δ* strains.

By inserting the *CLB2* promoter in front of *SGS1*, transcription of *SGS1* should occur during mitosis but not during meiosis, enabling Sgs1 to carry out its mitotic functions in the cell. If this is the case, we should see a noticeable improvement in spore viability for the *pCLB2-SGS1* strain when compared to *sgs1Δ*. As shown in **Table 3.3** and **Figure 3.4**, we see this improvement in spore viability (similar to that observed by Jessop *et al* (2006)) which can be attributed to the absence of mitotic problems. Interestingly, we also see a significant improvement in spore viability of the

pCLB2-SGS1 construct when compared to the C-terminal truncation *sgs1-ΔC795*. This provides evidence that the C-terminal construct does not allow Sgs1 to fully fulfil its mitotic obligations, and, as a result, it still leads to some mitotic death.

Although the data imply that there is expression of the Sgs1 protein during mitosis, the data cannot confirm that the Sgs1 protein is not expressed during the onset of meiosis. Jessop *et al* (2006) carried out Western blotting on samples obtained at various time points after the onset of sporulation. Their data suggest that the expression of the Sgs1 protein is repressed approximately 2 hours after the onset of sporulation, with no noticeable traces of the protein after 4 hours. We attempted to repeat the time course experiments carried out by Jessop *et al* (2006), but were unsuccessful, as we were unable to identify the 3HA-tagged Sgs1 protein on our Western blots. Instead, we continuously saw 'smearing' of the samples, suggesting that the protein was being degraded. We attribute our inability to carry out Western blotting using our *pCLB2-SGS1* strains to the strain background we use in our laboratory. We suggest that tagging Sgs1 in the Y55 background renders the protein unstable and therefore leads to its degradation. We propose this based on previous work, where we attempted to tag the C-terminus of Sgs1 with GFP in order to monitor the nuclear localisation of the protein (Amin, *unpublished*). Confocal microscopy revealed that the Sgs1-GFP protein was targeted to the lysosome for degradation, suggesting that this construct was unstable (Amin, *unpublished*). A way in which to test whether the strain background was in fact the issue would be to tag the Sgs1 protein in another strain background and assess whether this construct is more stable than its Y55 counterpart.

As mentioned above, we saw a statistically significant increase in spore viability for *pCLB2-SGS1* when compared to an *sgs1Δ* diploid. We interpret that this improvement in spore viability is caused by the presence of Sgs1 during mitosis, allowing it to carry out its mitotic roles. However, as shown in **Table 3.3** and **Figure 3.4**, the spore viability for the *pCLB2-SGS1* strain is significantly decreased when compared to *SGS1*. Assuming Sgs1 is expressed during mitosis, we propose that the decrease in spore viability compared to *SGS1* can be attributed to meiotic defects caused by the absence of Sgs1. This implies that the *CLB2* promoter is functioning as expected, and is repressing the transcription of the Sgs1 protein during meiosis. In addition to this, sequencing data revealed that the *CLB2* promoter in both strains did not contain any mutations, and was cloned in-frame of *SGS1*. Therefore, we concluded that this construct was suitable for use in our experiments.

Chapter 4: Does the Mismatch Repair Complex recruit Sgs1 to the sites of homeologous recombination?

4.1 Introduction

As described in **Section 1.8**, work by Chambers *et al* (1996), Malik (*unpublished*) and Chaix (2007) implicated the MMR system and Sgs1 in the suppression of meiotic homeologous recombination. We proposed a model in which this suppression is achieved via the assessment of sequence divergence between SEI events taking place between diverged sequences (**Figure 1.17**). In addition to this, Msh2 and Pms1 have also been implicated in the rejection of strand capture when sequences are diverged (Chambers et al., 1996) (**Figure 1.18**).

4.1.1 Aim

The aim of this study was to determine whether the MMR complex was responsible for the recruitment of Sgs1 to the sites of homeologous recombination at the SEI stage. We aimed to assess whether Mlh1, in particular, was responsible for this recruitment, due to its role as a molecular matchmaker (Sancar and Hearst, 1993, Wang and Kung, 2002). In order to do this, we used a strain containing several point mutations in *SGS1* that abolished the interaction between Sgs1 and Mlh1 (Dherin et al., 2009, Gellon et al., 2002) (**Figure 1.19A** and described in **Section 4.2.1**). We mated this *sgs1-mlh1-id* strain to the partial hybrid strain in which *sgs1* had been deleted (*sgs1Δ*) (ACT 2), as well as to a *S. cerevisiae sgs1Δ* strain (ACT 56) (as a homologous control). After tetrad dissection and G-test analysis, we were able to assess the rates of crossing over and rates of meiosis I non-disjunction in the partial hybrid cross. By comparing the data obtained from the *sgs1-mlh1-id/sgs1Δ* diploid to an *SGS1*/*SGS1* diploid, an *sgs1Δ*/*sgs1Δ* diploid as well as a *pCLB2-MSH2/msh2Δ* diploid, we were able to assess the effects of the *sgs1-mlh1-id* mutant on the ability of Sgs1 to suppress meiotic homeologous recombination. We used the *pCLB2-MSH2/msh2Δ* data as a positive control, as Chambers *et al* (1996) and Malik (*unpublished*) have previously shown that Msh2 plays a role in the suppression of meiotic homeologous recombination.

In addition to this, we wanted to determine whether Sgs1 also played a role in the strand capture stage when sequences are diverged, along with Msh2 and Pms1 (**Figure 1.18**). To do this, we assessed whether the dead spore in the three viable spore class of tetrads for a meiotic null of *sgs1* was recombinant in a given interval by predicting the genotype of the dead spore using the *2nd Law of Mendel* which defines independent assortment. This assessment was made using the χ^2-test,

which allowed us to ascertain whether the number of recombinant events in the dead spore deviated from the expected 50:50 ratio of recombinants : non-recombinants if death was random.

Previously, it has been shown that deletion of Sgs1 results in an increase in unequal recombination events, suggesting that Sgs1 plays a role in maintaining the barrier to sister-chromatid recombination (Chaix, 2007, Onoda et al., 2000). We therefore also wanted to determine whether the interaction between Sgs1 and Mlh1 was important for this role. We assessed the rates of unequal recombination using the Unequal Recombination Assay described in **Section 2.2.13** and illustrated in **Figure 2.2**. By mating a strain containing a HYG-CYH/HYG cassette on chromosome III, where a HYG-CYH cassette is inserted upstream of *HIS4* and a HYS cassette is inserted downstream of *LEU2*, to a strain that does not, we were able to monitor the rates of unequal recombination using CHEF Gels (**Section 2.2.2**) and Southern blotting (**Section 2.2.8**) and compare them to *sgs1Δ* and *SGS1* homozygous diploids.

4.2 Experimental Procedures

4.2.1 Mutation of the Mlh1 interacting domain of Sgs1

Gellon *et al* (2002) mapped the Mlh1 interacting domain of Sgs1 to a serine residue at position 1383 and two phenylalanine residues at positions 1385 and 1386. Mutation of these sites has been shown to abolish the interaction between Sgs1 and Mlh1 (Dherin et al., 2009). Using site directed mutagenesis, R. Mason (*unpublished*) changed each of these sites to alanine residues. The resulting strain, which contained all three point mutations, was named *sgs1-mlh1-id* (mutation of the Mlh1 interaction domain of Sgs1). This was achieved by firstly creating the point mutations on a plasmid copy of *SGS1* and disrupting the region with a *KANMX4-K.lactis-URA3* cassette in the genome. The corresponding region was amplified from the plasmid by PCR and transformed into the genome, screening for 5-FOA resistant colonies that were sensitive to G418. 5-FOA is counter-selective for uracil. Therefore, 5-FOA resistant colonies that were also G418 sensitive indicate that the *KANMX4-K.lactis-URA3* cassette has been replaced by the PCR amplified sequence from the plasmid. After subsequent sequencing to confirm this, three independent transformants, were obtained – Y55 3543, Y55 3544 and Y55 3535 (**Table 2.1**) – and were used in this study.

4.3 Results

4.3.1 MMS sensitivity assay

We wanted to determine whether the *sgs1-mlh1-id* point mutations affected the ability of Sgs1 to restart replication forks that have stalled due to the presence of lesions caused by exposure to MMS. Resistance to MMS indicates that the point mutations do not inhibit this role of Sgs1 (Ui et al., 2001). As in **Section 3.3.2.1**, we used the *SGS1* strain ACT53 as a positive control, and the *sgs1Δ* strain ACT56 as a negative control.

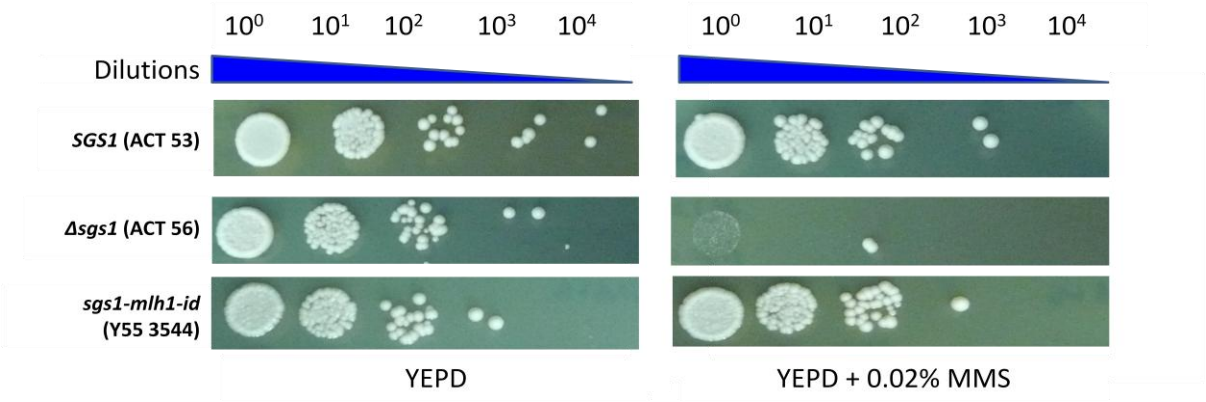


Figure 4.1: Testing the growth of the *sgs1-mlh1-id* mutant with respect to MMS resistance by spotting serial dilutions onto YEPD plates (as a control) and YEPD plates supplemented with 0.02% MMS. Failure to grow on YEPD media supplemented with 0.02% MMS is indicative of an inability to repair lesions which lead to the stalling of replication forks during mitosis. Only one of the three *sgs1-mlh1-id* transformants, Y55 3544, is shown here. The other two transformants produced a similar pattern of growth. The photographs for the *SGS1* strain (ACT 53) and the *sgs1Δ* strain (ACT 56) are the same as in **Figure 3.3** and are shown here for comparison.

As shown in **Figure 4.1**, the *sgs1-mlh1-id* point mutation confers wild-type resistance to MMS. This suggests that mutating these sites in *SGS1* does not affect the role of Sgs1 in restarting stalled replication forks during mitosis.

4.3.2.1 Disrupting the interaction between Sgs1 and Mlh1 does not affect homologous recombination

To assess whether disrupting the interaction between Sgs1 and Mlh1 affected the ability of Sgs1 to suppress homeologous recombination, we mated *sgs1-mlh1-id* to ACT56 (*sgs1Δ*) and also to ACT2 (*sgs1Δ* in the partial hybrid strain). The *pCLB2-SGS1* strains were not used, as they had not yet been constructed. After tetrad dissection, the distribution of Parental Ditypes (PD), Non-Parental Ditypes (NPD) and TetraTypes (TT) for the four viable spore tetrads across three intervals – *HML-HIS4*, *HIS4-LEU2* and *LEU2-MAT* located on chromosome III were calculated according to the Perkins formula (Perkins, 1949) (**Section 2.2.11.5**) using the *MacTetrad 6.9* software. We tested the three independent transformants of the *sgs1-mlh1-id* mutation (Y55 3543, Y55 3544 and Y55 3545) for homogeneity by comparing the distribution of PDs, NPDs and TTs using the G-test. As multiple data sets were compared, the Bonferroni correction (**Section 2.2.12**) was applied, and therefore p-values that were less than 0.0167 allowed us to reject the null hypothesis, H_0: 'the sets of data are the same'.

Table 4.1: Map distances on chromosome III in homologous *sgs1-mlh1-id* strains

	Homologous Chromosome III Diploids	PD	NPD	TT	Four Viable Spore Tetrads	Map Distance (cM)	p-values
HML-HIS4	*sgs1-mlh1-id 1.6 / sgs1Δ* (ADA 4)	76	4	138	218	37.2	0.634
	sgs1-mlh1-id 2.14 / sgs1Δ (ADA 6)	72	6	110	188	38.8	
	sgs1-mlh1-id 6.10 / sgs1Δ (ADA 8)	65	3	125	193	37	
HIS4-LEU2	*sgs1-mlh1-id 1.6 / sgs1Δ* (ADA 4)	153	0	65	218	14.9	0.363
	sgs1-mlh1-id 2.14 / sgs1Δ (ADA 6)	135	0	53	188	14.1	
	sgs1-mlh1-id 6.10 / sgs1Δ (ADA 8)	145	1	45	191	13.4	
LEU2-MAT	*sgs1-mlh1-id 1.6 / sgs1Δ* (ADA 4)	141	2	81	224	20.8	0.893
	sgs1-mlh1-id 2.14 / sgs1Δ (ADA 6)	122	2	73	197	21.6	
	sgs1-mlh1-id 6.10 / sgs1Δ (ADA 8)	118	1	80	199	21.6	

Using the G-test, we compared the distribution of PDs, NPDs and TTs for *sgs1-mlh1-id/sgs1Δ* homologous diploids. p-values < 0.0167 were considered significant allowing us to reject the null hypothesis, H_0: 'the sets of data are the same'.

Table 4.2: Map distances on chromosome III in homeologous *sgs1-mlh1-id* strains

	Homeologous Chromosome III Diploids	PD	NPD	TT	Four Viable Spore Tetrads	Map Distance (cM)	p-values
HML-HIS4	*sgs1-mlh1-id 1.6 / sgs1Δ* (ADA 5)	235	0	5	240	1	0.818
	sgs1-mlh1-id 2.14 / sgs1Δ (ADA 7)	293	0	3	296	0.5	
	sgs1-mlh1-id 6.10 / sgs1Δ (ADA 9)	216	0	5	221	1.1	
HIS4-LEU2	*sgs1-mlh1-id 1.6 / sgs1Δ* (ADA 5)	241	0	0	241	0	0.812
	sgs1-mlh1-id 2.14 / sgs1Δ (ADA 7)	297	0	1	298	0.2	
	sgs1-mlh1-id 6.10 / sgs1Δ (ADA 9)	218	0	1	219	0.2	
LEU2-MAT	*sgs1-mlh1-id 1.6 / sgs1Δ* (ADA 5)	228	1	12	241	3.7	0.0761
	sgs1-mlh1-id 2.14 / sgs1Δ (ADA 7)	293	0	6	299	1	
	sgs1-mlh1-id 6.10 / sgs1Δ (ADA 9)	207	0	13	220	3	

Using the G-test, we compared the distribution of PDs, NPDs and TTs for *sgs1-mlh1-id*/*sgs1Δ* homeologous diploids. p-values < 0.0167 were considered significant allowing us to reject the null hypothesis, H_0: 'the sets of data are the same'.

The data for these three transformants were not significantly different from each other for either the homologous and homeologous diploids (**Tables 4.1** and **4.2**). We therefore pooled the results and analysed the data from the three *sgs1-mlh1-id* mutants collectively.

The distribution of PDs, NPDs and TTs for the collective *sgs1-mlh1-id/sgs1Δ* data were compared to data obtained for *sgs1Δ* by Chaix (2007) and for *pCLB2-MSH2* by Malik (*unpublished*) using the G-test (**Table 4.3** and **Figure 4.1**). We compared our data to *pCLB2-MSH2* as data by both Chambers *et al* (1996) and Malik (*unpublished*) showed that Msh2 is involved in the suppression of meiotic homeologous recombination, and therefore this data serves as a positive control. As we were comparing the distribution of multiple data sets, we applied the Bonferroni correction, and so p-values that were less than 0.01 allowed us to reject the null hypothesis, H_0: 'the sets of data are the same'.

Table 4.3: Crossing over data for intervals along chromosome III for homologous diploids

	Homologous Chromosome III Diploids	**PD**	**NPD**	**TT**	**Total Number of Four Viable Spore Tetrads**
HML-HIS4	*SGS1*/*SGS1* (ACD 97)	196	20	363	579
	SGS1/ *sgs1Δ* (ACD 116)	77	7	129	213
	sgs1Δ/ *sgs1Δ* (ACD 95)	85	9	109	203
	pCLB2-SGS1/ *sgs1Δ* (ADA 1)	16	2	31	49
	pCLB2-MSH2 / *msh2Δ* (QMD 1)	68	3	125	196
	sgs1-mlh1-id/ *sgs1Δ* (ADA 4+6+8)	213	13	373	599
HIS4-LEU2	*SGS1*/*SGS1* (ACD 97)	404	2	165	571
	SGS1/ *sgs1Δ* (ACD 116)	163	1	49	213
	sgs1Δ/ *sgs1Δ* (ACD 95)	154	5	43	202 *
	pCLB2-SGS1/ *sgs1Δ* (ADA 1)	38	0	10	48
	pCLB2-MSH2 / *msh2Δ* (QMD 1)	141	0	52	193
	sgs1-mlh1-id/ *sgs1Δ* (ADA 4+6+8)	433	1	163	597 #
LEU2-MAT	*SGS1*/*SGS1* (ACD 97)	340	15	250	605
	SGS1/ *sgs1Δ* (ACD 116)	133	3	92	228
	sgs1Δ/ *sgs1Δ* (ACD 95)	124	7	100	231
	pCLB2-SGS1/ *sgs1Δ* (ADA 1)	29	2	21	52
	pCLB2-MSH2 / *msh2Δ* (QMD 1)	104	8	98	210
	sgs1-mlh1-id/ *sgs1Δ* (ADA 4+6+8)	381	5	234	620 ‡

Using the G-test, we compared the distribution of PDs, NPDs and TTs for homologous diploids. p-values < 0.01 were considered significant allowing us to reject the null hypothesis, H_0: 'the sets of data are the same'. The *SGS1*/*SGS1* (ACD 97) and *sgs1Δ*/*sgs1Δ* (ACD 116) diploid data were provided by Chaix (2007) and the *pCLB2-MSH2*/*msh2Δ* (QMD 1) diploid data were provided by Malik (*unpublished*). The *sgs1-mlh1-id/sgs1Δ* data is the pooled data from the diploid crosses ADA 4, ADA 6 and ADA 8 (see **Table 4.1**). The *SGS1*/*SGS1* (ACD 97) and heterozygote (ACD 116) data are the same as in **Table 3.1** and are shown here for comparison.

* =significantly different from *SGS1*/*SGS1*; # = significantly different from *sgs1Δ/sgs1Δ*;

‡ = significantly different from *pCLB2-MSH2/msh2Δ*

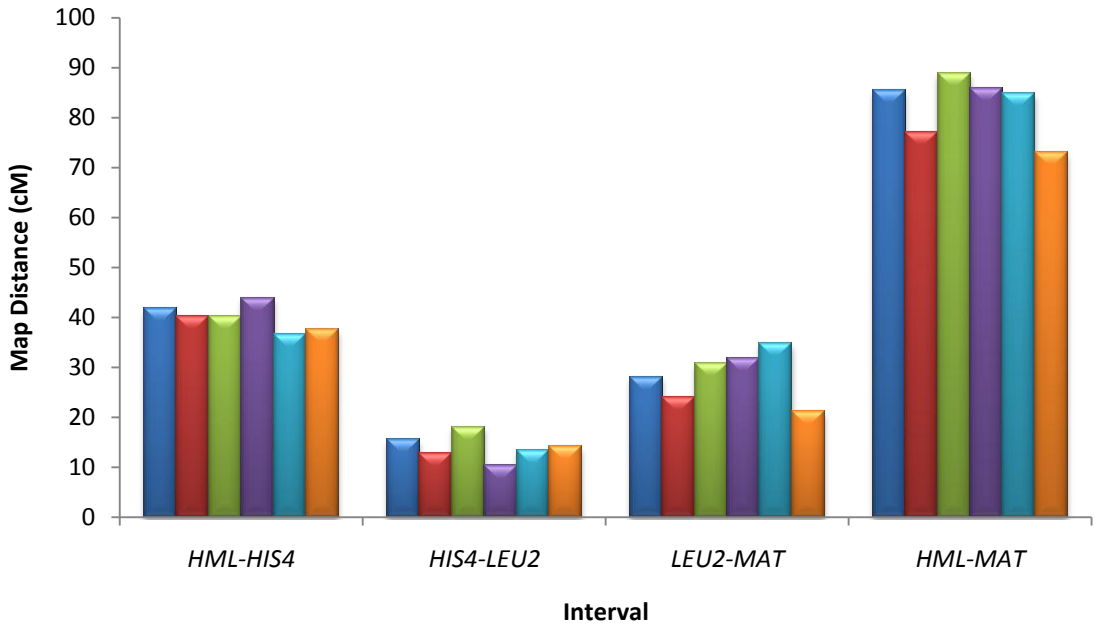


Figure 4.1: Genetic map distances for intervals along chromosome III for homologous diploids.
The map distances (in centiMorgans) for the *HML-HIS4*, *HIS4-LEU2* and *LEU2-MAT* intervals were calculated using the following formula (using the PD, NPD and TT data shown in **Table 4.3**):

$$\frac{\frac{1}{2}(TT + 6NPD)}{PD + NPD + TT}$$

The map distances for the *HML-MAT* interval were calculated by summing the map distances for the other three intervals. The *SGS1/SGS1* (ACD 97) and *sgs1Δ/sgs1Δ* (ACD 116) diploid data were provided by Chaix (2007) and the *pCLB2-MSH2/msh2Δ* (QMD 1) diploid data were provided by Malik (*unpublished*). The *sgs1-mlh1-id/sgs1Δ* data is the pooled data from the diploid crosses ADA 4, ADA 6 and ADA 8 (see **Table 4.1**).

