The Roles of Tel1, Srs2 and Rad6 During Meiotic DSB Repair

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Thank you to my family, for being my family.

Contents

Summary

Meiosis is a cell division in which one diploid parent cell produces four haploid daughter cells. Accurate alignment and segregation of homologous chromosomes during metaphase is critical for a successful meiotic division and viable gametes. Three concomitant events are required for a successful meiotic division: chromosome pairing, synapsis and recombination. Recombination is initiated by programmed induction of DNA double-strand breaks (DSBs). Interchromosomal repair of meiotic DSBs can form a crossover leading to genetic diversity by modifying linkage groups. Crossovers also tether the homologous chromosomes and help resist the tension of the first meiotic spindle. Controlled recombination is required for a successful meiotic division and segregation, however, recombination has to be tightly regulated. This work investigates the roles of Tel1, Rad6 and Srs2 during meiotic homologous DSB repair.

Tel1 is protein kinase required for initiating a signalling cascade in response to many forms of DNA damage. Tel1 has also been proven to function during meiosis and has been shown in some conditions to initiate a signalling cascade after the initiation of meiotic DSBs. In this work Tel1 is shown to influence the early stages of DSB repair during meiosis, however this is not in response to the formation of Spo11-DSB.

Recombination ensures genetic variation and correct homologue alignment during meiosis I therefore is extremely important and tightly controlled. Srs2 is known to be a negative regulator of recombination and is important for normal sporeulation and viability in yeast. Analysis of an experimental site specific DSB (made by VDE) and at natural Spo11-DSBs indicates that in the absence of Srs2 the rate of repair can be increased at Spo11-DSBs and decreased at the VDE-DSB. One potential role for Srs2 during meiosis is to dismantle recombination intermediates formed between the sister chromatids.

Rad6 is required for wild type amounts of Spo11-DSB formation. This work investigated the VDE-DSB repair in the absence of Rad6, and discovered that

Rad6 has a role in the initiation of repair. Rad6 ubiquitinates histone H2B, and further analyses suggest that this modification is required for repair at the VDE-DSB.

Each of the genes studied is required for wild type repair of VDE-DSBs and Spo11-DSBS, even though they come from widely different functional groups. This illustrates the diversity of cellular pathways controlling the initiation and regulation of meiotic recombination.

Abbreviations

Ade	adenine
amp	ampicillin
C. elegans	*Caenorhabditis elegans*
°C	degrees Celsius
CFU	colony forming unit
ChIP	chromatin immunoprecipitation
CTAB	hexadecyltrimethylammonium bromide
C-terminal	carboxy-terminal
D. melanogaster	*Drosophila melanogaster*
dAG	diploid strain number
DAPI	4′,6′–diamidino–2–phenylindoline
dH_20	deionised water
DNA	deoxyribonucleic acid
dNTP	deoxynucleotide triphosphate
DSB	double strand break
E. coli	Escherichia coli
EDTA	ethylene-diaminetetraacetic acid
FISH	fluorescence *in situ* hybridisation
5-FOA	5-fluoroorotic acid
G418	G418 Disulphate
hAG	haploid strain number
hph^R	hygromycin resistance gene
HR	homologous recombination
h	hour
IR	ionising radiation
kb	kilobases
l	litre
µg	microgram
µl	microlitre
M	molar
mM	millimolar
mg	milligram
ml	millilitre
MI/II	first/second meiotic division
MMS	methyl methanesulphonate
MRX	Mre11/Rad50/Xrs2 complex
NHEJ	non-homologous end joining
ORF	open reading frame
pAG	plasmid strain number
PCR	polymerase chain reaction
rpm	revolutions per minute
RT	room temperature
S. cerevisiae	*Saccharomyces cerevisiae*
S. pombe	*Schizosaccharomyces pombe*
Sc	synthetic complete (medium)
SC	synaptonemal complex
sec	seconds
ss	single stranded

SSA	single strand annealing
Ura	uracil
UV	ultraviolet
UP-H_20	ultra pure water
VDE	*VMA1*–derived endonuclease
v/v	volume by volume
w/o	without
w/w	weight by weight

Chapter One – General Introduction

Saccharomyces cerevisiae

The budding yeast *Saccharomyces cerevisiae* is a model organism for higher eukaryotic cellular processes. This yeast is used extensively as an experimental system for molecular biology because it is unicellular, straightforward to culture, the genome is fully sequenced and efficiently curated, mutants are easily generated and yeast genes have homologues in higher eukaryotes. Both the mitotic cycle and the meiotic cell divisions have been well characterised. The rapidly sporulating SK1 strain is widely used by many groups for meiotic studies because the meiotic division is relatively synchronous, completed within twenty four hours and is easily induced using starvation media (Kane and Roth, 1974).

The meiotic division: a general introduction

Most eukaryotes spend a greater part of the life cycle as diploids, which is perpetuated by the mitotic cell cycle. Through the alternation of generations specialised cells pass into a temporary haploid phase (Fig 1.1). The haploid phase is created by meiosis, which causes a diploid cell to produce four recombinant haploid daughter cells such as gametes or spores (in females only one gamete is maintained).

In both mitosis and meiosis genomic DNA is replicated so that each maternal and paternal chromosomes consists of two identical chromatids (sister chromatids; Fig 1.2). In the mitotic cell cycle a single division follows replication; in meiosis however two divisions follow DNA replication (Fig 1.2). The first meiotic division is termed reductional because the homologues are separated to different daughter cells. Consequently the daughter cells contain half the parental number of chromosomes. The second, equational, meiotic division separates sister chromatids After the two meiotic divisions there are four genetically distinct haploid daughter cells each containing one of the original

Alternation of generations

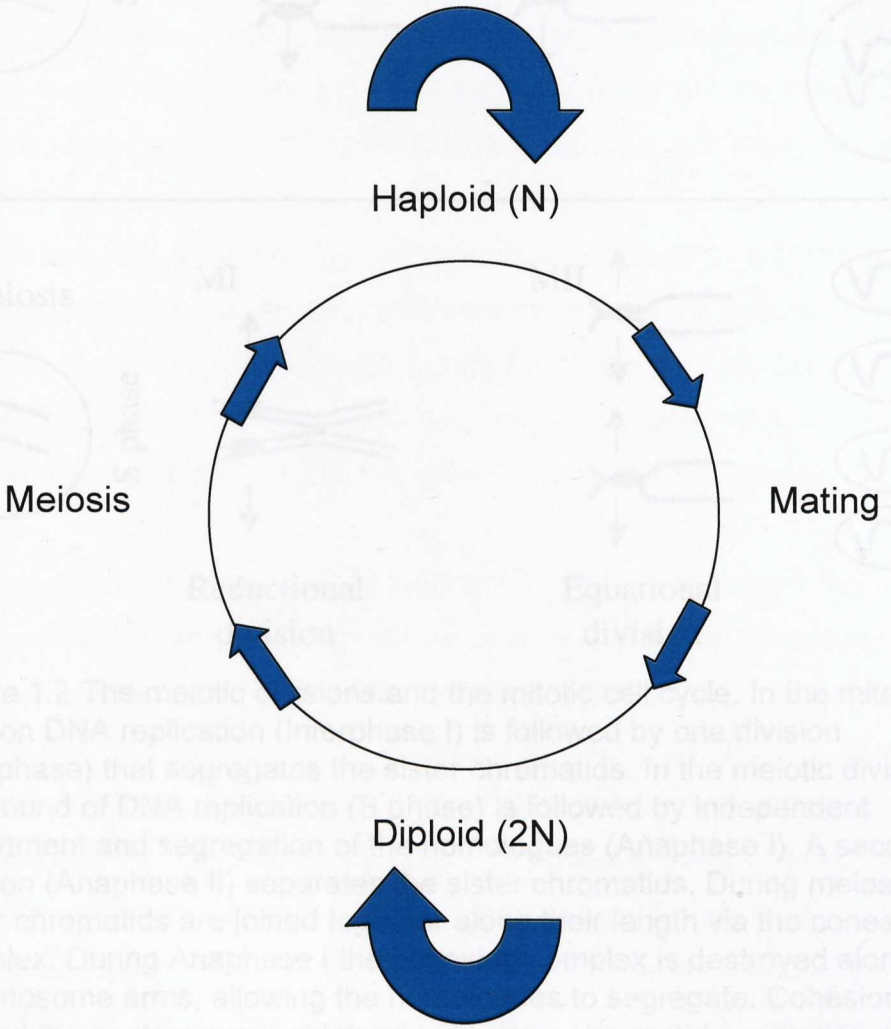


Figure 1.1 The alternation of generations. The meiotic division is seen in eukaryotes that replicate by sexual reproduction. In this life cycle the diploid cell divides via meiosis to produce genetically distinct haploid daughter cells. The haploid daughter cells mate with another haploid cell to form a diploid cell. In yeast the meiotic cycle can be induced in response to starvation conditions. In mammals meiosis is part of reproduction creating genetically diverse offspring.