SGS1/SGS1 (ACD 97); *SGS1/sgs1Δ* (ACD 116); *sgs1Δ/sgs1Δ* (ACD 95); *pCLB2-SGS1/sgs1Δ* (ADA 1); *pCLB2-MSH2/msh2Δ* (QMD 1); *sgs1-mlh1-id/sgs1Δ* (ADA 4, 6, 8)

As shown in **Table 4.3** and **Figure 4.1**, *sgs1-mlh1-id* does not affect the rate of homologous recombination, as no significant difference is seen from the *SGS1* homozygous diploid for any of the three intervals. Previously, it has been shown that a deletion of *sgs1* leads to a modest yet significant increase in homologous recombination (Jessop et al., 2006, Rockmill et al., 2003, 2006). Our data (**Table 4.3** and **Figure 4.1**) do not agree with this in two of the three intervals. We suggest that this discrepancy may be due to the large number of tetrads dissected by these groups. Alternatively, this difference may be due to the different strain backgrounds used in these studies.

4.3.2.2 The presence of increased sequence divergence leads to an overall decrease in the levels of recombination

The distribution of PDs, NPDs and TTs for the collective *sgs1-mlh1-id/sgs1Δ* data in the partial hybrid were compared to data obtained for *sgs1Δ* in the partial hybrid and for *pCLB2-MSH2* in the partial hybrid (as a positive control) by Malik (*unpublished*) using the G-test (**Table 4.4** and **Figure 4.2**). As multiple data sets were compared, the Bonferroni correction was applied, and so p-values that were less than 0.01 allowed us to reject the null hypothesis, H_0: 'the sets of data are the same'.

Table 4.4: Crossing over data for intervals along chromosome III for homeologous diploids

	Homeologous Chromosome III Diploids	PD	NPD	TT	Total Number of Four Viable Spore Tetrads
HML-HIS4	*SGS1/SGS1* (ACD 94)	626	0	4	630
	SGS1/sgs1Δ (ACD 117)	221	0	1	222
	sgs1Δ/ sgs1Δ (ACD 96)	95	0	5	100
	pCLB2-SGS1/ sgs1Δ (ADA 2)	194	0	11	205 * ±
	pCLB2-MSH2 / msh2Δ (QMD 2)	210	1	7	218 *
	sgs1-mlh1-id/ sgs1Δ (ADA 5+7+9)	744	0	13	757
HIS4-LEU2	*SGS1/SGS1* (ACD 94)	630	0	1	631
	SGS1/sgs1Δ (ACD 117)	221	0	1	222
	sgs1Δ/ sgs1Δ (ACD 96)	97	0	3	100
	pCLB2-SGS1/ sgs1Δ (ADA 2)	202	0	3	205
	pCLB2-MSH2 / msh2Δ (QMD 2)	219	0	2	221
	sgs1-mlh1-id/ sgs1Δ (ADA 5+7+9)	756	0	2	758
LEU2-MAT	*SGS1/SGS1* (ACD 94)	611	1	19	631
	SGS1/sgs1Δ (ACD 117)	218	1	5	224
	sgs1Δ/ sgs1Δ (ACD 96)	90	1	9	100
	pCLB2-SGS1/ sgs1Δ (ADA 2)	179	2	24	205 * ±
	pCLB2-MSH2 / msh2Δ (QMD 2)	204	2	17	223 *
	sgs1-mlh1-id/ sgs1Δ (ADA 5+7+9)	728	1	31	760 †

Using the G-test, we compared the distribution of PDs, NPDs and TTs for homeologous diploids. p-values < 0.01 were considered significant allowing us to reject the null hypothesis, H_0: 'the sets of data are the same'. The *pCLB2-MSH2/msh2Δ* (QMD 2) diploid data were provided by Malik (*unpublished*). The *sgs1-mlh1-id/sgs1Δ* data is the pooled data from the diploid crosses ADA 5, ADA 7 and ADA 9 (see **Table 4.2**). The *SGS1/SGS1* (ACD 94) and heterozygote (ACD 117) data are the same as in **Table 3.2** and are shown here for comparison.

* =significantly different from *SGS1/SGS1*; ± = significantly different from *SGS1/sgs1Δ*;

† = significantly different from *pCLB2-SGS1/sgs1Δ*

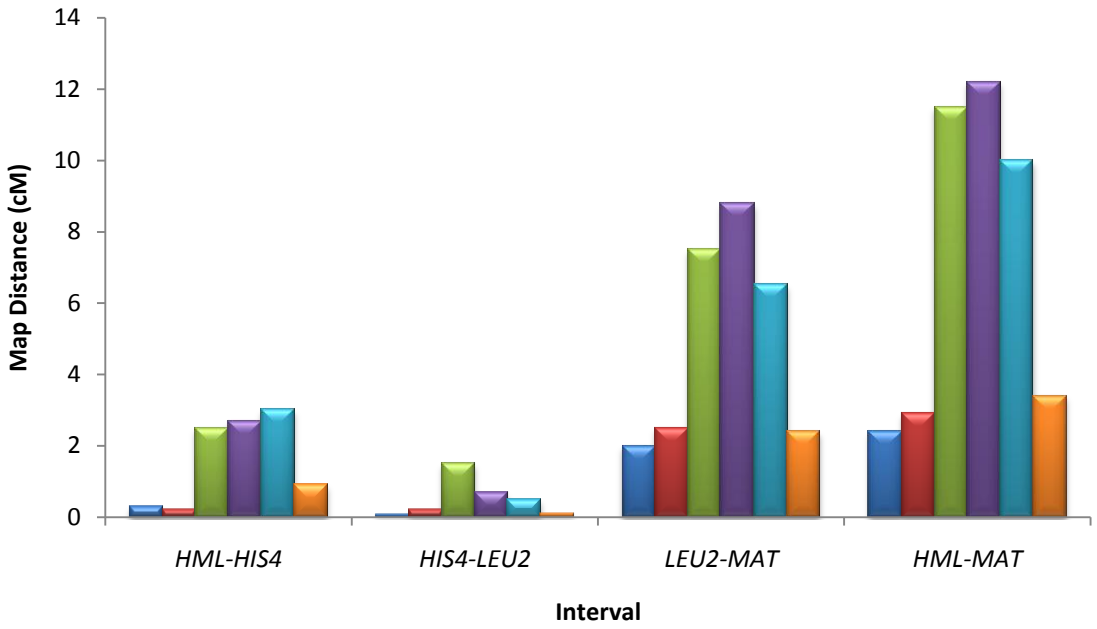


Figure 4.2: Genetic map distances for intervals along chromosome III for homeologous diploids.
The map distances (in centiMorgans) for the *HML-HIS4*, *HIS4-LEU2* and *LEU2-MAT* intervals were calculated using the following formula (using the PD, NPD and TT data shown in **Table 4.4**):

$$\frac{\frac{1}{2}(TT + 6NPD)}{PD + NPD + TT}$$

The map distances for the *HML-MAT* interval were calculated by summing the map distances for the other three intervals. The *pCLB2-MSH2/msh2Δ* (QMD 2) diploid data were provided by Malik (*unpublished*). The *sgs1-mlh1-id/sgs1Δ* data is the pooled data from the diploid crosses ADA 5, ADA 7 and ADA 9 (see **Table 4.2**).

SGS1/SGS1 (ACD 94); *SGS1/sgs1Δ* (ACD 117); *sgs1Δ/sgs1Δ* (ACD 96); *pCLB2-SGS1/sgs1Δ* (ADA 2); *pCLB2-MSH2/msh2Δ* (QMD 2); *sgs1-mlh1-id/sgs1Δ* (ADA 5, 7, 9)

By comparing the homologous (**Figure 4.1**) and homeologous (**Figure 4.2**) diploid data sets, we observe a significant decrease in the map distances in the partial hybrid. We see map distances of up to approximately 90cM for the homologous chromosome III diploids (**Figure 4.1**) compared to map distances of up to approximately 12cM for the homeologous chromosome III diploids (**Figure 4.2**). This decrease is suggested to be due to the presence of sequence divergence, which will result in fewer successful strand invasion events, and therefore a lower level of recombination. The greatest decrease is seen in the *HIS4-LEU2* interval. This is most probably due to the fact that this is the most diverged region out of the three intervals analysed. Although the complete aligned sequence of *S. paradoxus* is not yet available, contigs are available from the NCBI website. By piecing these contigs together and blasting them against the corresponding interval from *S. cerevisiae* (from the *Saccharomyces Genome Database* - http://yeastgenome.org/) using the Fungal BLAST tool on the *Saccharomyces Genome Database* (http://www.yeastgenome.org/cgi-bin/blast-fungal.pl), we can estimate the degree of similarity for each interval (**Figure 4.3**). The *HML-HIS4* interval between *S. paradoxus* and *S. cerevisiae* are approximately 86% identical. The *LEU2-MAT*

interval is approximately 83% identical between *S. paradoxus* and *S. cerevisiae*. However, the *HIS4-LEU2* interval shares only 52% identity. DasGupta and Radding (1982) saw that the formation of heteroduplex DNA was dependent on RecA in *E. coli*, which can facilitate heteroduplex formation for sequences that are up to 30% divergent. We propose that in our assay, the *HIS4-LEU2* interval, which shows the greatest degree of divergence between *S. cerevisiae* and *S. paradoxus*, contains stretches of sequences that are too diverged for successful Rad51- and/or Dmc1- catalysed heteroduplex DNA formation (Rad51 and Dmc1 are the *S. cerevisiae* homologs to RecA and are depicted in **Figure 1.2**). This is proposed to account for the greatest decrease in homeologous recombination that is seen in this interval.

The data shown in **Table 4.4** and **Figure 4.2** suggest that *sgs1-mlh1-id* has no obvious effect on the rates of homeologous recombination as no significant increase in the rates of homeologous recombination were observed when compared to *SGS1* for any of the three intervals. This is in contrast to both *pCLB2-SGS1* and *pCLB2-MSH2*, which have been previously implicated in the suppression of homeologous recombination. In this study, both *pCLB2-SGS1* and *pCLB2-MSH2* exhibit a significant increase in homeologous recombination for two of the three intervals when compared to *SGS1* (**Table 4.4**). No significant increase in recombination was observed from *SGS1* in the *HIS4-LEU2* interval for either *pCLB2-SGS1* or *pCLB2-MSH2* in this study (**Table 4.4**). For reasons discussed above and illustrated in **Figure 4.3**, we propose that this is due to the high degree of sequence divergence in this region between *S. cerevisiae* and *S. paradoxus*, which will result in fewer successful strand invasions.

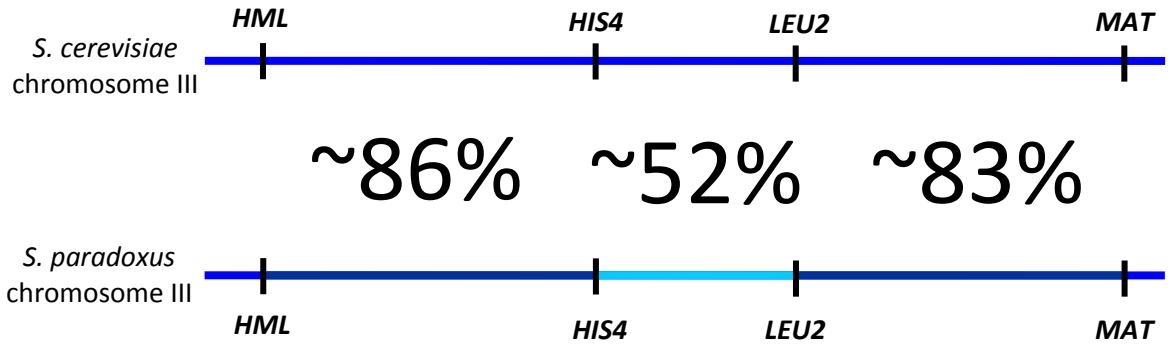


Figure 4.3: Sequence comparison between chromosome III of *S. cerevisiae* and *S. paradoxus*
Using the Fungal BLAST tool on the *Saccharomyces Genome Database* (http://www.yeastgenome.org/cgi-bin/blast-fungal.pl), we can estimate the degree of similarity for each of the three intervals assessed along chromosome III. *HML-HIS4* shares approximately 86% sequence identity, *HIS4-LEU2* shares approximately 52% sequence identity, and *LEU2-MAT* shares approximately 83% sequence identity between *S. cerevisiae* and *S. paradoxus*. There are no essential genes located in the *HIS4* and *LEU2* interval.

4.3.3 Meiosis I non-disjunction events increase with a decrease in recombination

We assessed the levels of meiosis I non-disjunction (discussed in **Section 2.2.11.7** and illustrated in **Figure 1.4**). As shown in **Figure 1.4**, meiosis I non-disjunction results in the death of two spores. The two remaining, viable spores are disomic, containing information from both parents. As the mating-type cassettes are located on chromosome III, strains that are disomic for chromosome III will be non-maters. Therefore, the number of meiosis I non-disjunction events that have occurred were measured by assessing the number of non-maters amongst the two viable spore class of tetrads (**Table 4.5** and **Figure 4.4**).

Previously, Chaix (2007) showed that the levels of meiosis I non-disjunction are increased when homeologous sequences are present during meiosis (also repeated for this study and shown in **Table 4.5** and **Figure 4.4**). This high level of meiosis I non-disjunction was interpreted to indicate a failure of crossing over due to the presence of sequence divergence. Chaix (2007) proposed that the presence of sequence divergence will decrease the levels of crossing over. This is consistent with the crossing over data shown in **Table 4.4** and **Figure 4.2**. The decrease in crossing over was proposed to increase the likelihood of missegregation events from occurring, and therefore, increase the likelihood of meiosis I non-disjunction. As a result, the *SGS1* homeologous diploid led to 11.5% of meiosis I non-disjunction events (**Table 4.5** and **Figure 4.4**). In addition to this, Chaix (2007) also showed that by deleting *sgs1*, the levels of meiosis I non-disjunction were significantly decreased from *SGS1* (also repeated for this study and shown in **Table 4.5** and **Figure 4.4**). This was proposed to further support a role for Sgs1 in the suppression of meiotic homeologous recombination. By deleting Sgs1, the levels of homeologous recombination are increased (**Table 4.4** and **Figure 4.2**). Increasing the levels of crossing over were hypothesised to decrease the likelihood of missegregation events (Chaix, 2007). As a result, only 0.8% of meiosis I non-disjunction events were recovered for the *sgs1Δ* homeologous diploid (**Table 4.5** and **Figure 4.4**). In agreement with this, Malik (*unpublished*) showed that the levels of meiosis I non-disjunction were significantly decreased from *SGS1*, further supporting a role for both Msh2 and Sgs1 in the suppression of homeologous recombination (also shown in **Table 4.5** and **Figure 4.4** for comparison).

We assessed the levels of meiosis I non-disjunction for the *pCLB2-SGS1/sgs1Δ* and *sgs1-mlh1-id/sgs1Δ* homeologous diploids and compared them to the data obtained by Chaix (2007) and Malik (*unpublished*) (**Table 4.5** and **Figure 4.4**). For *pCLB2-SGS1* we recovered 4.6% meiosis I non-disjunction events. This significantly differs from *SGS1*, which agrees with the observations by Chaix (2007) for the *sgs1* deletion. However, the data obtained for *pCLB2-SGS1* also significantly differs

from *sgs1Δ*. This discrepancy is proposed to reflect the presence of mitotic, as well as meiotic, defects that will be present in the *sgs1Δ* mutant. However, the mitotic defects are predicted to be absent in the *pCLB2-SGS1* strain, as sufficient amounts of Sgs1 protein are proposed to be present to fulfil the mitotic roles of Sgs1 (as discussed in **Chapter 3**). In agreement with this, the *pCLB2-MSH2/msh2Δ* data provided by Malik (*unpublished*) is also significantly different from both *SGS1* and *sgs1Δ*, whereas it is not significantly different from *pCLB2-SGS1*.

Table 4.5: Levels of meiosis I non-disjunction events for the homeologous diploids

Homeologous Chromosome III Diploids	Meiosis I Non-Disjunction Events	Total Number of Tetrads	Percentage of Meiosis I Non-Disjunction Events
SGS1 /SGS1 (ACD 94)	120	1040	11.5%
sgs1Δ / sgs1Δ (ACD 96)	5	620	0.8% *
pCLB2-SGS1 / sgs1Δ (ADA 2)	25	539	4.6% *#
pCLB2-MSH2 / msh2Δ (QMD 2)	19	390	4.9% *#
sgs1-mlh1-id / sgs1Δ (ADA 5+7+9)	80	1140	7.0% *#

Using the G-test, we compared the numbers of meiosis I non-disjunction events (by summing the number of non-maters for the two viable spore class of tetrads) for homeologous diploids. As multiple data sets were compared, the Bonferroni correction was applied and so p-values < 0.01 were considered significant, allowing us to reject the null hypothesis, H_0: 'the sets of data are the same'. The *pCLB2-MSH2/msh2Δ* (QMD 2) diploid data were provided by Malik (*unpublished*). The *sgs1-mlh1-id/sgs1Δ* data is the pooled data from the diploid crosses ADA 5, ADA 7 and ADA 9 (see **Table 4.2**).
* =significantly different from *SGS1/SGS1*; # = significantly different from *sgs1Δ/ sgs1Δ*

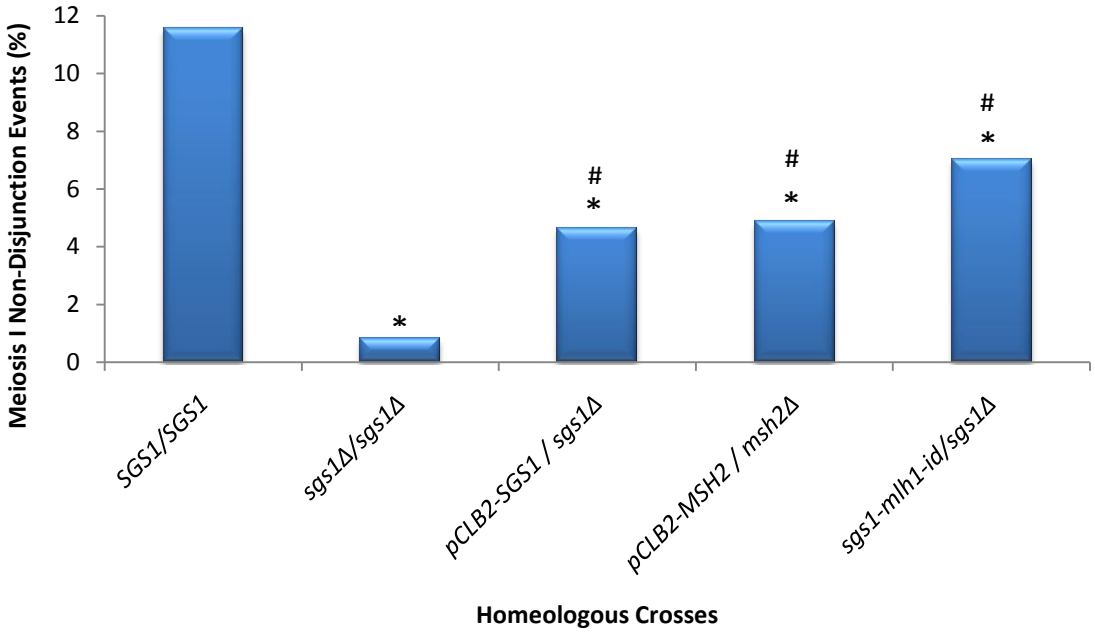


Figure 4.4: Graphical representation of the rates of meiosis I non-disjunction events

As described for **Table 4.5**, the G-test was used to compare the rates of meiosis I non-disjunction for the homeologous chromosome III diploids. p-values < 0.01 were considered significant allowing us to reject the null hypothesis, H_0: 'the sets of data are the same'. The *pCLB2-MSH2/msh2Δ* (QMD 2) diploid data were provided by Malik (*unpublished*). The *sgs1-mlh1-id/sgs1Δ* data is the pooled data from the diploid crosses ADA 5, ADA 7 and ADA 9 (see **Table 4.2**).
* =significantly different from *SGS1/SGS1*; # = significantly different from *sgs1Δ/ sgs1Δ*

As shown in **Table 4.5** and **Figure 4.4**, *sgs1-mlh1-id* shows a significant decrease in the levels of meiosis I non-disjunction when compared to *SGS1*. However, *sgs1-mlh1-id* does not significantly differ from *pCLB2-SGS1*. Assuming the hypothesis by Chaix (2007) (discussed above), the data suggest that *sgs1-mlh1-id* does affect the ability of Sgs1 in suppressing meiotic homeologous recombination. In further support of this, *sgs1-mlh1-id* is not significantly different to *pCLB2-MSH2* (Malik, *unpublished*), which has been previously implicated in the suppression of homeologous recombination (Chambers et al., 1996).

However, the data contradict the crossing over data (**Table 4.4** and **Figure 4.2**). Chaix (2007) proposed that the decrease in meiosis I non-disjunction observed when *sgs1* was deleted is a consequence of an increase in crossing over between diverged sequences that is permitted in the absence of the helicase. The crossing over data shown in **Table 4.4** and **Figure 4.2** shows that abolishing the interaction between Sgs1 and Mlh1 does not lead to a significant increase from *SGS1*.

One could argue that the reason for this is that the crossing over data only measures crossovers across three intervals, while the meiosis I non-disjunction gives an indication of a failure of crossing over across the entire length of chromosome III. Therefore, it could be argued that the meiosis I non-disjunction data is a more sensitive approach to assessing the effects of sequence divergence on recombination. If this is true, then the data implicate the interaction between Sgs1 and Mlh1 as being important in the suppression of meiotic homeologous recombination.

Alternatively, this meiosis I non-disjunction data may be representative of another role of Sgs1 during meiosis, in the segregation of chromosomes. We recovered 11.5% of meiosis I non-disjunction events for the *SGS1* homeologous diploid (**Table 4.5** and **Figure 4.4**). This can be interpreted as the segregation of homologs being random in 23% of the cells, with half of these not suffering from non-disjunction by chance. This means that the majority of cells (77%) were still able to segregate their homologs in an ordered manner. This segregation may be achieved via a crossover, as even though the levels of crossing over were decreased in the *SGS1* homeologous diploid, they were not eliminated (**Table 4.4** and **Figure 4.2**). Alternatively, the ordered segregation of homologs may be achieved by an alternative mechanism, such as distributive segregation. Distributive segregation is a phenomenon originally reported in *Drosophila* where chromosomes of the same size are seen to segregate correctly despite the absence of crossovers. Distributive segregation has also been reported to occur in *S. cerevisiae* but has been reported to not be dependent on chromosome size (Dawson et al., 1986, Guacci and Kaback, 1991, Mann and Davis,

1986, Kaback et al., 1989). Taking this into account, we recovered 4.6% of meiosis I non-disjunction events for the *pCLB2-SGS1/sgs1Δ* diploid. Therefore, 9.2% of cells exhibited random segregation, whereas 90.8% of the cells were able to efficiently segregate their homologs. This increase in ordered segregation in the absence of Sgs1 may reflect a role for Sgs1 in the suppression of distributive disjunction. This possibility is addressed in **Section 4.4.1**.

4.3.4 The majority of dead spores in the three viable class of tetrads are recombinant when *sgs1* is absent during meiosis

As discussed in **Section 1.8**, Chambers *et al* (1996) saw an increase in the number of three viable spore tetrads in the partial hybrid strain. By predicting the genotype of the dead spore using the *2nd Law of Mendel* (which defines independent assortment), the majority of dead spores were found to be recombinant. Assuming death occurs randomly, the numbers of recombinant : non-recombinant spores would be expected to be approximately 50:50, as was confirmed by assessing the three viable spore class of tetrads for the *SGS1* homologous control (Chambers et al., 1996). The fact that the introduction of sequence divergence led to an almost six-fold increase in the number of recombinants led Chambers *et al* (1996) to propose that it was the attempt at recombination between diverged sequences which led to spore death. The model proposed by Chambers *et al* (1996) states that attempted recombination, leading to the formation of heteroduplex DNA, but a failure to complete a reciprocal crossover, is the underlying cause of spore death in these tetrads (**Figure 1.18**).

In an attempt to further characterise this predicted defect in strand capture of the reciprocal product during recombination, Chambers *et al* (1996, 1999) investigated the effects of deleting the MMR proteins Msh2, Pms1 and Msh6 on the recombinant : non-recombinant ratio. Deletion of *msh2* and *pms1* (Chambers et al., 1996), but not *msh6* (Chambers, 1999), restored the recombinant : non-recombinant ratio to equal frequencies. This suggests that the absence of Msh2 and Pms1, but not Msh6, facilitate strand capture of the reciprocal product despite the presence of sequence divergence. Therefore, these data implicate Msh2 and Pms1 in assessing that there is sufficient sequence similarity for the resolution of recombination (**Figure 1.18**).

To assess the potential role of Sgs1 in this process, we assessed the effects of deleting *sgs1* on the recombinant : non-recombinant ratio for the dead spores in the three viable spore class of tetrads. We first determined the rate of recombination in the three viable spore class of tetrads and saw that the rate of recombination was elevated when compared to the four viable spore class of tetrads

(**Table 4.6**). We then compared the ratio of recombination in the four viable spore class of tetrads to recombination in the three viable spore class of tetrads (**Table 4.7**). In addition to this, we assessed the proportion of three viable spore events where the dead spore was recombinant (**Table 4.7**). By predicting the genotype of the dead spore using *2nd Law of Mendel*, we were able to ascertain the number of NPD and TT events that would have taken place. This indicates whether the dead spore in the three viable spore class of tetrads had undergone a recombinant event. We compared the number of these recombinant events to the number of non-recombinant events, and assessed whether the ratio of recombinants : non-recombinants deviated from 50:50 using the χ^2 test (**Table 4.7**).

Table 4.6: Meiotic recombination in the three and four viable spore class of tetrads

Homeologous Chromosome III Diploid	***HML-HIS4***		***HIS4-LEU2***		***LEU2-MAT***		**Total Recombination (%)**	
	4 spores	3 spores	4 spores	3 spores	4 spores	3 spores	4 spores	3 spores
SGS1/*SGS1* (ACD 94)	0.63% (4/631)	5.88% (3/51)	0.16% (1/631)	3.92% (2/51)	3.33% (21/631)	29.11 (15/51)	4.12% (26/631)	39.26% (20/51)
pCLB2-SGS1/ sgs1Δ (ADA 2)	5.37% (11/205)	7.36% (12/163)	1.46% (3/205)	4.3% (7/163)	13.66% (28/205)	15.85% (26/164)	20.49% (42/205)	27.44 (45/164)
sgs1-mlh1-id/ sgs1Δ (ADA 5+7+9)	1.72% (13/758)	8.86% (14/158)	0.26% (2/758)	3.16% (5/158)	4.35% (33/758)	18.35% (29/158)	6.33% (48/758)	30.38% (48/158)

For the four viable spore class of tetrads, the rates of recombination were calculated by pooling the number of TTs with twice the number of NPDs for each interval. For the three viable class of tetrads, the genotype of the dead spore was predicted by using the *2nd Law of Mendel* which defines independent assortment. The rate of recombination for the three viable spore class of tetrads was then calculated as with the four viable spore class of tetrads (by pooling the number of TTs with twice the number of NPDs for each interval). The rates of recombination were elevated in the three viable spore class of tetrads for all three intervals.

Table 4.7: Distribution of recombination events in the three and four viable spore class of tetrads

Homeologous Chromosome III Diploid	**Total Recombination (%)**		**Ratio of recombination in the 4 viable class to recombination in the 3 viable class of tetrads** [a]	**Three viable spores with an exchange in which the dead spore is recombinant (%)** [b]
	4 spores	3 spores		
SGS1/*SGS1* (ACD 94)	4.12% (26/631)	39.26% (20/51)	1 : 9.53	80% (16/20)*
pCLB2-SGS1/ sgs1Δ (ADA 2)	20.49% (42/205)	27.44 (45/164)	1 : 1.34	66.7% (30/45)*
sgs1-mlh1-id/ sgs1Δ (ADA 5+7+9)	6.33% (48/758)	30.38% (48/158)	1 : 4.8	93.8% (45/48)*

[a] The rates of recombination (calculated in **Table 4.6**) were expressed as a ratio comparing recombination in the four viable spore class of tetrads to recombination in the three viable spore class of tetrads.

[b] By predicting the genotype of the dead spore using the *2nd Law of Mendel*, we were able to calculate the number of NPDs and TTs that would have occurred had all four spores been viable. This enabled us to assess the proportion of dead spores in the three viable spore class of tetrads which exhibited a recombinant event. Using the χ^2 test, we were able to calculate whether the numbers of recombinant : non-recombinant events deviated from the expected 50:50 ratio if death was random. p-values < 0.05 were considered significant using χ^2 test (significance denoted by *) which indicated that the recombinant : non-recombinant ratio deviated significantly from 50:50.

In agreement with the data presented by Chambers *et al* (1996), the majority of dead spores in the *SGS1* three viable spore class of tetrads exhibits a recombination event when sequence divergence is introduced, suggesting that death is caused by the attempt at recombination between diverged sequences (**Table 4.7**). However, the data suggests that unlike *msh2* or *pms1*, deletion of *sgs1* during meiosis does not restore the recombinant : non-recombinant ratio to approximately equal frequencies as significant deviation is observed from 50:50. This suggests that despite the absence of Sgs1 during meiosis, strand capture of the reciprocal product is still rejected.

However, the proportion of recombinants to non-recombinants obtained for the *pCLB2-SGS1/sgs1Δ* homeologous diploid is close to 50:50, despite being significantly different using the χ^2-test. Due to this, it remains possible that Sgs1 does have a role in the rejection of strand capture of the reciprocal product during recombination. However, the sample size tested in this study may be too small to reflect this. Assuming that this is the case, and therefore Sgs1 does act in the rejection of strand capture, the data shown in **Table 4.7** suggests that the interaction between Sgs1 and Mlh1 is not important for Sgs1 to carry out this role, as we see significant deviation from 50:50.

4.3.5 The rates of unequal recombination are increased in the absence of Sgs1

We analysed the rates of unequal recombination events for *sgs1-mlh1-id* using the unequal recombination assay as described in **Section 2.2.13** and illustrated in **Figure 2.2**. Briefly, a HYG-CYH cassette was inserted upstream of *HIS4* and a HYG cassette was inserted downstream of *LEU2* on chromosome III. By mating this strain to a strain that does not contain this HYG-CYH/HYG cassette, we were able to assess the rates of unequal recombination following tetrad dissection. As shown in **Figure 2.2**, unequal recombination (caused by either unequal sister chromatid exchange or by a deletion) can be analysed by assessing the number of four viable spore tetrads exhibiting 3:1 hygromycin resistance : sensitivity and 2:2 cycloheximide resistance : sensitivity. However, 3:1 hygromycin resistance : sensitivity and 2:2 cycloheximide resistance : sensitivity can also be caused by gene conversion events (**Figure 2.2**). CHEF Gel (**Section 2.2.2**) and Southern Blot (**Section 2.2.8**) analysis allows us to differentiate between these events (examples shown in **Figure 2.2**).

We wanted to assess whether the interactions between Sgs1 and Mlh1 are important in the suppression of unequal recombination events as deletion of *sgs1* has been shown to increase the rate of unequal recombination (Chaix, 2007, Onoda et al., 2000) (also shown in **Table 4.9** for comparison). We therefore mated strains carrying the *sgs1-mlh1-id* mutation (which also contains the HYG-CYH/HYG cassette) to the partial hybrid strain (ADA 5+7+9) and the *S. cerevisiae* homologous control (ADA 4+6+8) and carried out tetrad dissection. We selected every four viable spore tetrad that exhibited 3:1 hygromycin resistance : sensitivity and 2:2 cycloheximide resistance : sensitivity and subjected them to CHEF Gel and Southern Blot analysis in order to differentiate the types of recombination events that occurred (as described in **Section 2.2.13** and illustrated in **Figure 2.2**). An example of a CHEF Gel from the homologous crosses ADA 4+6+8 is illustrated in **Figure 4.5**.

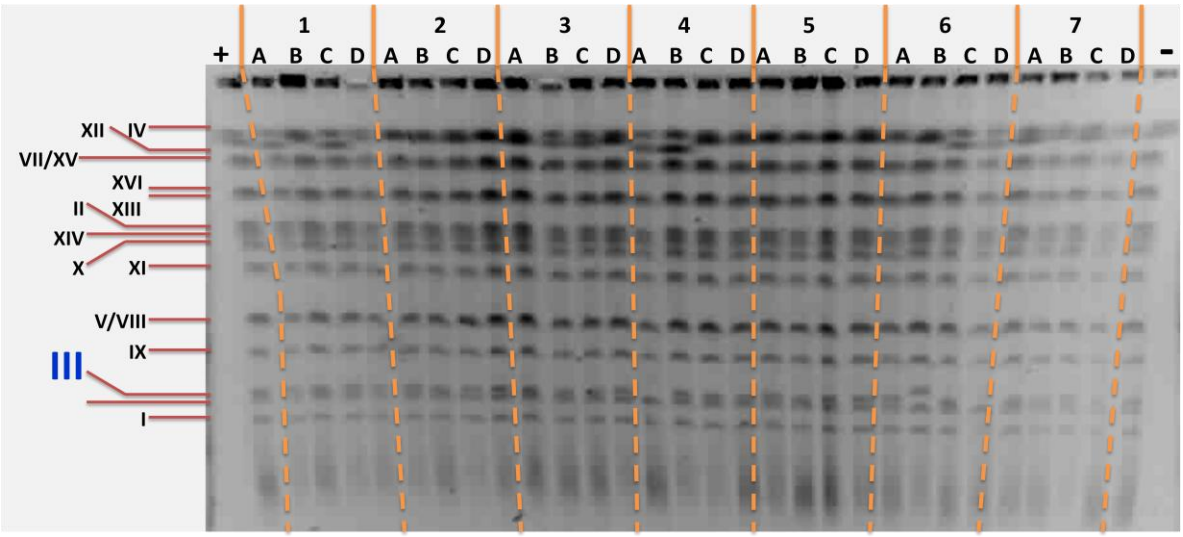


Figure 4.5: An example of a CHEF Gel from the *sgs1-mlh1-id/sgs1Δ* homologous cross

The separation of yeast chromosomes is achieved using CHEF Gel analysis. Seven four viable spore tetrads (that were 3:1 hygromycin resistant : sensitive and 2:2 cycloheximide resistant : sensitive) can be run on one CHEF Gel (A-D represent the four spores that make up the tetrad), as well as a positive control (which is a strain containing the HYG-CYH/HYG cassette) and a negative control (a strain which does not contain the HYG-CYH/HYG cassette). The positive and negative controls are used as controls for the Southern Blot, as the *URA3-CYH2* probe (described in **Section 2.2.13**) will produce a band for the positive control and not for the negative control. In this example, tetrads 1, 2, 3, 5 and 7 are gene conversions; tetrad 4 is a deletion; and tetrad 6 is a USCE event.

We compared the effects of the *sgs1-mlh1-id* mutation on the distribution of gene conversion events (**Table 4.8**) and unequal recombination events (**Table 4.9**) separately, using the G-test. As we were compared the distribution of multiple data sets, we applied the Bonferroni correction, and so p-values that were less than 0.01 allowed us to reject the null hypothesis, H_0: 'the sets of data are the same'.

Table 4.8: Gene conversion events for homologous diploids

Homologous Chromosome III Diploid	Number of gene conversion events	Tetrads that did not exhibit a gene conversion	Total Number of tetrads	Percentage of gene conversion events
SGS1/SGS1 (ACD 97)	11	244	255	4.3%
sgs1Δ/ sgs1Δ (ACD 116)	5	173	178	2.8%
sgs1-ΔC795/ sgs1Δ (ADA 3)	4	173	177	2.6%
sgs1-mlh1-id/ sgs1Δ (ADA 4+6+8)	8	413	421	1.9%

Using the G-test, we compared the distribution of gene conversion events for homologous diploids (by comparing the number of gene conversions vs the number of tetrads that did not exhibit a gene conversion amongst the homologous diploids). p-values < 0.01 were considered significant allowing us to reject the null hypothesis, H_0: 'the sets of data are the same'. The *SGS1/SGS1* (ACD 97) and *sgs1Δ/sgs1Δ* (ACD 116) diploid data were provided by Chaix (2007). The *sgs1-mlh1-id/sgs1Δ* data is the pooled data from the diploid crosses ADA 4, ADA 6 and ADA 8 (see **Table 4.1**). No significance was observed for any of the diploids.

Table 4.9: Unequal recombination events for homologous diploids

Homologous Chromosome III Diploid	Unequal Recombination Events		Tetrads that did not exhibit an unequal recombination event	Total Number of tetrads	Percentage of unequal recombination events
	USCE Events	Deletion Events			
SGS1/*SGS1* (ACD 97)	6	0	249	255	2.4%
sgs1Δ/ sgs1Δ (ACD 116)	13	5	160	178	10.1% *
sgs1-ΔC795/ sgs1Δ (ADA 3)	4	8	165	177	6.8% *
sgs1-mlh1-id/ sgs1Δ (ADA 4+6+8)	8	10	403	421	4.3% *#

Using the G-test, we compared the distribution of unequal recombination events for homologous diploids (by comparing the number of unequal recombination events vs the number of tetrads that did not exhibit unequal recombination amongst the homologous diploids). p-values < 0.01 were considered significant allowing us to reject the null hypothesis, H_0: 'the sets of data are the same'. The *SGS1*/*SGS1* (ACD 97) and *sgs1Δ*/*sgs1Δ* (ACD 116) diploid data were provided by Chaix (2007). The *sgs1-mlh1-id/sgs1Δ* data is the pooled data from the diploid crosses ADA 4, ADA 6 and ADA 8 (see **Table 4.1**).

* =significantly different from *SGS1*/*SGS1*; # = significantly different from *sgs1Δ*/*sgs1Δ*

As shown in **Table 4.8**, the number of gene conversion events obtained for the homologous *sgs1-mlh1-id/sgs1Δ* diploid did not significantly differ from any of the other homologous diploids. However, we observe a significant increase in the number of unequal recombination events for *sgs1-mlh1-id/sgs1Δ* when compared to *SGS1*/*SGS1* (**Table 4.9**). The data therefore suggest that the increases in the rates of unequal recombination that are caused by a deletion of Sgs1 are partially dependent on its interactions with Mlh1. The importance of this interaction in the suppression of unequal recombination is discussed in **Section 4.4.3**.

We also assessed the effects of the *sgs1-mlh1-id* mutation on unequal recombination in the partial hybrid strain. An example of a CHEF Gel from the homeologous crosses ADA 5+7+9 is illustrated in **Figure 4.6**.

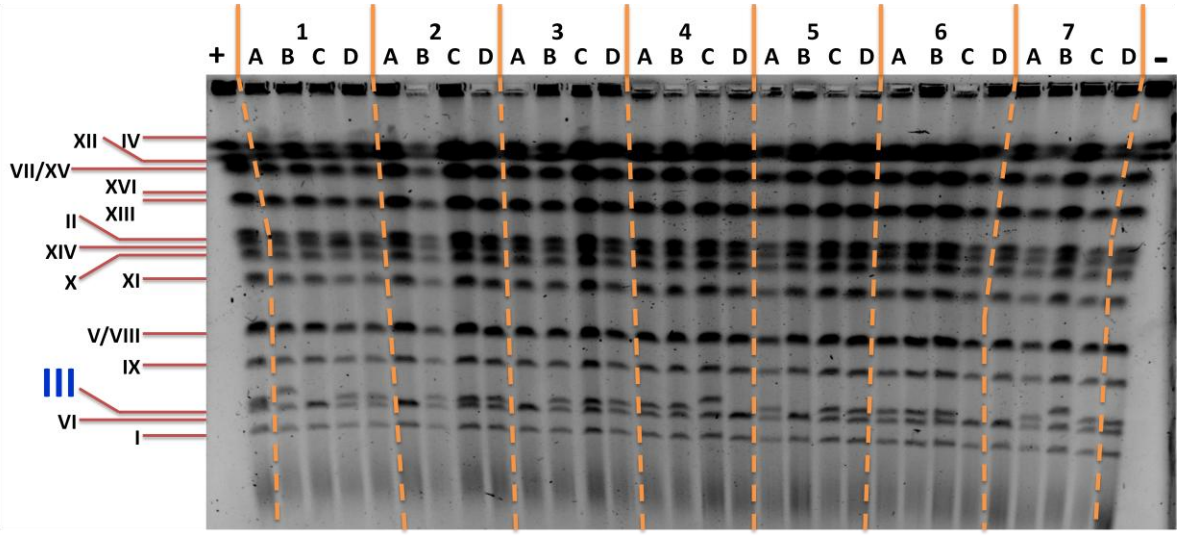


Figure 4.6: An example of a CHEF Gel from the *sgs1-mlh1-id/sgs1Δ* homeologous cross

As described for **Figure 4.5**, seven four viable spore tetrads (that were 3:1 hygromycin resistant : sensitive and 2:2 cycloheximide resistant : sensitive) were run on one CHEF Gel (A-D represent the four spores that make up the tetrad), as well as a positive control (which is a strain containing the HYG-CYH/HYG cassette) and a negative control (a strain which does not contain the HYG-CYH/HYG cassette). In this example, tetrads 2, 3, 5 and 6 are deletions; and tetrads 1, 4 and 7 are USCE events. As discussed in the text, no gene conversion events were observed for any of the homeologous diploids.

As with the homologous diploids, we compared the effects of the *sgs1-mlh1-id* mutation on the distribution of gene conversion events (**Table 4.10**) and unequal recombination events (**Table 4.11**) separately, using the G-test. The Bonferroni correction was applied as multiple comparisons were carried out, and so p-values that were less than 0.0167 allowed us to reject the null hypothesis, H_0: 'the sets of data are the same'.

Table 4.10: Gene conversion events for homeologous diploids

Homeologous Chromosome III Diploid	Number of gene conversion events	Tetrads that did not exhibit a gene conversion	Total Number of tetrads	Percentage of gene conversion events
SGS1/SGS1 (ACD 94)	0	222	222	0%
pCLB2-SGS1/ sgs1Δ (ADA 2)	0	301	301	0%
sgs1-mlh1-id/ sgs1Δ (ADA 5+7+9)	0	539	539	0%

Using the G-test, we compared the distribution of gene conversion events for homeologous diploids (by comparing the number of gene conversions vs the number of tetrads that did not exhibit a gene conversion amongst the homeologous diploids). p-values < 0.0167 were considered significant allowing us to reject the null hypothesis, H_0: 'the sets of data are the same'. The *sgs1-mlh1-id/sgs1Δ* data is the pooled data from the diploid crosses ADA 5, ADA 7 and ADA 9 (see **Table 4.2**). No gene conversions were observed for any of the homeologous diploids, and therefore, no significance was observed.

Table 4.11: Unequal recombination events for homeologous diploids

Homeologous Chromosome III Diploid	Unequal Recombination Events		Tetrads that did not exhibit an unequal recombination event	Total Number of tetrads	Percentage of unequal recombination events
	USCE Events	Deletion Events			
SGS1/*SGS1* (ACD 94)	11	17	194	222	12.6%
pCLB2-SGS1/ sgs1Δ (ADA 2)	28	24	249	301	17.3%
sgs1-mlh1-id/ sgs1Δ (ADA 5+7+9)	29	21	489	539	9.3% †

Using the G-test, we compared the distribution of unequal recombination events for homeologous diploids (by comparing the number of unequal recombination events vs the number of tetrads that did not exhibit unequal recombination amongst the homeologous diploids). p-values < 0.0167 were considered significant allowing us to reject the null hypothesis, H_0: 'the sets of data are the same'. The *sgs1-mlh1-id/sgs1Δ* data is the pooled data from the diploid crosses ADA 5, ADA 7 and ADA 9 (see **Table 4.2**).

† =significantly different from *pCLB2-SGS1*/*sgs1Δ*

No gene conversions were detected for any of the homeologous diploids (**Table 4.10**). *sgs1-mlh1-id*/*sgs1Δ* shows a significant decrease in unequal events when compared to *pCLB2-SGS1*/*sgs1Δ* but is not statistically different from *SGS1*/*SGS1*. The data suggest that the interaction between Sgs1 and Mlh1 is not important in maintaining the barrier to sister chromatid recombination in response to sequence divergence. However, we speculate that the assay used for this investigation is not sensitive enough for this analysis due to the large amount of sequence divergence present in the *HIS4-LEU2* interval (**Figure 4.3**). This is discussed further in **Section 4.4.3**.

4.4 Discussion

4.4.1 The interaction between Sgs1 and Mlh1 may be important in the suppression of homeologous recombination at the SEI stage, or may be important in the suppression of distributive segregation

We proposed that Mlh1 recruited Sgs1 to the sites of homeologous recombination due to its role as a molecular matchmaker (Sancar and Hearst, 1993, Wang and Kung, 2002). However, the crossing over data (**Table 4.4** and **Figure 4.2**) suggest that the interaction between Sgs1 and Mlh1 is not important for the ability of Sgs1 to suppress meiotic homeologous recombination, as the rates of crossing over for *sgs1-mlh1-id* do not significantly differ from *SGS1*. However, it remains possible that the interaction between Sgs1 and Mlh1 is important. In the absence of this interaction, other proteins may act to recruit Sgs1 to the sites of homeologous recombination. As a result, the data for *sgs1-mlh1-id* obtained does not differ significantly from *SGS1*, as Sgs1 would still be able to unwind SEI events that are occurring between diverged sequences. These other proteins may include other members of the MMR complex that have been implicated in studies by Hunter *et al* (1996) and Chambers *et al* (1996) in the suppression of homeologous recombination in *S. cerevisiae*.

As described by Chaix (2007), the meiosis I non-disjunction data (**Table 4.5** and **Figure 4.4**) can be interpreted as a measure for the failure of crossing over across the entire chromosome III. Chaix (2007) showed that the presence of sequence divergence leads to an increase in the levels of meiosis I non-disjunction events (also repeated in this study and shown in **Table 4.5** and **Figure 4.4**). This was proposed to reflect a decrease in the levels of crossing over caused by the presence of mismatches sequences. Chaix (2007) suggested that the increased levels of meiosis I non-disjunction could be attributed to a decreased level of crossing over, which would result in an increase in the likelihood of missegregation events. Chaix (2007) also showed that a deletion of *sgs1* leads to a significant decrease in the levels of meiosis I non-disjunction when compared to *SGS1* (also shown in this study for the meiotic null *pCLB2-SGS1* - **Table 4.5** and **Figure 4.4**). This was suggested to be caused by an increase in the levels of crossing over between diverged sequences, which would be facilitated in the absence of Sgs1, which would decrease the probability of missegregation events.

If the meiosis I non-disjunction data truly represents a failure of crossing over across the entire chromosome, one could suggest that this is a more sensitive measure of the effects of the *sgs1-mlh1-id* mutation on homeologous recombination, as the crossing over data only measures crossing over across three intervals, one of which is highly diverged (**Figure 4.3**). If the meiosis I non-

disjunction data is interpreted in this way, it would suggest that the interaction between Sgs1 and Mlh1 is important in the ability of Sgs1 to suppress meiotic homeologous recombination. This is because the data show that the levels of meiosis I non-disjunction for the *sgs1-mlh1-id* mutant are significantly decreased when compared to *SGS1*, but do not differ from *pCLB2-SGS1* or the positive control *pCLB2-MSH2* (**Table 4.5** and **Figure 4.4**). This would suggest that abolishing the interaction between Sgs1 and Mlh1 facilitates crossovers between diverged sequences, which decrease the probability of missegregation events from occurring.

However, the meiosis I non-disjunction data could be interpreted in another way which may reflect a role for Sgs1 in homolog segregation. As discussed in **Section 4.3.3**, the fact that we recovered only 11.5% of meiosis I non-disjunction events for the *SGS1* homeologous diploid means that the majority of the cells are still able to segregate their homologs in an ordered manner. We propose that this segregation may be achieved by a crossover, as crossovers were not completely eliminated by the introduction of sequence divergence (**Table 4.4** and **Figure 4.2**). To assess whether crossovers are still occurring, which would explain the high levels of ordered homolog segregation, we could design homeolog-specific probes at the telomeric ends of the chromosomes in an effort assess crossovers along the entire chromosome. Assuming the high levels of ordered homolog segregation are not caused by crossovers, we suggest that an alternative mechanism, such as distributive segregation (Dawson et al., 1986, Guacci and Kaback, 1991, Mann and Davis, 1986, Kaback et al., 1989) may be responsible for this observation. Taking this into account, the fact that a greater number of cells are able to segregate their homologs efficiently in the *pCLB2-SGS1*/*sgs1Δ* homeologous diploid and also in the *sgs1-mlh1-id/sgs1Δ* (as discussed in **Section 4.3.3**) may reflect a role for Sgs1 in the suppression of distributive disjunction. As the presence of sequence divergence would decrease the levels of crossing over, we propose that various factors may act to promote distributive segregation in an effort to carry out homolog segregation. These factors may be similar to the genes involved in the SOS response in *E. coli* (discussed in **Section 1.2.1.1**). However, due to the presence of sequence divergence, carrying out recombination using this mechanism may be detrimental to the cell. We can speculate that Sgs1 is therefore required to counteract these factors, perhaps by unwinding physical interactions along the chromosome that would lead to distributive segregation. This putative role may be related to the anti-recombination role of Sgs1 in suppressing the formation of closely-spaced double crossovers, which would lead to the formation of inter-homolog and multi-chromatid joint molecules (Jessop and Lichten, 2008) (discussed in **Section 1.3.2**). The importance of the interaction between Sgs1 and Mlh1 in this process may be related to physical interactions that form between mismatched sequences along the chromosome. Therefore, we still propose that the

interaction between Sgs1 and Mlh1 is important in allowing Mlh1 to recruit Sgs1 its desired cellular location.

4.4.2 Sgs1 might be required to reject strand capture of the reciprocal product during recombination in response to sequence divergence

Chambers *et al* (1996) saw that the presence of sequence divergence leads to an increase in the three viable spore class of tetrads, with the majority of dead spores exhibiting a recombinant event. They attributed the increase in spore death to the attempt at recombination where strand capture of the reciprocal product would be aborted due to the presence of mismatches. By deleting *msh2* and *pms1*, Chambers *et al* (1996) noted that the recombinant : non-recombinant ratio was restored to 50:50. They therefore suggested that Msh2 and Pms1 act in the suppression of strand capture of the reciprocal product in the presence of high levels of sequence divergence. As shown in **Table 4.7**, deletion of *sgs1* leads to the majority of dead spores in the three viable spore class of tetrads being recombinant, as the data significantly differs from 50:50. This therefore suggests that unlike Msh2 and Pms1, Sgs1 does not act in the rejection of second strand capture during recombination.

However, we speculate that Sgs1 does play a role in this process, as the data obtained does not drastically deviate from 50:50. We suggest that the data obtained in **Table 4.7** might be due to the sample size being too small. Therefore, it would be of interest to repeat this experiment with a greater number of tetrads. Assuming this experiment still results in an elevation in dead spores that exhibit a recombinant event, it still does not necessarily mean that Sgs1 is not involved in this process. The mechanism by which homeologous recombination is suppressed may be different for the SEI stage of recombination and the second strand capture stage of recombination. Therefore, Sgs1 may act independently to Msh2 and Pms1. Therefore, it would be interesting to assess the effects of an *sgs1Δ msh2Δ* or *sgs1Δ pms1Δ* double mutation. Alternatively, other helicases may act alongside Sgs1 in the rejection of second strand capture. Therefore, by deleting Sgs1, the cell is able to compensate for its loss by recruiting other factors, and so we would still see an elevation in the number of dead spores which exhibit a recombinant event.

4.4.3 In the absence of homology, repair is carried out using the sister chromatid

Chaix (2007) showed that deleting *sgs1* leads to an increase in unequal recombination events (**Table 4.9**) which reflected a role for Sgs1 in maintaining a barrier towards sister chromatid recombination. We show that this role of Sgs1 may be dependent on its interactions with Mlh1, as *sgs1-mlh1-id* also leads to an increase in the number of unequal events (**Table 4.9**). This suggests that both Sgs1 and

Mlh1 are required in maintaining the barrier to sister chromatid recombination. This is somewhat surprising, as the MMR protein would not be expected to play a role in this process, as sister chromatids normally share perfect sequence identity. We speculate that the role of Mlh1 may be to suppress sister-chromatid recombination between repeats that contain single nucleotide polymorphisms. Although the HYG and CYH cassettes used in this study were sequenced, it is possible that a silent mutation had been acquired in one of the two HYG cassettes. This may be why we saw a role for both Sgs1 and Mlh1 in the suppression of unequal recombination.

When divergence was introduced in this unequal recombination assay, we expected to see a decrease in the number of unequal events. This is because we hypothesised that the absence of the Sgs1 helicase would allow strand invasion, and subsequent recombination, to occur between diverged sequences. As a result, we would expect repair to be carried out using the 'homologous' strand, despite the presence of mismatches, which would lead to an increase in the gene conversion events. In contrast to this, we saw no gene conversion events for any of the homeologous crosses (**Table 4.10**). A potential explanation that may account for this is the additional role of Sgs1 in the suppression of sister chromatid recombination, as shown in **Table 4.9**. Therefore, deletion of *sgs1* would be expected to increase sister chromatid recombination, perhaps at the expense of gene conversion events. However, we also saw no gene conversions for the *SGS1* homeologous cross (**Table 4.10**). This suggests that this additional role of Sgs1 in the suppression of sister chromatid recombination is not the reason as to why we see no gene conversion events, as the presence of Sgs1 does not suppress unequal events in favour of gene conversions. We propose that the absence of gene conversions is due to the high degree of sequence divergence present in the *HIS4-LEU2* interval, which is the interval used in this assay (**Figure 4.3**). We suggest that this interval is so diverged that strand invasions are drastically reduced. In the absence of successful strand invasions, repair must be carried out using the sister chromatid. If this is true, then it would mean that this assay, which investigates unequal recombination events initiated in the *HIS4-LEU2* interval, is not sensitive enough. Therefore, while we propose that while *SGS1* does play a role in the suppression of homeologous recombination, it is not required in this interval. This is because strand invasions, which would ultimately lead to recombination, do not take place.

Chapter 5: Which domains of Sgs1 are required for the suppression of homeologous recombination?

5.1 Introduction

Chaix (2007) showed that Sgs1 is required for the suppression of meiotic homeologous recombination (as discussed in **Section 1.8**). The aim of this investigation was to assess which domains of Sgs1 are required for this suppression (**Figure 1.19B**).

As discussed in **Sections 1.6** and **1.7**, there is an evolutionary conserved interaction between the RecQ helicases and the type IA topoisomerase Top3. In yeast, Sgs1 and Top3 have been shown to act in the intra-S checkpoint response (**Section 1.7.1**), in DSB strand resection (**Section 1.7.3**) and in the stabilisation and processing of stalled replication forks (**Section 1.7.4**) during mitosis. Both Sgs1 and Top3 have also been implicated in the acting in the same pathway to suppress mitotic homeologous recombination (Myung et al., 2001b, Putnam et al., 2009) (**Section 1.7.6**).

Sgs1 and Top3 also have a role in the suppression of meiotic crossovers by resolving double Holliday junctions into non-crossover products (**Section 1.7.5**). In support of this, Chaix (2007) also suggested a late meiotic role for Sgs1 in the decatenation of double Holliday junction structures by assessing the frequency of non-sister spores using the centromere marker *TRP1* (**Section 2.2.11.6**). Chaix (2007) showed that a deletion of *sgs1*, resulted in an elevation in the number of non-sister spores compared to sister spores for the two viable spore class of tetrads. Chaix (2007) proposed that this genotype was dependent on the interaction between Sgs1 and Top3, as in the wild-type, both Sgs1 and Top3 act in the dissolution of double Holliday junction structures (Chaix, 2007). However, when *sgs1* is deleted, resulting in the absence of this interaction, it was proposed that these structures cannot be dissolved. Chaix (2007) suggested that this leads to the death of these spores which contain the entangled chromosomes. The two surviving spores will therefore be non-sisters (Chaix, 2007) (**Figure 5.1**).

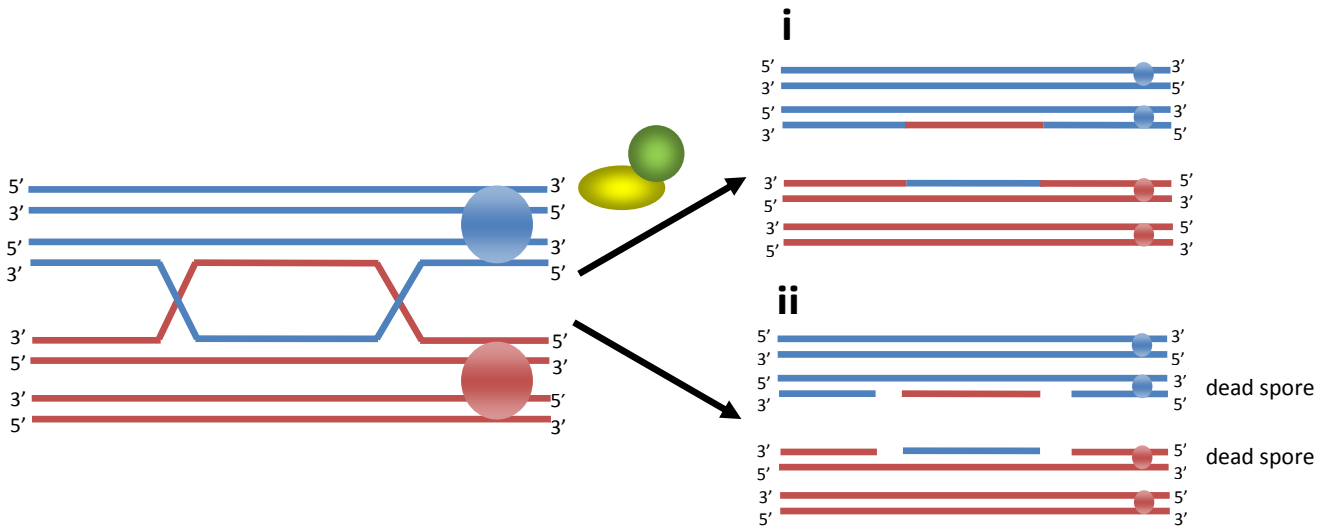


Figure 5.1: Model proposed to account for the late meiotic role of Sgs1 in the decatenation of dHJ structures with Top3

Chaix (2007) proposed that Sgs1 and Top3 act to dissolve double Holliday junctions (**i**). In the absence of this interaction, the double Holliday junction is not dissolved leading to destruction of the entangled chromosomes (**ii**).

As discussed in **Section 1.6**, the RecQ helicases share a number of conserved domains, including a helicase domain which acts to unwind DNA structures in an ATP-dependent manner as well as a conserved C-terminal HRDC domain that is important in facilitating DNA binding. As discussed in **Section 1.6.3**, there is some controversy regarding the function of the helicase domain of Sgs1. While some researchers have shown that a point mutation which inactivates the helicase activity of Sgs1 is able to rescue the slow growth phenotype exhibited by *sgs1 top1* double mutants and complement the poor sporulation phenotype exhibited by *sgs1Δ* cells (Lu et al., 1996, Miyajima et al., 2000a, Miyajima et al., 2000b), another group (Weinstein and Rothstein, 2008) have provided contradictory results. Weinstein and Rothstein (2008) showed that the *sgs1-K706A* point mutation was only able to partially rescue the *sgs1 top1* slow growth and also showed that this point mutation exhibited a poor sporulation phenotype. As discussed in **Section 1.6.3**, the discrepancies seen between these studies is suggested to be due to the experimental approach utilised by these groups. The approach carried out by Weinstein and Rothstein (2008) involved transplacing the *sgs1-K706A* mutation at the endogenous site within the genome, whereas the observations made by the other studies described above were carried out using plasmid-based complementation studies.

5.1.1 Aim

In order to assess which domains of Sgs1 are required for the suppression of meiotic homeologous recombination, we assessed the effects of three different mutations (**Figure 1.19B**) - an *sgs1-top3-id* (*sgs1-K4AP5AL9A*) mutation, which disrupted the interaction between Sgs1 and Top3; an *sgs1-K706A* point mutation, which inactivates the helicase domain of Sgs1; and an *sgs1ΔHRDC* mutation, which was a truncation of the 360 amino acids of the Sgs1 protein that deleted the HRDC domain. The point mutations used in this study were transplaced into the genome at their endogenous locations. Each of these mutations was crossed to the *pCLB2-SGS1* or *sgs1Δ* to assess their impact on the ability of Sgs1 to suppress meiotic homeologous recombination.

In addition to this, we wanted to test the model proposed by Chaix (2007) that suggested a late role for Sgs1 and Top3 in the decatenation of dHJ structures. As shown in **Figure 5.1**, this model was based on observations that the two viable spore class of tetrads were enriched for non-sisters when *sgs1* was deleted. To elucidate whether this increase in non-sister spores was dependent on the interaction between Sgs1 and Top3, we assessed whether the frequency of non-sister spores was elevated compared to the frequency of sister spores amongst the two viable spore class of tetrads for the *sgs1-top3-id* mutant.

5.2 Experimental Procedures

5.2.1 Truncation of Sgs1 to delete the HRDC domain

The last 360 amino acid residues were deleted from the C-terminal end of Sgs1. This truncation deletes the HRDC domain of Sgs1. This truncation mutant was made by amplifying the *KANMX4* cassette from plasmid *pFA6KANMX4* (**Table 2.4**) using the *sgs1ΔHRDC* trunc Fwd and *sgs1ΔHRDC* trunc Rev primers (**Table 2.3**). These primers were designed so that the C-terminal most 360 residues of Sgs1 would be replaced by the *KANMX4* cassette upon transformation (**Chapter 2**). The PCR products were transformed into ACT 65 to create Y55 3588 (*sgs1-ΔHRDC*) and also into Y55 3540 to create Y55 3589 (*sgs1-K706AΔHRDC*). Transformants were plated on YEPD media that was supplemented with Geneticin. After DNA extraction was carried out (**Chapter 2**), the successful insertion of the construct was confirmed by PCR using primers *sgs1ΔHRDC* trunc A1 and *sgs1ΔHRDC* trunc A4 (**Table 2.3**).

5.3 Results

5.3.1 MMS sensitivity assay

We wanted to determine whether the various mutations in *sgs1* affected the ability of Sgs1 to restart replication forks that have stalled due to the presence of lesions caused by exposure to MMS. We assessed the effects of the *sgs1-top3-id* mutation (Y55 3541), which abolishes the interaction between Sgs1 and Top3. We also tested the *sgs1-K706A* point mutation (Y55 3540) which mutates the helicase activity of Sgs1. In addition to these mutations we tested a C-terminal truncation of Sgs1 which deletes the 360 amino acids, including the HRDC domain (*sgs1-ΔHRDC* – Y55 3588). Finally, we tested a strain that carries the *sgs1-K706A* point mutation in combination with the C-terminal 360 amino acid truncation (*sgs1-K706AΔHRDC* – Y55 3589). Resistance to MMS indicates that the mutations do not inhibit this mitotic role of Sgs1 (Ui et al., 2001). As in **Section 3.3.2.1**, we used the *SGS1* strain ACT53 as a positive control, and the *sgs1Δ* strain ACT56 as a negative control.

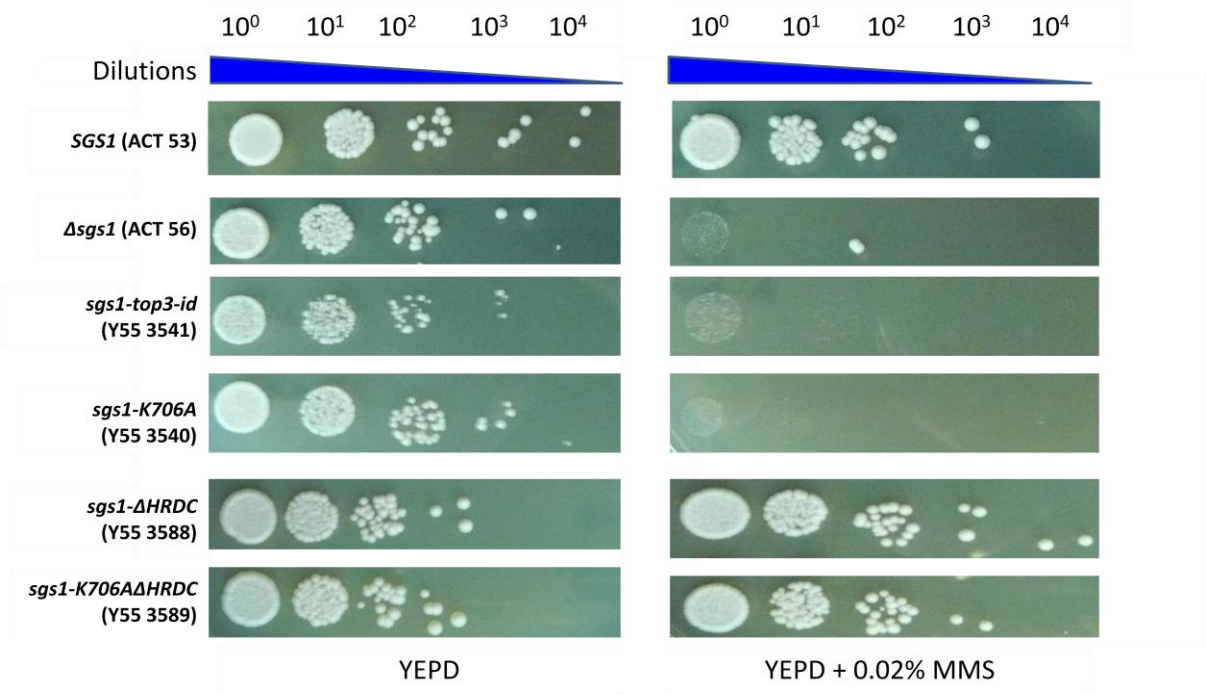


Figure 5.2: Testing the growth of the *sgs1-top3-id*, *sgs1-K706A*, *sgs1-ΔHRDC* and *sgs1-K706AΔHRDC* mutant with respect to MMS resistance by spotting serial dilutions onto YEPD plates (as a control) and YEPD plates supplemented with 0.02% MMS. Failure to grow on YEPD media supplemented with 0.02% MMS is indicative of an inability to repair lesions which lead to the stalling of replication forks during mitosis. The photographs for the *SGS1* strain (ACT 53) and the *sgs1Δ* strain (ACT 56) are the same as in **Figure 3.3** and are shown here for comparison.

In agreement with observations by several groups (Miyajima et al., 2000a, Miyajima et al., 2000b, Mullen et al., 2000, Ui et al., 2001, Weinstein and Rothstein, 2008), we showed that *sgs1-K706A* is sensitive to MMS (**Figure 5.2**). These same groups also showed that deletion of part of the N-terminus of Sgs1, responsible for binding Top3, renders the strains sensitive to MMS (Miyajima et al., 2000a, Miyajima et al., 2000b, Mullen et al., 2000, Ui et al., 2001, Weinstein and Rothstein, 2008). In agreement with this, we saw that a mutation which results in the inability of Sgs1 to bind Top3 leads to sensitivity to MMS. Miyajima *et al* (2000b) and Ui *et al* (2001) showed that a truncation of Sgs1 that deleted the C-terminal most 254 amino acids resulted in wild-type resistance to MMS. Our C-terminal truncation (*sgs1-ΔHRDC*), which deletes 360 amino acids, is in agreement with their observations, as it also shows resistance to MMS (**Figure 5.2**). Surprisingly, deletion of the C-terminal 360 amino acids in a helicase defective mutation of Sgs1 (*sgs1-K706AΔHRDC*) also rescues the sensitivity of the *sgs1-K706A* mutation to MMS. This suggests that the actions of the helicase domain are dependent on an active HRDC domain (this is discussed further in **Section 5.4**).

5.3.2 Preventing Sgs1 from interacting with Top3 may affect the ability of Sgs1 to inhibit homeologous recombination

5.3.2.1 *sgs1-top3-id* does not affect the rates of crossing over

To assess whether disrupting the interaction between Sgs1 and Top3 affected the ability of Sgs1 to suppress homeologous recombination, we mated the *sgs1-top3-id* mutation (Y55 3541) to a *sgs1Δ* strain (ACT 56) to create the homologous diploid ADA 12. During this time, we created the *pCLB2-SGS1* strains Y55 3565 and Y55 3567. We therefore also mated the *sgs1-top3-id* mutation (Y55 3541) to the *pCLB2-SGS1* (Y55 3567) to create the homologous diploid ADA 13. We determined that the data obtained from the homologous diploids ADA 12 (*sgs1-top3-id / sgs1Δ*) and ADA 13 (*sgs1-top3-id / pCLB2-SGS1*) showed homogeneity, and therefore both could be used to assess the effects of the *sgs1-top3-id* mutation on the rates of homologous recombination. To assess the effects of sequence divergence, we mated the *sgs1-top3-id* mutation (Y55 3541) to *pCLB2-SGS1* in the partial hybrid (Y55 3565) to create the homologous diploid ADA 14.

After tetrad dissection, the distribution of Parental Ditypes (PD), Non-Parental Ditypes (NPD) and TetraTypes (TT) for the four viable spore tetrads across three intervals – *HML-HIS4*, *HIS4-LEU2* and *LEU2-MAT* located on chromosome III were calculated according to the Perkins formula (Perkins, 1949) (**Section 2.2.11.5**) using the *MacTetrad 6.9* software. The data obtained for the homologous diploids ADA 12 (*sgs1-top3-id/sgs1Δ*) and ADA 13 (*sgs1-top3-id/pCLB2-SGS1*) were compared to *sgs1Δ/sgs1Δ* (Chaix, 2007) and to *pCLB2-SGS1/sgs1Δ* using the G-test (**Table 5.1** and **Figure 5.3**). As multiple data sets were compared, the Bonferroni correction (**Section 2.2.12**) was applied, and therefore p-values that were less than 0.01 allowed us to reject the null hypothesis, H_0: 'the sets of data are the same'.

Table 5.1: Crossing over data for intervals along chromosome III for homologous diploids

	Homologous Chromosome III Diploids	PD	NPD	TT	Four Viable Spore Tetrads
HML-HIS4	*SGS1/SGS1* (ACD 97)	196	20	363	579
	SGS1/ sgs1Δ (ACD 116)	77	7	129	213
	sgs1Δ/ sgs1Δ (ACD 95)	85	9	109	203
	pCLB2-SGS1/ sgs1Δ (ADA 1)	16	2	31	49
	sgs1-top3-id/sgs1Δ (ADA 12)	86	8	113	207
	sgs1-top3-id/pCLB2-SGS1 (ADA 13)	15	4	20	39
HIS4-LEU2	*SGS1/SGS1* (ACD 97)	404	2	165	571
	SGS1/ sgs1Δ (ACD 116)	163	1	49	213
	sgs1Δ/ sgs1Δ (ACD 95)	154	5	43	202 *
	pCLB2-SGS1/ sgs1Δ (ADA 1)	38	0	10	48
	sgs1-top3-id/sgs1Δ (ADA 12)	144	2	55	201
	sgs1-top3-id/pCLB2-SGS1 (ADA 13)	27	0	11	38
LEU2-MAT	*SGS1/SGS1* (ACD 97)	340	15	250	605
	SGS1/ sgs1Δ (ACD 116)	133	3	92	228
	sgs1Δ/ sgs1Δ (ACD 95)	124	7	100	231
	pCLB2-SGS1/ sgs1Δ (ADA 1)	29	2	21	52
	sgs1-top3-id/sgs1Δ (ADA 12)	112	7	99	218
	sgs1-top3-id/pCLB2-SGS1 (ADA 13)	18	0	23	41

Using the G-test, we compared the distribution of PDs, NPDs and TTs for homologous diploids. p-values < 0.01 were considered significant allowing us to reject the null hypothesis, H_0: 'the sets of data are the same'. The *SGS1/SGS1* (ACD 97) and *sgs1Δ/sgs1Δ* (ACD 116) diploid data were provided by Chaix (2007). The *SGS1/SGS1* (ACD 97), heterozygote (ACD 116), *sgs1Δ/sgs1Δ* (ACD 95) and *pCLB2-SGS1/sgs1Δ* (ADA 1) data are the same as in **Table 4.3** and are shown here for comparison.

* =significantly different from *SGS1/SGS1*

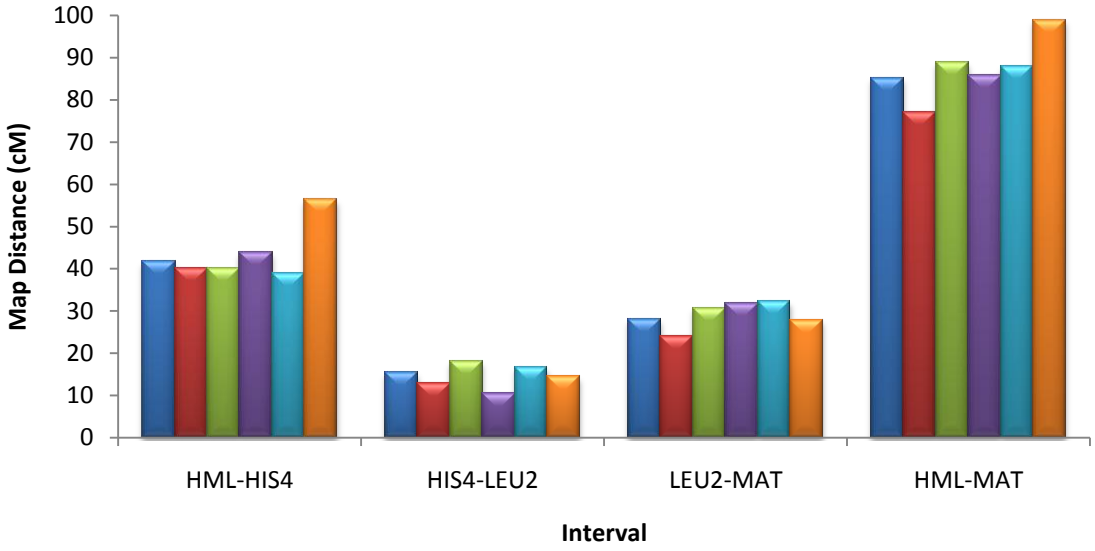


Figure 5.3: Genetic map distances for intervals along chromosome III for homologous diploids.
The map distances (in centiMorgans) for the *HML-HIS4*, *HIS4-LEU2* and *LEU2-MAT* intervals were calculated using the following formula (using the PD, NPD and TT data shown in **Table 5.1**):

$$\frac{\frac{1}{2}(TT + 6NPD)}{PD + NPD + TT}$$

The map distances for the *HML-MAT* interval were calculated by summing the map distances for the other three intervals. The *SGS1*/*SGS1* (ACD 97) and *sgs1Δ*/*sgs1Δ* (ACD 116) diploid data were provided by Chaix (2007). The *SGS1*/*SGS1* (ACD 97), heterozygote (ACD 116), *sgs1Δ*/*sgs1Δ* (ACD 95) and *pCLB2-SGS1*/*sgs1Δ* (ADA 1) data are the same as in **Figure 4.1** and are shown here for comparison.

SGS1/*SGS1* (ACD 97); *SGS1*/*sgs1Δ* (ACD 116); *sgs1Δ*/*sgs1Δ* (ACD 95); *pCLB2-SGS1*/*sgs1Δ* (ADA 1); *sgs1-top3-id*/*sgs1Δ* (ADA 12); *sgs1-top3-id*/*pCLB2-SGS1* (ADA 13)

As shown in **Table 5.1** and **Figure 5.3**, *sgs1-top3-id* does not affect the rate of homologous recombination, as no significant difference is seen from *SGS1* for any of the three intervals for either homologous diploids ADA 12 or ADA 13.

The distribution of PDs, NPDs and TTs for the *sgs1-top3-id/pCLB2-SGS1* (ADA 14) data in the partial hybrid were compared to data obtained for *sgs1Δ* and for *pCLB2-SGS1* in the partial hybrid using the G-test (**Table 5.2** and **Figure 5.4**). As multiple data sets were compared, the Bonferroni correction was applied, and so p-values that were less than 0.01 allowed us to reject the null hypothesis, H_0: 'the sets of data are the same'.

Table 5.2: Crossing over data for intervals along chromosome III for homeologous diploids

	Homeologous Chromosome III Diploids	**PD**	**NPD**	**TT**	**Four Viable Spore Tetrads**
HML-HIS4	*SGS1/SGS1* (ACD 94)	626	0	4	630
	SGS1/sgs1Δ (ACD 117)	221	0	1	222
	sgs1Δ/ sgs1Δ (ACD 96)	95	0	5	100
	pCLB2-SGS1/ sgs1Δ (ADA 2)	194	0	11	205 *
	sgs1-top3-id/pCLB2-SGS1 (ADA 14)	210	0	6	216
HIS4-LEU2	*SGS1/SGS1* (ACD 94)	630	0	1	631
	SGS1/sgs1Δ (ACD 117)	221	0	1	222
	sgs1Δ/ sgs1Δ (ACD 96)	97	0	3	100
	pCLB2-SGS1/ sgs1Δ (ADA 2)	202	0	3	205
	sgs1-top3-id/pCLB2-SGS1 (ADA 14)	214	0	0	214
LEU2-MAT	*SGS1/SGS1* (ACD 94)	611	1	19	631
	SGS1/sgs1Δ (ACD 117)	218	1	5	224
	sgs1Δ/ sgs1Δ (ACD 96)	90	1	9	100
	pCLB2-SGS1/ sgs1Δ (ADA 2)	179	2	24	205 *
	sgs1-top3-id/pCLB2-SGS1 (ADA 14)	202	2	10	214

Using the G-test, we compared the distribution of PDs, NPDs and TTs for homeologous diploids. p-values < 0.01 were considered significant allowing us to reject the null hypothesis, H_0: 'the sets of data are the same'. The *SGS1/SGS1* (ACD 94), heterozygote (ACD 117), *sgs1Δ/sgs1Δ* (ADA 96) and *pCLB2-SGS1/sgs1Δ* (ADA 2) data are the same as in **Table 4.4** and are shown here for comparison.

* =significantly different from *SGS1/SGS1*

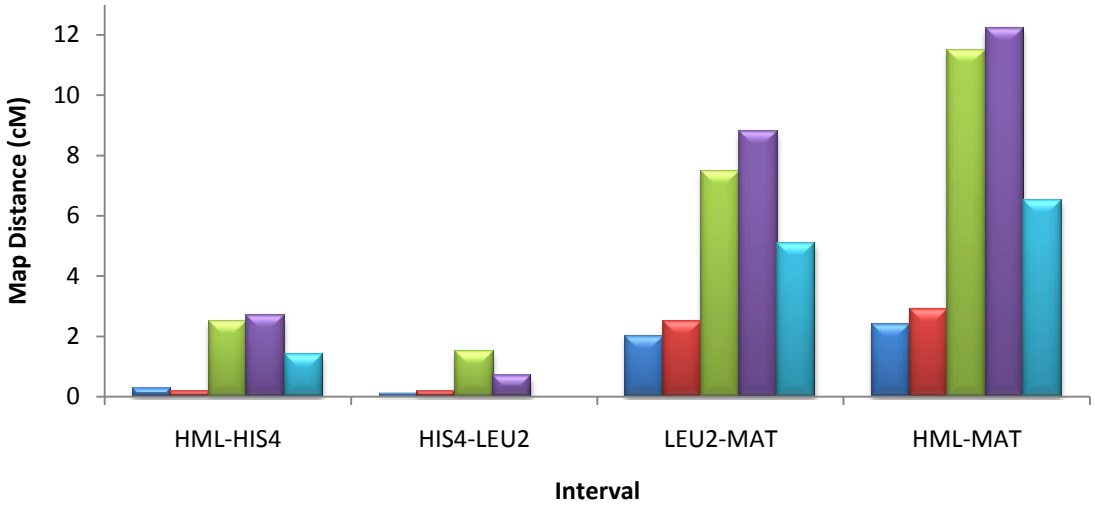


Figure 5.4: Genetic map distances for intervals along chromosome III for homeologous diploids.

The map distances (in centiMorgans) for the *HML-HIS4*, *HIS4-LEU2* and *LEU2-MAT* intervals were calculated using the following formula (using the PD, NPD and TT data shown in **Table 5.2**):

$$\frac{\frac{1}{2}(TT + 6NPD)}{PD + NPD + TT}$$

The map distances for the *HML-MAT* interval were calculated by summing the map distances for the other three intervals. The *SGS1/SGS1* (ACD 94), heterozygote (ACD 117), *sgs1Δ/sgs1Δ* (ACD 96) and *pCLB2-SGS1/sgs1Δ* (ADA 2) data are the same as in **Figure 4.2** and are shown here for comparison.

SGS1/SGS1 (ACD 94); *SGS1/sgs1Δ* (ACD 117); *sgs1Δ/sgs1Δ* (ACD 96); *pCLB2-SGS1/sgs1Δ* (ADA 2); *sgs1-top3-id/pCLB2-SGS1* (ADA 14)

As with the data obtained for *sgs1-mlh1-id* (**Table 4.4** and **Figure 4.2**), the data shown in **Table 5.2** and **Figure 5.4** suggest that *sgs1-top3-id* has no obvious effect on the rates of homeologous recombination as no significant increase in the rates of homeologous recombination were observed when compared to *SGS1* for any of the three intervals. Although the data in **Figure 5.4** suggest that the map distance is increased in two intervals – *HML-HIS4* and *LEU2-MAT*. The fact that no significant difference was observed from *SGS1* in these intervals may be a reflection on the sample size tested.

5.3.2.2 *sgs1-top3-id* decreases the rates of meiosis I non-disjunction

We assessed the levels of meiosis I non-disjunction caused by the *sgs1-top3-id* mutation (discussed in **Section 2.2.11.7** and illustrated in **Figure 1.4**). Meiosis I non-disjunction leads to the death of two spores. The two remaining spores are disomic and hence contain information from both parents (**Figure 1.4**). These disomes will be non-maters due to the presence of both mating-type cassettes on chromosome III. Therefore, we were able to assess the frequency of meiosis I non-disjunction events that have occurred by noting the number of non-maters amongst the two viable spore class of tetrads (**Table 5.3** and **Figure 5.5**).

As discussed in **Chapter 4**, Chaix (2007) suggested that meiosis I non-disjunction represents a failure of crossovers across the whole chromosome. Chaix (2007) proposed that the increase in meiosis I non-disjunction seen by the presence of sequence divergence is a direct consequence of a decrease in the rates of crossing over. This decrease in the rates of crossing over is suggested to increase the probability of missegregation events. Deletion of *sgs1*, leads to a decrease in the rates of meiosis I non-disjunction, which was hypothesised to reflect an increase of recombination between diverged sequences in the absence of the helicase (Chaix, 2007). We therefore wanted to assess whether the *sgs1-top3-id* mutation resulted in a similar decrease in the rates of meiosis I non-disjunction (**Table 5.3** and **Figure 5.5**).

Table 5.3: Levels of meiosis I non-disjunction events for the homeologous crosses

Homeologous Chromosome III Diploids	Meiosis I Non-Disjunction Events	Total Number of Tetrads	Percentage
SGS1 /SGS1 (ACD 94)	120	1040	11.54%
pCLB2-SGS1 / sgs1Δ (ADA 2)	25	539	4.64% *
sgs1-top3-id/pCLB2-SGS1 (ADA 14)	27	398	6.78% *

Using the G-test, we compared the numbers of meiosis I non-disjunction events (by summing the number of non-maters for the two viable spore class of tetrads) for homeologous diploids. As multiple data sets were compared, the Bonferroni correction was applied and so p-values < 0.01 were considered significant, allowing us to reject the null hypothesis, H_0: 'the sets of data are the same'. The *SGS1/SGS1* (ACD 94) and *pCLB2-SGS1/sgs1Δ* (ADA 2) data are the same as in **Table 4.5** and are shown here for comparison.

* =significantly different from *SGS1/SGS1*

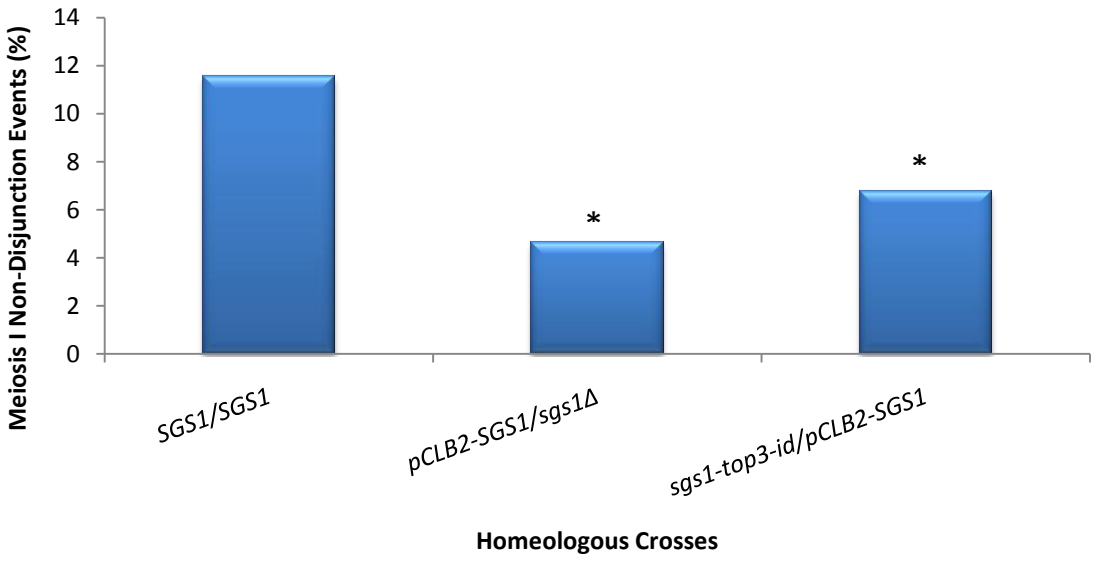


Figure 5.5: Graphical representation of the rates of meiosis I non-disjunction events

As described for **Table 5.3**, the G-test was used to compare the rates of meiosis I non-disjunction for the homeologous diploids. p-values < 0.01 were considered significant allowing us to reject the null hypothesis, H_0: 'the sets of data are the same'. The *SGS1/SGS1* (ACD 94) and *pCLB2-SGS1/sgs1Δ* (ADA 2) data are the same as in **Figure 4.4** and are shown here for comparison.

* =significantly different from *SGS1/SGS1*

As shown in **Table 5.3** and **Figure 5.5**, *sgs1-top3-id* shows a significant decrease in the levels of meiosis I non-disjunction when compared to *SGS1*. However, *sgs1-top3-id* does not significantly differ from *pCLB2-SGS1*. Assuming the hypothesis by Chaix (2007) (discussed above), the data suggest that *sgs1-top3-id* does affect the ability of Sgs1 in suppressing meiotic homeologous recombination.

However, as with *sgs1-mlh1-id* (**Chapter 4**), the data contradict the crossing over data (**Table 5.2** and **Figure 5.4**). As discussed in **Chapter 4**, this may be due to crossovers only being assessed across three intervals, one of which is highly diverged (**Figure 4.3**). Therefore, one could argue that the meiosis I non-disjunction data is a more sensitive method of assessing the effects of sequence divergence on recombination, as it may reflect a failure in crossing over across the entire chromosome. If this hypothesis is correct, then the data imply that the interaction between Sgs1 and Top3 is important for the suppression of meiotic homeologous recombination.

Alternatively, as addressed in **Chapter 4**, the meiosis I non-disjunction data may implicate Sgs1 in the suppression of distributive segregation. This is because the majority of the cells are still able to segregate their homologs in an ordered manner despite the presence of sequence divergence (discussed in detail in **Section 4.3.3**). If this is the case, then the interaction between Sgs1 and Top3 may be important in the suppression of distributive segregation. This possibility is discussed further in **Section 5.4**.

5.3.2.3 The interaction between Sgs1 and Top3 is not important in the suppression of unequal recombination events

We analysed the rates of unequal recombination events for *sgs1-top3-id* using the unequal recombination assay as described in **Section 2.2.13** and illustrated in **Figure 2.2**. Briefly, a strain carrying a HYG-CYH/HYG cassette (where a HYG-CYH cassette was inserted upstream of *HIS4* and a HYG cassette was inserted downstream of *LEU2* on chromosome III) was mated to a strain that does not contain this HYG-CYH/HYG cassette. Following tetrad dissection, we were able to assess the rates of unequal recombination. As shown in **Figure 2.2**, unequal recombination (caused by either unequal sister chromatid exchange or by a deletion) can be analysed by assessing the number of four viable spore tetrads exhibiting 3:1 hygromycin resistance : sensitivity and 2:2 cycloheximide resistance : sensitivity. However, 3:1 hygromycin resistance : sensitivity and 2:2 cycloheximide resistance : sensitivity can also be caused by gene conversion events (**Figure 2.2**). CHEF Gel (**Section**

2.2.2) and Southern Blot (**Section 2.2.8**) analysis allows us to differentiate between these events (examples shown in **Figure 2.2**).

Sgs1 has been shown to be important in maintaining the barrier to sister chromatid recombination, as deletion of *sgs1* has been shown to increase the rate of unequal recombination (Chaix, 2007) (also shown in **Table 5.5** for comparison). We therefore wanted to assess whether the interaction between Sgs1 and Top3 was important for this role of Sgs1. We therefore mated the *sgs1-top3-id* mutation (which also contains the HYG-CYH/HYG cassette) to *pCLB2-SGS1* in the partial hybrid strain (ADA 14). We also mated the *sgs1-top3-id* to *S. cerevisiae sgs1Δ* and *pCLB2-SGS1* homologous control strains (ADA 12 and ADA 13). Following tetrad dissection, we selected every four viable spore tetrad that exhibited 3:1 hygromycin resistance : sensitivity and 2:2 cycloheximide resistance : sensitivity and subjected them to CHEF Gel and Southern Blot analysis. This was carried out in order to differentiate the types of recombination events that occurred (as described in **Section 2.2.13** and illustrated in **Figure 2.2**). An example of a CHEF Gel from a homologous cross is shown in **Figure 4.5**.

We compared the effects of the *sgs1-top3-id* mutation on the distribution of gene conversion events (**Table 5.4**) and unequal recombination events (**Table 5.5**) separately, using the G-test. As we were compared the distribution of multiple data sets, we applied the Bonferroni correction, and so p-values that were less than 0.01 allowed us to reject the null hypothesis, H_0: 'the sets of data are the same'.

Table 5.4: Gene conversion events for homologous diploids

Homologous Chromosome III Diploid	**Number of gene conversion events**	**Tetrads that did not exhibit a gene conversion**	**Total Number of tetrads**	**Percentage of gene conversion events**
SGS1/*SGS1* (ACD 97)	11	244	255	4.3%
sgs1Δ/ sgs1Δ (ACD 116)	5	173	178	2.8%
sgs1-ΔC795/ sgs1Δ (ADA 3)	4	173	177	2.6%
sgs1-top3-id /sgs1Δ (ADA 12)	4	221	225	1.8%
sgs1-top3-id / pCLB2-SGS1 (ADA 13)	2	40	42	4.8%

Using the G-test, we compared the distribution of gene conversion events for homologous diploids (by comparing the number of gene conversions vs the number of tetrads that did not exhibit a gene conversion amongst the homologous diploids). p-values < 0.01 were considered significant allowing us to reject the null hypothesis, H_0: 'the sets of data are the same'. The *SGS1*/*SGS1* (ACD 97) and *sgs1Δ*/*sgs1Δ* (ACD 116) diploid data were provided by Chaix (2007). The *SGS1*/*SGS1* (ACD 97), *sgs1Δ*/*sgs1Δ* (ACD 116) and *sgs1-ΔC795*/*sgs1Δ* (ADA 3) diploid data are the same as in **Table 4.8** and are shown here for comparison. No significance was observed for any of the diploids.

Table 5.5: Unequal recombination events for homologous diploids

Homologous Chromosome III Diploid	Unequal Recombination Events		Tetrads that did not exhibit an unequal recombination event	Total Number of tetrads	Percentage of unequal recombination events
	USCE Events	Deletion Events			
SGS1/*SGS1* (ACD 97)	6	0	249	255	2.35%
sgs1Δ/ sgs1Δ (ACD 116)	13	5	160	178	10.11% *
sgs1-ΔC795/ sgs1Δ (ADA 3)	4	8	165	177	6.78% *
sgs1-top3-id /sgs1Δ (ADA 12)	9	4	212	225	5.78%
sgs1-top3-id / pCLB2-SGS1 (ADA 13)	2	1	39	42	7.14%

Using the G-test, we compared the distribution of unequal recombination events for homologous diploids (by comparing the number of unequal recombination events vs the number of tetrads that did not exhibit unequal recombination amongst the homologous diploids). p-values < 0.01 were considered significant allowing us to reject the null hypothesis, H_0: 'the sets of data are the same'. The *SGS1*/*SGS1* (ACD 97) and *sgs1Δ*/*sgs1Δ* (ACD 116) diploid data were provided by Chaix (2007). The *SGS1*/*SGS1* (ACD 97), *sgs1Δ*/*sgs1Δ* (ACD 116) and *sgs1-ΔC795*/*sgs1Δ* (ADA 3) diploid data are the same as in **Table 4.9** and are shown here for comparison.

* =significantly different from *SGS1*/*SGS1*

As shown in **Tables 5.4** and **5.5**, the number of gene conversion events and unequal recombination events obtained for the homologous *sgs1-top3-id/sgs1Δ* diploid and the *sgs1-top3-id/pCLB2-SGS1* diploid did not significantly differ from any of the other homologous diploids. The data therefore suggest that the interaction between Sgs1 and Top3 is not important in the suppression of unequal recombination events. However, as with the crossing over data, the data presented in **Table 5.5** suggest that the percentage of unequal recombination events may be elevated for the *sgs1-top3-id* mutation when compared to *SGS1*. The fact that we do not see a significant difference from *SGS1* may be a reflection on the sample size tested in this experiment.

We also assessed the effects of the *sgs1-top3-id* mutation on unequal recombination in the partial hybrid strain. An example of a CHEF Gel from a homeologous cross is illustrated in **Figure 4.6**. As with the homologous diploids, we compared the effects of the *sgs1-top3-id* mutation on the distribution of gene conversion events (**Table 5.6**) and unequal recombination events (**Table 5.7**) separately, using the G-test. The Bonferroni correction was applied as multiple comparisons were carried out, and so p-values that were less than 0.0167 allowed us to reject the null hypothesis, H_0: 'the sets of data are the same'.

Table 5.6: Gene conversion events for homeologous diploids

Homeologous Chromosome III Diploid	Number of gene conversion events	Tetrads that did not exhibit a gene conversion	Total Number of tetrads	Percentage of gene conversion events
SGS1/*SGS1* (ACD 94)	0	222	222	0%
pCLB2-SGS1/ sgs1Δ (ADA 2)	0	301	301	0%
sgs1-top3-id / pCLB2-SGS1 (ADA 14)	0	218	218	0%

Using the G-test, we compared the distribution of gene conversion events for homeologous diploids (by comparing the number of gene conversions vs the number of tetrads that did not exhibit a gene conversion amongst the homeologous diploids). p-values < 0.0167 were considered significant allowing us to reject the null hypothesis, H_0: 'the sets of data are the same'. The *SGS1*/*SGS1* (ACD 94) and *pCLB2-SGS1*/*sgs1Δ* (ADA 2) diploid data are the same as in **Table 4.10** and are shown here for comparison. No gene conversions were observed for nay of the homeologous diploids, and therefore, no significance was observed.

Table 5.7: Unequal recombination events for homeologous diploids

Homeologous Chromosome III Diploid	Unequal Recombination Events		Tetrads that did not exhibit an unequal recombination event	Total Number of tetrads	Percentage of unequal recombination events
	USCE Events	Deletion Events			
SGS1/*SGS1* (ACD 94)	11	17	194	222	12.61%
pCLB2-SGS1/ sgs1Δ (ADA 2)	28	24	249	301	17.28%
sgs1-top3-id / pCLB2-SGS1 (ADA 14)	8	10	200	218	8.26% †

Using the G-test, we compared the distribution of unequal recombination events for homeologous diploids (by comparing the number of unequal recombination events vs the number of tetrads that did not exhibit unequal recombination amongst the homeologous diploids). p-values < 0.0167 were considered significant allowing us to reject the null hypothesis, H_0: 'the sets of data are the same'. The *SGS1*/*SGS1* (ACD 94) and *pCLB2-SGS1*/*sgs1Δ* (ADA 2) diploid data are the same as in **Table 4.11** and are shown here for comparison.

† =significantly different from *pCLB2-SGS1*/*sgs1Δ*

No gene conversions were detected for any of the homeologous diploids (**Table 5.6**). *sgs1-top3-id* shows a significant decrease in unequal events when compared to *pCLB2-SGS1* but is not statistically different from *SGS1*. The data suggest that the interaction between Sgs1 and Top3 is not important in maintaining the barrier to sister chromatid recombination in response to sequence divergence. However, as discussed in **Section 4.4.3**, we speculate that the assay used for this investigation is not sensitive enough for this analysis due to the large amount of sequence divergence present in the *HIS4-LEU2* interval (**Figure 4.3**).

5.3.2.4 The interaction between Sgs1 and Top3 is not responsible the increase in non-sister spores in the 2 viable spore class of tetrads

Deletion of *sgs1* leads to an increase in non-sister spores compared to sister spores for the two viable spore class of tetrads (Chaix, 2007). Chaix (2007) proposed that this represented a late role for Sgs1 in meiosis, in which it interacts with Top3 in the decatenation of double Holliday junction structures. The model (**Figure 5.1**) suggested that in the absence of their interaction double Holliday junctions are not dissolved, leading to the death of the two spores which contain the entangled chromosomes.

To assess whether the increase in non-sister spores seen by deletion of *sgs1* was dependent on the interaction between Sgs1 and Top3 (**Figure 5.1**), we examined the effects of the *sgs1-top3-id* mutation on the number of sister and non-sister spores. We mated the *sgs1-top3-id* mutation to *sgs1Δ* (ADA 12) and also to *pCLB2-SGS1* (ADA 13) and carried out tetrad dissection. As described in **Section 2.2.11.6**, the frequencies of sister and non-sister spores can be assessed by analysing the segregation of the centromere marker *TRP1* for the two viable spore class of tetrads. Sisters were identified if both viable spores were auxotrophic or prototrophic for tryptophan, whereas non-sister spores were identified if one spore was auxotrophic and the other was prototrophic for tryptophan. We compared the numbers of sister and non-sister spores for the *sgs1-top3-id* homologous diploids (ADA 12 and ADA 13) to *sgs1*Δ (ACD 95), *sgs1-ΔC795* (ADA 3) and *pCLB2-SGS1* (ADA 1) homologous diploids using the χ^2 test. This test was used as random death would result in equal frequencies of sister to non-sister spores. Therefore, this test enabled us to determine whether disrupting the interaction between Sgs1 and Top3 resulted in a significant deviation from this 50:50 distribution.

Table 5.8: Distribution of sister and non-sister spores for homologous diploids

Homologous Chromosome III Diploids	**2 viable spore class of tetrads**		**p-value**
	Sisters	**Non-Sisters**	
sgs1Δ / sgs1Δ (ACD 95)	110 (36.54%)	191 (63.46%)	$3x10^{-6}$ *
sgs1-ΔC795 / sgs1Δ (ADA 3)	56 (38.62%)	89 (61.38%)	0.006 *
pCLB2-SGS1 / sgs1Δ (ADA 1)	3 (14.29%)	18 (85.71%)	0.001 *
sgs1-top3-id / sgs1Δ (ADA 12)	47 (43.52%)	61 (56.48%)	0.178
sgs1-top3-id / pCLB2-SGS1 (ADA 13)	3 (42.46%)	4 (57.54%)	0.705

Using the χ^2 test, we were able to calculate whether the numbers of sister : non-sister spores deviated from the expected 50:50 ratio if death was random. p-values < 0.05 were considered significant using χ^2 test (significance denoted by *) which indicated that the ratio of sister : non-sister spores deviated significantly from 50:50. The *sgs1Δ/sgs1Δ* and *sgs1-ΔC795/sgs1Δ* data were provided by Chaix (2007)

The data for both *sgs1-top3-id/sgs1Δ* and *sgs1-top3-id/pCLB2-SGS1* (**Table 5.8**) show no significant difference between the numbers of sister and non-sister spores. This suggests that the inability of

Top3 to interact with Sgs1 is not the reason as to why *sgs1Δ* cells exhibit an increase in non-sister spores in the two viable spore class of tetrads. An alternative model is proposed in **Section 5.4**.

5.3.3 The helicase activity of Sgs1 is required for sporulation

In order to assess the effects of inactivating the helicase domain of Sgs1 on homeologous recombination, we mated a strain carrying the *sgs1-K706A* mutation to *pCLB2-SGS1* in the partial hybrid (ADA 16) and *pCLB2-SGS1* in a *S. cerevisiae* homologous control (ADA 15). We aimed to dissect the tetrads and assess the distribution of PDs, NPDs and TTs as described above for the *sgs1-top3-id* mutation and in **Chapter 4** for the *sgs1-mlh1-id* mutation. However, we were unable to detect many tetrads after the crosses were sporulated.

We needed to be able to dissect the homeologous *sgs1-K706A/pCLB2-SGS1* diploid (ADA 16) in order to assess whether *sgs1-K706A* affected the ability of Sgs1 to suppress homeologous recombination. We carried out DAPI staining analysis (described in **Section 2.211.4**) which enables us to count the number of nuclei present per cell. The DAPI staining analysis (**Table 5.9**) shows that the *sgs1-K706A/pCLB2-SGS1* homologous diploid (ADA 15) produces very few cells containing 4 nuclei, with the majority of cells present containing only one nuclei. This suggests that the *sgs1-K706A* mutant results in a sporulation defect. In order to assess whether this was a dominant-negative phenotype, we set up the control cross ADA 21 where *sgs1-K706A* was crossed to an *SGS1* strain. As the cross is able to produce a greater number of cells containing 4 nuclei, the sporulation defect conferred by the *K706A* point mutation is not dominant-negative.

This sporulation defect is in contrast to what has been reported by Miyajima *et al* (2000a, 2000b) who show that the *sgs1-K706A* mutation was able to complement the poor sporulation phenotype exhibited by *sgs1Δ* cells. However, our observations are in agreement with Weinstein and Rothstein (2008) who showed that a homozygous *sgs1-K706A* diploid exhibits poor sporulation. As discussed in **Section 1.6.3**, the different sporulation phenotypes that were observed can be attributed to the experimental approach utilised by these groups. Miyajima *et al* (2000a, 2000b) used a plasmid-based complementation study, whereas Weinstein and Rothstein (2008) transplaced the *K706A* point mutation at the appropriate place in the genome. Our studies also use this latter approach. The potential implications of these different phenotypes have been discussed briefly in **Section 1.6.3** and will be discussed further in **Section 5.4**.

Table 5.9: DAPI staining analysis

Homologous Chromosome III Diploid	**Number of cells with 1 nuclei**	**Number of cells with 2 nuclei**	**Number of cells with 4 nuclei**	**TOTAL**
SGS1/*sgs1Δ* homologous (ADA 23)	28 (13.2%)	5 (2.4%)	180 (84.5%)	213
pCLB2-SGS1/sgs1Δ homologous (ADA 1)	75 (32.3%)	10 (4.3%)	147 (63.4%)	232
sgs1-K706A/pCLB2-SGS1 homologous (ADA 15)	171 (80.7%)	3 (1.4%)	38 (17.9%)	212
sgs1-K706A/SGS1 homologous (ADA 21)	34 (15.8%)	0 (0%)	181 (84.2%)	215

DAPI staining (described in **Section 2.2.11.4**) fluorescently labels the DNA which enables examination of the nuclei. The *sgs1-K706A/pCLB2-SGS1* mutation leads to very few cells with four nuclei which is indicative of a sporulation defect. However, when *sgs1-K706A* is mated to an *SGS1* strain, the proportion of cells with four nuclei resembles the *SGS1*/*sgs1Δ* homologous diploid. This indicates that the observed sporulation defect caused by the *sgs1-K706A* mutation is not dominant negative.

This phenotype exhibited by the *K706A* mutation suggests that inactivation of the helicase domain of Sgs1 results in a defect in sporulation. However, the *sgs1-ΔC795* truncation, which deletes the C-terminus of Sgs1 including the helicase domain, is able to sporulate more efficiently than an *sgs1* deletion (Rockmill et al., 2003) and has been used by several laboratories. We hypothesised that this discrepancy was due to the presence of an intact HRDC domain in the *sgs1-K706A* mutant.

We proposed a model (**Figure 5.6**) where the helicase domain of Sgs1 is necessary for sporulation. As discussed in **Chapter 1**, a pre-meiotic checkpoint exists which ensures cell cycle arrest to repair DNA lesions caused by replication prior to entry into meiosis. As Sgs1 has been shown to act in mitotic cell cycle checkpoints to repair DNA lesions, we propose that Sgs1 is also involved in this pre-meiotic checkpoint. We suggest that the wild-type Sgs1 is recruited via its HRDC domain to lesions that arise during DNA replication prior to meiosis. We hypothesise that correction of these lesions is required for the process of sporulation. The HRDC domain is proposed to function by facilitating Sgs1 to stably bind DNA. On binding to the DNA, Sgs1 uses its helicase activity to correct these lesions. Despite the fact that the helicase domain is inactivated in the *sgs1-K706A* mutant, we suggested that Sgs1 is still recruited to these lesions on the DNA via its HRDC domain. However, despite being at the desired location and despite stably binding to DNA, the lack of helicase activity of Sgs1 results in the inability to repair these DNA lesions, resulting in poor sporulation. In contrast, as the *sgs1-ΔC795* truncation not only deletes the helicase domain, but also deletes the HRDC domain, we propose that Sgs1 cannot be recruited to these DNA. As a result, we suggest that in the absence of Sgs1, alternative pathways, which may use alternative helicases, are used in the repair of DNA lesions for the process of sporulation. We suggest that in the *sgs1-K706A* mutant, the recruitment of a helicase-defective Sgs1 protein to these DNA lesions will block other repair proteins

or alternative helicases from being recruited. Therefore, the DNA lesions will not be repaired leading to poor sporulation.

To test this hypothesis, we constructed a truncation of Sgs1 that deleted the HRDC domain. We replaced the 360 residues from the start of the HRDC domain to the stop codon of Sgs1 with a KANMX4 cassette. This was carried out in a wild-type strain of Sgs1 (Y55 3588) as well as in the *sgs1-K706A* strain (Y55 3589) (**Section 5.2.2.1**). If our hypothesis is correct, both of these truncation strains should be able to sporulate, as the inability to recruit Sgs1 via its HRDC domain should activate this minor pathway and recruit an alternative helicase to Sgs1.

However, both of these strains sporulated very poorly. Although this highlights the importance of the HRDC domain in sporulation, presumably in the correction of DNA lesions (**Figure 5.6A**), it disproves our theory as to why the *sgs1-ΔC795* mutation is able to sporulate (**Figure 5.6C**). Further to this, Malik (*unpublished*) created several mutations in the HRDC domain of Sgs1 that disrupted the DNA binding ability of Sgs1. This *sgs1-K1329V,R1333S,K1336S,K1339S,I1342R,L1345R* mutation also was unable to sporulate, further disproving our theory (**Figure 5.6C**). Malik (*unpublished*) showed that for the *sgs1-K1329V,R1333S,K1336S,K1339S,I1342R,L1345R/pCLB2-SGS1* homologous cross, only 4.21% of the cells contained 4 nuclei. As with the *sgs1-K706A* mutation, this phenotype was not dominant-negative. Consistent with our MMS sensitivity assay (**Figure 5.2**), Malik (*unpublished*) showed that this *sgs1-K1329V,R1333S,K1336S,K1339S,I1342R,L1345R* mutation was resistant to MMS and was therefore able to repair lesions that lead to replication fork stalling and collapse.

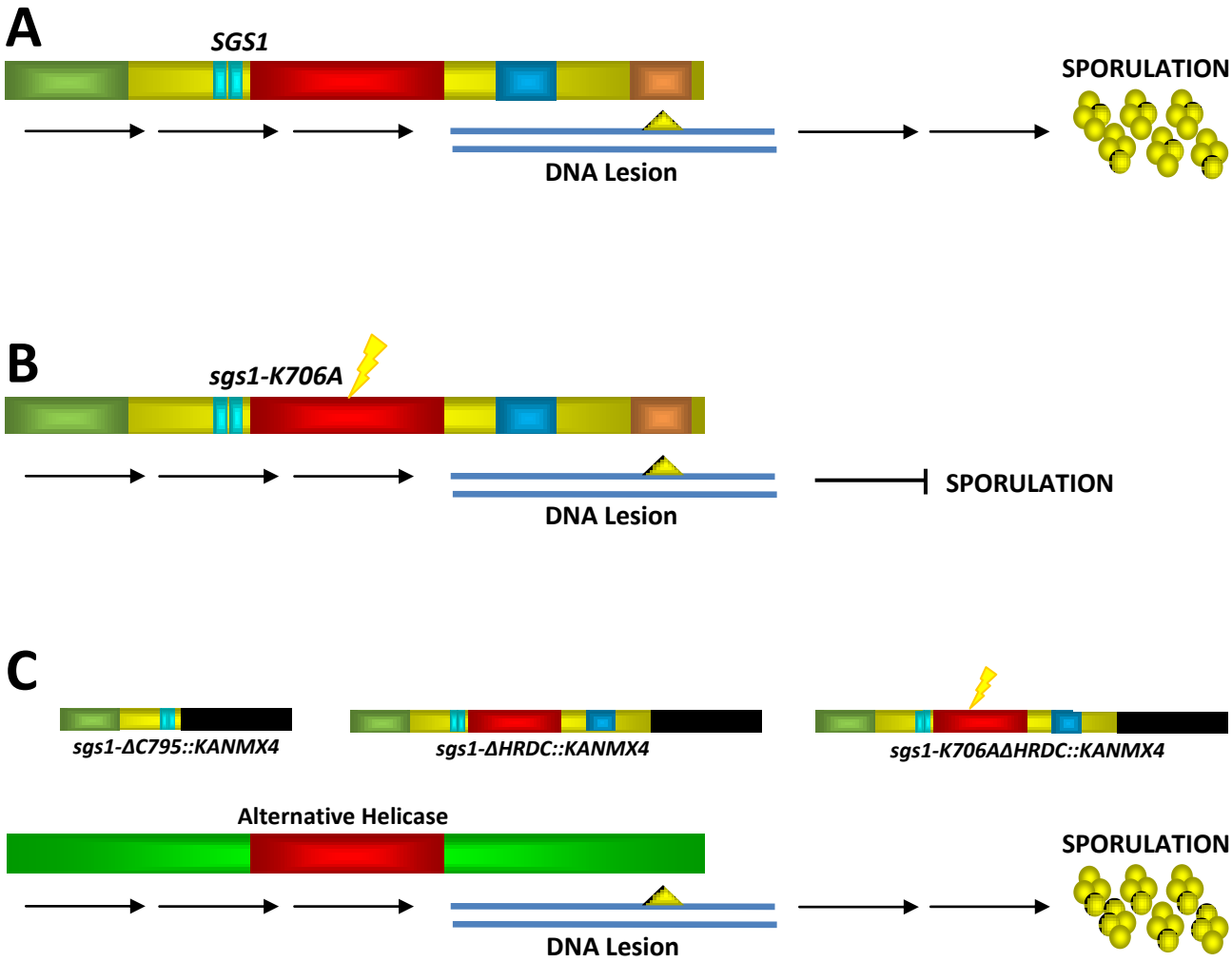


Figure 5.6: Model suggesting the predicted the role of Sgs1 in sporulation

Under wild-type conditions, the helicase activity of Sgs1 is recruited to lesions that arise during DNA replication prior to meiosis via the HRDC domain. Cell cycle arrest allows for the repair of these lesions which is essential for the process of sporulation. This repair is carried out by Sgs1 who binds to the DNA via its HRDC domain and corrects the DNA lesions using its helicase activity (**A**). Despite inactivation of the helicase domain (*sgs1-K706A*), Sgs1 is still recruited via its HRDC domain. However, despite being able to stably bind to the DNA, the lack of helicase activity results in the inability to correct the DNA lesions, resulting in poor sporulation (**B**). In addition to this, the recruitment of the helicase-defective Sgs1 protein via the HRDC domain will prevent the recruitment of additional factors that may act in the repair of these DNA lesions, therefore leading to poor sporulation (**B**). For *sgs1-ΔC795* (where Sgs1 is truncated deleting both the helicase domain and the HRDC domain), *sgs1-ΔHRDC* (where Sgs1 has been truncated deleting the HRDC domain) and *sgs1-K706AΔHRDC* (where Sgs1 has been truncated deleting the HRDC domain and the helicase domain has been inactivated), Sgs1 cannot be recruited to the DNA lesions due to the absence of the HRDC domain. As a result, an alternative helicase is recruited, which enables sporulation (**C**).

5.4 Discussion

5.4.1.1 The ability of Sgs1 to suppress 'homeologous' recombination may be partially dependent on its ability to interact with Top3

The crossing over data (**Table 5.2** and **Figure 5.4**) suggest that the interaction between Sgs1 and Top3 is not important for the ability of Sgs1 to suppress meiotic homeologous recombination, as the rates of crossing over for *sgs1-top3-id* do not significantly differ from *SGS1*. However, as discussed in **Section 4.4.1**, this does not necessarily mean that the interaction between Sgs1 and Top3 is not important for this process. It is possible that many factors act independently in the regulation of meiotic homeologous recombination. Therefore, despite abolishing the interactions between Sgs1 and Top3, homeologous recombination may be suppressed in an alternative way, which may explain why we do not see a significant increase in the levels of crossing over. This alternative mechanism may still involve a role for Sgs1, suggesting that it can still be recruited to the sites of homeologous recombination and interact with other protein to act in its suppression. As we suspect that the interaction between Sgs1 and Mlh1 is also important for this role (**Chapter 4**), it would be interesting to create a double *sgs1-(mlh1+top3)-id* mutation and assess its effects on the ability of Sgs1 to suppress meiotic homeologous recombination.

As was seen with the *sgs1-mlh1-id* mutation (**Chapter 4**), the *sgs1-top3-id* mutation exhibits a significant decrease in the rates of meiosis I non-disjunction events when compared to *SGS1* (**Table 5.3** and **Figure 5.5**). As suggested by Chaix (2007), the meiosis I non-disjunction data can be interpreted as a measure for the failure of crossing over across the entire chromosome III. If this is the case, then one could argue that this data shows that the interaction between Sgs1 and Top3 is important in the suppression of homeologous recombination. This is because the decrease in the levels of meiosis I non-disjunction seen for the *sgs1-top3-id* mutation (**Table 5.3** and **Figure 5.5**) may reflect an increase in crossing over between diverged sequences that is facilitated by the absence of interactions between Sgs1 and Top3 (as discussed in **Section 4.4.1** for the data obtained for the *sgs1-mlh1-id* mutation).

If the interaction between Sgs1 and Top3 is important in the suppression of meiotic homeologous recombination, we propose that the nature of this interaction is different to the interaction between Sgs1 and Mlh1. We propose that Mlh1 potentially recruits Sgs1 to the sites of homeologous recombination, so that the Sgs1 helicase can carry out its unwinding actions at SEI events occurring between diverged sequences. We suggest that in addition to this, Sgs1 also recruits Top3 to these

sites. We propose that successful strand invasions may occur if the degree of sequence divergence is low at the sites of initiation. When the degree of sequence divergence is extremely low, the helicase activity of Sgs1 may not be utilised in the unwinding of the SEI. This would therefore result in a dHJ structure that may contain mismatches. We hypothesise that the role of Top3, in association with Sgs1, is to resolve these mismatch-containing dHJ structures as non-crossovers. In the absence of interactions between Sgs1 and Top3, these intermediates are resolved as crossovers, which leads to an increase in 'homeologous' recombination. This model suggests that the roles of Sgs1 at the sites of homeology are different depending on the degree of sequence divergence present. If this is the case, then perhaps the recently identified third component to the Sgs1-Top3 complex, Rmi1, may also play a role in aiding Sgs1 in the suppression of homeologous recombination. As mentioned above, it still remains possible however that additional proteins act in the recruitment of Sgs1 to maintain the barrier to inter-species recombination (**Chapter 6**).

As discussed in **Section 4.4.1**, the meiosis I non-disjunction data may reflect another role for Sgs1, in the suppression of distributive segregation (Dawson et al., 1986, Guacci and Kaback, 1991, Mann and Davis, 1986, Kaback et al., 1989). We suggest that in the absence of perfect homology, the cell may act to segregate homologs using the distributive segregation pathway in an effort to promote recombination. We suggest that this response is similar to the SOS response in *E. coli* which promotes inter-species mating (discussed in **Section 1.2.1.1**). There is an increase in the number of cells that are able to segregate their homologs in an ordered manner for the *sgs1-top3-id* mutant when compared to *SGS1*. This suggests that both Sgs1 and Top3 may act in the suppression of events that may lead to distributive segregation. We proposed in **Section 4.4.1** that Sgs1 required to counteract this mechanism, perhaps by unwinding physical interactions along the chromosome that would lead to distributive segregation. We likened this hypothesised role of Sgs1 to its role in suppressing the formation of closely-spaced double crossovers, which result in the formation of inter-homolog and multi-chromatid joint molecules (Jessop and Lichten, 2008) (discussed in **Section 1.3.2**). We speculate that the unwinding of physical interactions that may lead to distributive segregation by Sgs1 may generate torsional stress, which is relieved by Top3.

5.4.1.2 Sgs1 interacts with Top2 to resolve errors that occur during DNA replication prior to meiosis

Chaix (2007) showed that the absence of Sgs1 leads to a increase in the number of non-sister spores for the two viable spore class of tetrads (**Table 5.8**). It was proposed that this was due to the absence of interactions between Sgs1 and Top3 which act to dissolve double Holliday junction

structures at a late stage during meiosis (**Figure 5.1**). The data obtained for the *sgs1-top3-id* mutation show that the absence of any interactions between Sgs1 and Top3 does not lead to any significant differences between the numbers of sister and non-sister spores (**Table 5.8**). The data obtained for the *sgs1-ΔC795* mutant also show an increase in the number of non-sister spores compared to sister spores (**Table 5.8**). This mutant deleted the C-terminal 795 amino acids, but retains the Top3-interacting domain of Sgs1. This also suggests that the interaction between Top3 and Sgs1 is not important in suppressing the number of non-sister spores. Although the Top3-interacting domain of Sgs1 is present in the *sgs1-ΔC795* mutation, the Top2-interacting domain of Sgs1 may have been deleted. Sgs1 has been shown to interact with Top2, and this interaction partially overlaps with the helicase domain of Sgs1 (Watt et al., 1995) (**Figure 1.19B**). Therefore, it is possible that the inability of Sgs1 to interact with Top2 in both the *sgs1Δ* and *sgs1-ΔC795* mutants leads to the increase in non-sister spores. Therefore, we propose that Sgs1 and Top2 act together in the decatenation of pre-meiotic replication errors (**Figure 5.7**). The absence of this interaction leads to the destruction of the two entangled spores when a crossover occurs between the entangled region and the centromere. If this is the case, the two remaining spores will be non-sisters. This destruction is proposed to be similar to that seen in *A. thaliana* cells where entangled chromosomes are torn apart during anaphase I (Chan et al., 2007) (as discussed in **Section 1.7.5**). In agreement with this model, the *sgs1-top3-id* mutation, which disrupts the ability of Sgs1 to interact with Top3, but does not affect its interactions with Top2, does not lead to a significant difference in the number of sister and non-sister spores. If this model is true, it further implicates a role for Sgs1 in the pre-meiotic replication checkpoint to resolve errors that have occurred during DNA replication prior to meiosis. In order to confirm whether the interactions between Sgs1 and Top2 are important in the resolution of pre-meiotic errors, it would be interesting to create an *sgs1 top2* double mutant, perhaps using the temperature-sensitive *top2* mutant background (Jannatipour et al., 1993, Nitiss et al., 1993).

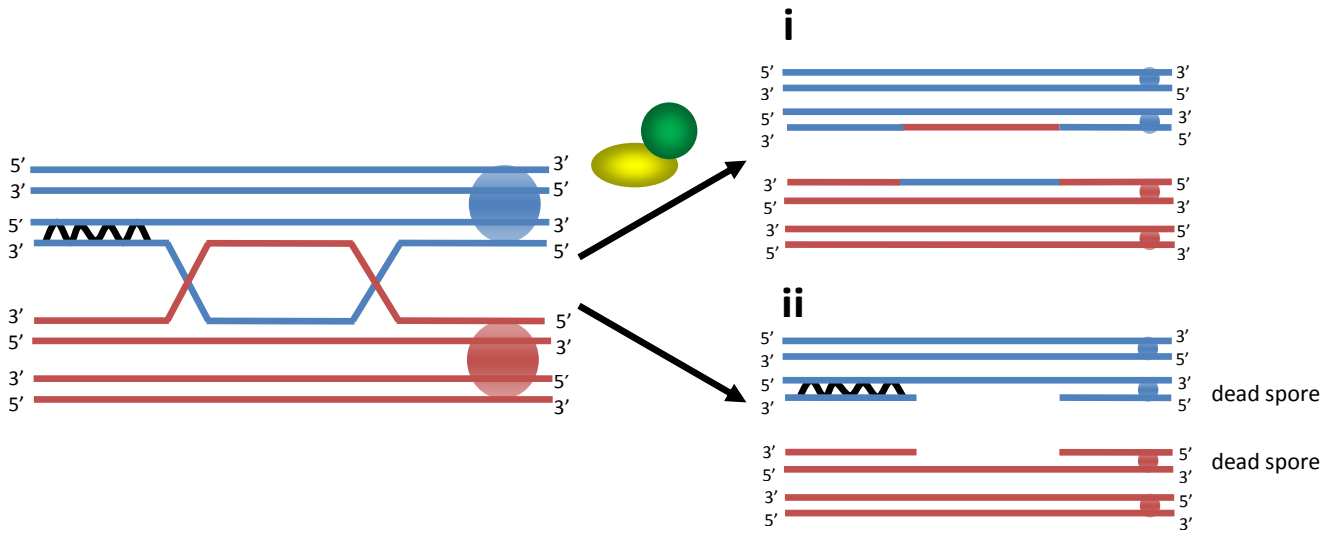


Figure 5.7: Sgs1 interacts with Top2 to pre-meiotic replication errors

Sgs1 acts with Top2 in the pre-meiotic checkpoint in the decatenation of DNA replication errors that occur prior to meiosis (**i**). In the absence of this interaction, the two chromosomes remain entangled, leading to spore death (**ii**).

5.4.2 Sgs1 acts in the activation of the pre-meiotic replication checkpoint to repair replication errors that prevent sporulation

By mutating the helicase domain of Sgs1 we saw a major defect in sporulation (**Table 5.9**), suggesting that the helicase activity of Sgs1 is required for this process. This is in contrast to the sporulation phenotype reported by Miyajima *et al* (2000a, 2000b) who used a plasmid-based complementation study to assess the effects of the *sgs1-K706A* mutation. However, our observations are in agreement with Weinstein and Rothstein (2008) who transplaced the *K706A* point mutation in the genome and also saw a sporulation defect. As discussed in **Section 1.6.3**, we suggest that the *sgs1-K706A* mutation may have been overexpressed in the studies by Miyajima *et al* (2000a, 2000b) which may account for the rescued sporulation phenotype. This would suggest that the *sgs1-K706A* mutation is hypomorphic, and therefore may lead to a decreased activity of the Sgs1 gene. In support of this, although the studies by Miyajima *et al* (2000a, 2000b) report that the *sgs1-K706A* mutation rescues the poor sporulation phenotype exhibited by *sgs1Δ* cells, the rate of rescue was slow. They showed that only 25% of cells sporulated by 12 hours, which was improved to 60% by 24 hours. To assess whether the *sgs1-K706A* mutation is hypomorphic, it would be interesting to quantify the amount of mRNA produced by carrying out Northern blot analysis so that we can compare the amounts of mRNA produced when the *sgs1-K706A* point mutation is transplaced into the genome to the amounts produced using a plasmid-based complementation assay.

As data by Malik (*unpublished*) showed that the HRDC domain of Sgs1 is also important for sporulation, as point mutations that disrupted the DNA binding activity of the HRDC domain lead to a major sporulation defect, we proposed that Sgs1 acts in the pre-meiotic checkpoint to repair DNA lesions that have occurred during DNA replication. The correction of these lesions was suggested to be essential for the process of sporulation. We proposed that these lesions induce a checkpoint-controlled cell cycle arrest, which leads to the recruitment of Sgs1 to these lesions. Sgs1 is recruited to via the HRDC domain, which facilitates stable binding to the DNA. The repair of the DNA lesions is then carried out by the helicase activity of Sgs1 (**Figure 5.6A**). Despite mutating the helicase domain in the *sgs1-K706A* mutant, the HRDC domain is still intact. Therefore, we suggest that Sgs1 is still recruited to the DNA lesions during cell cycle arrest. However, it cannot function in their repair due to the absence of any helicase activity, and may also block the recruitment of additional repair factors, leading to a poor sporulation phenotype (**Figure 5.6B**). As the HRDC domain mutation of Sgs1 also did not sporulate, we can postulate that the importance of the HRDC domain is in the sensing of DNA lesions, which leads to activation of the pre-meiotic checkpoint. This leads to cell

cycle arrest which allows for the repair of these lesions. This repair may involve the actions of Sgs1, as well as other proteins that are activated by this checkpoint activation.

However, our hypothesis does not explain why *sgs1-ΔC795* is able to sporulate despite the absence of any helicase activity or the HRDC domain (**Figure 5.6C**). It is possible that Sgs1 is recruited by unknown factors that bind within the region between the helicase domain and the HRDC domain to correct these DNA lesions, as this region is also deleted in *sgs1-ΔC795*. As shown in **Figure 1.19B**, the RQC (RecQ Conserved) domain lies within this region. It is therefore possible that this domain, which mediates protein-protein interactions, is responsible for the recruitment of Sgs1. If this is the case, we propose that the role of Sgs1 in sporulation may involve activation of the pre-meiotic checkpoint and this activity is mediated through protein-protein interactions. However, as the RQC domain is absent in the *sgs1-ΔC795* mutation, the cell would be unable to recruit Sgs1 and may therefore act to recruit an alternative helicase to carry out the repair required for sporulation (**Figure 5.7C**) (discussed further in **Chapter 6**). Therefore, it would be interesting to see whether mutations in the RQC domain also cause a sporulation defect. The importance of both the HRDC and RQC domains for the helicase activity of the RecQ helicases was highlighted by Macris *et al* (2006) who suggest that the absence of any helicase activity in RECQL4 is due to the absence of these domains in the protein (as discussed in **Chapter 1**).

This hypothesis proposes two distinct roles for Sgs1 in the pre-meiotic replication checkpoint. The first is in the activation of this checkpoint in response to aberrant structures that need to be repaired for efficient sporulation. The second is a role for Sgs1, together with Top2, in resolving entangled chromosomes that would lead to spore death (discussed in **Section 5.4.1.2** and shown in **Figure 5.7**). However, these two roles may not be mutually exclusive. As shown in **Figure 1.19B**, the *sgs1-K706A* mutation overlaps the Top2-interacting domain of Sgs1, which is predicted to span amino acid residues 466-746 (Duno et al., 2000, Watt et al., 1995). It is possible that the *sgs1-K706A* point mutation also disrupts the interaction between Sgs1 and Top2 and this is the underlying cause of the sporulation defect. It would be interesting to carry out yeast-two-hybrid and co-immunoprecipitation experiments to assess whether the interaction between Sgs1 and Top2 is disrupted by the *sgs1-K706A* mutation.

Chapter 6: Discussion and Future Perspectives

6.1 The role of Sgs1 in the suppression of meiotic homeologous recombination may be dependent on its interactions with Mlh1 and Top3 (at least) ... however, these interactions may be important for the suppression of distributive segregation

Data in our laboratory implicated components of the mismatch repair (MMR) complex, consisting of Msh2, Msh6 and Pms1, along with the Sgs1 helicase in the suppression of meiotic homeologous recombination (Chaix, 2007, Chambers et al., 1996, Hunter et al., 1996). We proposed a model where the MMR complex scans the genome, searching for single end invasion (SEI) events that are occurring between diverged sequences during meiosis. We hypothesise that the MMR complex binds to such SEI events and impedes their progression. We further suggest that components of the MMR complex recruit the RecQ helicase Sgs1, whose helicase activity unwinds the heteroduplex DNA, allowing the invading strand to continue its homology search (**Figure 1.17**).

The data presented in this investigation suggest that the interaction between Mlh1 and Sgs1 may be important in the suppression of homeologous recombination at the SEI stage. Although mutation of the Mlh1-interacting domain of Sgs1 does not lead to significant increases in the rates of crossing over, we do see a decrease in the number of meiosis I non-disjunction events (**Chapter 4**). If the meiosis I non-disjunction data is interpreted as a representation of a failure of crossing over along the entire chromosome, the interaction between Sgs1 and Mlh1 appears to be important for the ability of Sgs1 to suppress meiotic homeologous recombination. This is because, in the absence of this interaction, an increase in recombination between diverged sequences would result in a decreased probability of missegregation events, and hence a significant decrease in the rate of meiosis I non-disjunction (as shown in **Chapter 4**). Therefore, a role of Mlh1 may be in the recruitment of Sgs1 to the sites of homeology, which is consistent with its role as a molecular matchmaker (Modrich and Lahue, 1996, Sancar and Hearst, 1993). However, we cannot discount the activities of additional mismatch repair factors in the recruitment of Sgs1. For example, in the absence of interactions with Mlh1, other components of the MMR complex may still facilitate the recruitment of Sgs1. In addition to interacting with Mlh1 (Gellon et al., 2002), Sgs1 has also been shown to interact with Msh6 using yeast-two-hybrid analysis (Pedrazzi et al., 2003). The possibility that other proteins may facilitate the recruitment of Sgs1 to the sites of homeologous recombination may explain why we see no significant increases in the rates of crossing over between diverged sequences. To investigate this possibility, it would be interesting to create an *sgs1-msh6-id* mutation and investigate its effects on the suppression of homeologous recombination. However, the

interacting domain of Msh6 has not been mapped in Sgs1. Yeast-two-hybrid analysis using Sgs1 truncations may help define the Msh6-interacting domain of Sgs1, and would therefore allow construction of the *sgs1-msh6-id* mutation. If both Mlh1 and Msh6 are able to recruit Sgs1 to the sites of homeologous recombination, it would also be interesting to create an *sgs1-(mlh1+msh6)-id* double mutation, so that Sgs1 cannot interact with either MMR protein, and assess its effects on the suppression of homeologous recombination.

Data presented in **Chapter 5** suggest that the interaction between Top3 and Sgs1 may also important in the suppression of recombination between diverged sequences, as the *sgs1-top3-id* mutation showed a significant decrease in meiosis I non-disjunction events when compared to *SGS1*. However, we propose that the interaction between Sgs1 and Top3 is not important in the suppression of homeologous recombination at the SEI stage. Instead, we hypothesise that when recombination is initiated in regions of very low sequence divergence, strand invasions are successful. This will then lead to strand capture resulting in the formation of a double Holliday junction (dHJ) (**Figure 1.1**). Sgs1 is proposed to recruit Top3 to resolve these dHJ structures as non-crossover products (Wu et al., 2006). In the absence of this interaction between Sgs1 and Top3, the mismatch-containing dHJ is resolved as a crossover, which will lead to an increase in 'homeologous' recombination. To test this hypothesis we would need to investigate the effects of our point mutations using defined substrates, where we would introduce the desired number of mismatches. In addition to this, Sgs1 and Top3 have been shown to interact with Rmi1 in the dissolution of dHJ structures (**Chapter 5**). Therefore, it would also be interesting to assess the ability of an *rmi1Δ* mutant to suppress meiotic homeologous recombination.

We also propose that the meiosis I non-disjunction data can be interpreted in another way. As an increasing number of cells are still able to segregate their homologs in an ordered manner despite the presence of sequence divergence, we propose that one of the roles of Sgs1 may be in the suppression of distributive segregation. Distributive segregation is a mechanism that facilitates the segregation of homologs despite the absence of crossovers, and has been reported to occur in *S. cerevisiae* (Dawson et al., 1986, Guacci and Kaback, 1991, Mann and Davis, 1986, Kaback et al., 1989). We saw that deletion of *sgs1* leads to an increase in the number of cells that are able to segregate their homologs in an ordered fashion when compared to *SGS1*. This is also seen for the *sgs1-mlh1-id* and *sgs1-top3-id* mutations (**Chapters 4** and **5**). As the presence of sequence divergence decreases the rates of crossing over, we propose that factors in the cell promote distributive segregation in an effort to carry out homolog segregation. These factors may be similar

to the SOS response in *E. coli* which promotes inter-species mating (as reviewed by Michel (2005) as discussed in **Chapter 1**). We hypothesis that one of the roles of Sgs1 to is suppress this action, and liken this role of Sgs1 to its role in suppressing the formation of closely-spaced double crossovers (Jessop and Lichten, 2008) (discussed in **Chapter 1**). We therefore suggest that Sgs1 is required to unwind physical interactions between homologs that may lead to distributive segregation. If these physical interactions occur in mismatched regions, we suggest that Mlh1 recruits Sgs1 to these sites so that Sgs1 can carry out its unwinding activities. We also hypothesise that the unwinding caused by Sgs1 generates torsional stress that is relieved by Top3. However, the meiosis I non-disjunction may not be representative of an increase in distributive segregation caused by deletion of *sgs1*. Instead, the increased rates of ordered homolog segregation may be caused by crossovers, as although crossovers were significantly decreased, they were not eliminated. To assess whether this is the case, it would be interesting to design homeolog-specific probes at the ends of the chromosome in an effort to measure whether crossovers are occurring across this region.

6.2 Sgs1 may act in the pre-meiotic replication checkpoint

Chaix (2007) proposed a role late in meiosis for Sgs1, acting with Top3, in the decatenation of dHJs. This was based on the observation that a deletion of *sgs1* led to an increase in non-sister spores when compared to sister spores for the two viable class of tetrads. This increase was suggested to have been caused by the inability to dissolve dHJ structures which results in the death of the two entangled chromosomes (Chaix, 2007) (**Figure 5.1**). To test whether this hypothesis was correct, we assessed the distribution of sister and non-sister spores using the *sgs1-top3-id* point mutation. We found that preventing the interactions between Sgs1 and Top3 did not result in an excess in non-sister spores when compared to sister spores. The data suggest that interactions with Top3 are not responsible for the increase in non-sister spores seen when *sgs1* is deleted.

We hypothesise that the equal distribution of sister and non-sister spores in the *sgs1-top3-id* point mutation is due to the ability of this mutant to still interact with the type II topoisomerase Top2. We therefore propose that the increase in non-sister spores seen in the absence of *sgs1* is due to the inability of Sgs1 to interact with the Top2. Further support for this theory comes from observations that the *sgs1-ΔC795* mutation, which deletes the C-terminal 795 amino acids, including the helicase domain, leads to an increase in non-sister spores. The Top2 interacting domain of Sgs1 has been suggested to overlap with the helicase domain. Therefore, it is possible that truncation of the Sgs1 protein in the *sgs1-ΔC795* mutant deletes the Top2-interacting domain. We therefore propose that Sgs1 and Top2 act to resolve entangled chromosomes that occur during DNA replication prior to entry into meiosis. By preventing their interaction this resolution cannot be carried out, leading to the death of the two entangled chromosomes (**Figure 5.7**). To test this model, it would be interesting to test an *sgs1 top2* double mutant. However, deletion of *top2* confers several mitotic and meiotic defects making it challenging to use in the laboratory. Potential ways to overcome this may be to clone the *CLB2* promoter upstream of both *SGS1* and *TOP2*. Alternatively, researchers have used a temperature-sensitive *top2* conditional mutant that could potentially be introduced into a *pCLB2-SGS1* strain (Jannatipour et al., 1993, Nitiss et al., 1993). Another approach would be to further define the Top2-interacting domain of Sgs1 in order to create an *sgs1-top2-id* mutant using yeast-two-hybrid analysis.

This model suggests that the role of Sgs1 in resolving chromosomal entanglements is a very early role in meiosis. This role is proposed to occur even earlier than the role of Sgs1 at SEIs and in the dissolution of dHJ structures. As discussed in **Chapter 1**, a pre-meiotic replication checkpoint exists that ensures the repair of replication errors prior to entry into meiosis. If our model is correct, then

it implicates Sgs1, together with Top2, in acting in this pre-meiotic replication checkpoint to resolve entangled chromosomes that would lead to spore death.

One of the aims of this investigation was to assess whether the helicase domain of Sgs1 was important in the suppression of meiotic homeologous recombination. Our model proposes that the helicase activity of Sgs1 is responsible for unwinding the heteroduplex DNA (**Figure 1.17**). Therefore, we would predict that a mutation that disrupts the helicase domain would hinder the actions of Sgs1 and therefore allow homeologous recombination to occur. However, we were unable to analyse this mutation, as the introduction of a point mutation that ablated the helicase activity of Sgs1 resulted in a sporulation defect (**Chapter 5**). Malik (*unpublished*) also showed that a series of point mutations that disrupt the HRDC domain of Sgs1 also result in a sporulation defect. These observations highlight the importance of both the helicase and HRDC domains of Sgs1 in sporulation.

Rockmill *et al* (2003) suggested that deletion of *sgs1* leads to the accumulation of stalled replication forks, which triggers the activation of a checkpoint that prevents sporulation. Rockmill *et al* (2003) originally proposed a role for Sgs1 in the pachytene checkpoint. However, they dismissed this hypothesis as they saw that cell cycle arrest was not avoided in an *sgs1Δ* background by mutations that prevent the initiation of recombination (Rockmill et al., 2003). In addition to this, they saw that meiosis-specific genes are not required for cell cycle arrest in *sgs1Δ* cells, further challenging their hypothesis (Rockmill et al., 2003). We propose that the predicted role of Sgs1 in the pre-meiotic checkpoint, in the resolution of entangled chromosomes (discussed above), is one of two roles at this checkpoint. We hypothesise that Sgs1 also acts in the activation of this checkpoint in response to DNA replication errors that have formed prior to meiosis. This role of Sgs1 in the activation of the pre-meiotic replication checkpoint may involve the HRDC domain, to facilitate the stable binding of Sgs1 to the DNA lesions; the RQC domain, to facilitate protein-protein interactions with other proteins involved in this checkpoint response; and the helicase activity of Sgs1, to help restart stalled replication forks that have stalled due to the presence of these pre-meiotic errors. In order to test this hypothesis, it would be interesting to utilise a promoter that represses meiotic expression at a different time point than the *CLB2* promoter. By using this type of promoter, Sgs1 would be present in the cell to carry out its predicted roles in the pre-meiotic checkpoint. Alternatively, a temperature sensitive mutation of *sgs1* could be used as part of a temperature shift experiment. For this type of experiment, the cells would be placed at the restrictive temperature at the onset of meiosis, therefore ensuring that Sgs1 is present during pre-meiotic replication.

However, it remains unclear as to why cells containing the C-terminal truncation of Sgs1, *sgs1-ΔC795*, are still able to sporulate. Rockmill *et al* (2003) suggested that this mutation does not lead to an accumulation of stalled replication forks, which would normally activate a checkpoint that would impede meiosis. We suggest that in the absence of the HRDC and RQC domains in *sgs1-ΔC795*, Sgs1 is not be recruited to any pre-replication errors that need correction via activation of the pre-meiotic replication checkpoint. As a result, we propose that the cell recruits an alternative helicase that is able to substitute for the actions of Sgs1 in this pre-meiotic replication checkpoint (**Figure 5.6C**).

One of the most promising candidates for a helicase that acts in the absence of Sgs1 is Srs2. Like Sgs1, Srs2 is a 3'-to-5' helicase (Rong and Klein, 1993). Srs2 has been shown to be involved in the unwinding of replication intermediates (Van Komen et al., 2003). The fact that both *sgs1* and *srs2* results in a synthetically lethal phenotype (Lee et al., 1999) further implies that the two helicase may have overlapping roles in sporulation. This lethality can be suppressed by mutation in the homologous recombination genes *rad51*, *rad52*, *rad55* or *rad57* (Aboussekhra et al., 1992, Fabre et al., 2002, Gangloff et al., 2000, Lee et al., 1999, Symington, 2002). This suggests that the absence of both Sgs1 and Srs2 leads to an accumulation of toxic recombination intermediates, which lead to activation of checkpoints during DNA replication. Although these studies show roles for both Sgs1 and Srs2 in mitotic DNA replication, it is possible that both act during DNA replication that occurs prior to entry into meiosis.

Srs2 is important for sporulation, as deletion leads to decrease in spore viability (Palladino and Klein, 1992). Therefore, to assess whether Srs2 is able to substitute for Sgs1 in *sgs1Δ* or *sgs1-ΔC795* cells, it would be interesting to overexpress *SRS2* and assess whether this leads to an improvement in sporulation. It would also be interesting to see the effects of a double mutation of both *sgs1* and *srs2* on sporulation. However, as mentioned above, the *sgs1 srs2* mutant is synthetically lethal due to problems that arise during DNA replication. One potential method to overcome this would to place both genes under control of a promoter that represses meiotic expression at a different time point than the *CLB2* promoter, as discussed above. By using this type of promoter, both Sgs1 and Srs2 would be present in the cell to carry out their predicted roles in the activation of the pre-meiotic checkpoint and should therefore lead to wild-type levels of sporulation.

6.3 Concluding Remarks

The data presented in this investigation show that the interactions between Sgs1 and the MMR protein Mlh1, and also the type IA topoisomerase Top3, may be important in the ability for Sgs1 to suppress meiotic homeologous recombination. We suggest that the interaction with Mlh1 is important in suppressing recombination between diverged sequences at the SEI stage. We also suggest the interaction between Sgs1 and Top3 is important in resolving mismatch-containing dHJ structures as non-crossovers. This work therefore lays the foundation to further our understanding of how homeologous recombination may be prevented in cells to maintain the barrier to inter-species recombination. Failure to do so could lead to catastrophic results, including deletions, duplications and even chromosome loss.

We also suggest another role for Sgs1 in the pre-meiotic checkpoint. We suggest that Sgs1 acts with Top2 to resolve entangled chromosomes thereby preventing spore death. We also suggest a role for Sgs1 in the activation of this checkpoint to repair DNA lesions that occur during DNA replication, which would prevent sporulation if not repaired. This work therefore emphasises the importance of Sgs1 early during meiosis, and, in combination with the roles of Sgs1 during mitosis, shows how vital Sgs1 is for the functions of the cell.

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