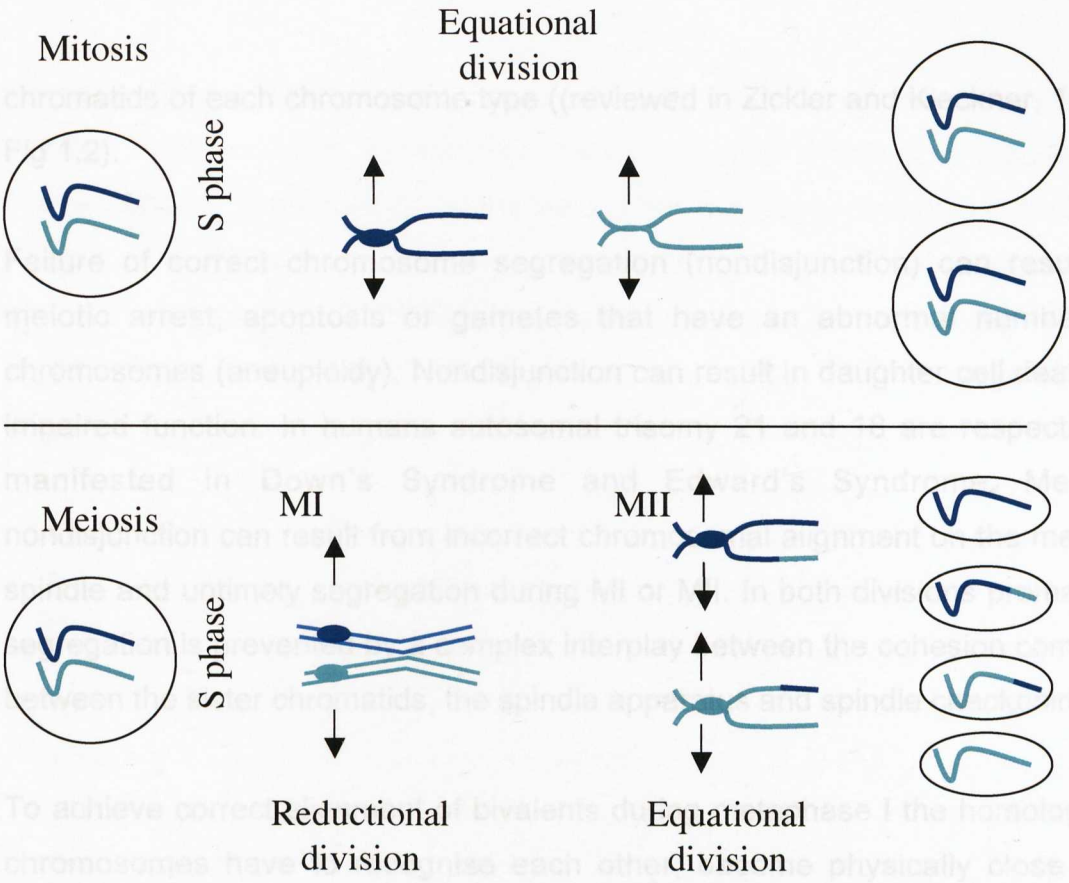


Figure 1.2 The meiotic divisions and the mitotic cell cycle. In the mitotic division DNA replication (Interphase I) is followed by one division (Anaphase) that segregates the sister chromatids. In the meiotic division one round of DNA replication (S phase) is followed by independent assortment and segregation of the homologues (Anaphase I). A second division (Anaphase II) separates the sister chromatids. During meiosis I, the sister chromatids are joined together along their length via the cohesion complex. During Anaphase I the cohesion complex is destroyed along the chromosome arms, allowing the homologues to segregate. Cohesion around the centromere is retained until sister chromatid separation at Anaphase II. In the first division, tethered homologous chromosomes (bivalents) align on the meiotic spindle and are segregated. This is dependent upon three concomitant events, homologue pairing, synapsis and recombination. During pairing the homologues become physically close. Synapsis is identified by the formation of a tripartite proteinaceous structure between homologues. Recombination is the induced DSB repair, a certain proportion of DSBs are repaired forming crossover events that tether homologue chromosomes together. The maternal and paternal kinetochores (protein complex to which microtubles attach) interact with tubulin filaments of the meiotic spindle (microtubles) from the opposing spindle pole. The COs and sister chromatid cohesion join the homologous chromosomes helping bivalent resist the spindle tension; preventing premature segregation and aneuploidy. The spindle assembly checkpoint is required to prevent disjunction. The spindle is under tension when homologues are correctly aligned, and the spindle checkpoint prevents progression unless tension is achived. Cohesion is lost along the arms of the chromosomes releasing the CO connection and allowing the homologues to move to opposite poles.

chromatids of each chromosome type ((reviewed in Zickler and Kleckner, 1998) Fig 1.2).

Failure of correct chromosome segregation (nondisjunction) can result in meiotic arrest, apoptosis or gametes that have an abnormal number of chromosomes (aneuploidy). Nondisjunction can result in daughter cell death or impaired function. In humans autosomal trisomy 21 and 18 are respectively manifested in Down's Syndrome and Edward's Syndrome. Meiotic nondisjunction can result from incorrect chromosomal alignment on the meiotic spindle and untimely segregation during MI or MII. In both divisions premature segregation is prevented by a complex interplay between the cohesion complex between the sister chromatids, the spindle apparatus and spindle checkpoints.

To achieve correct alignment of bivalents during metaphase I the homologous chromosomes have to recognise each other, become physically close and remain together until programmed segregation. In humans and yeast homologue recognition and juxtaposition are achieved by three events: homologue pairing, recombination and synapsis that are concomitant with each other during meiosis. Homologue pairing is the close alignment of homologues along the entire chromosome length. Homologues are physically joined to form bivalents by recombination products (called crossovers; COs) and intersister chromatid cohesion (Fig 1.2). Before recombination, bivalents also undergo synapsis; that is the formation of a proteinaceous structure that is necessary to maintain close and tight pairing and plays a role in regulating crossover formation, frequency and distribution. Crossovers are sites of reciprocal exchange that physically tether homologues, allowing the force of the meiotic spindle to be resisted until programmed segregation when homologues are able to migrate to opposite poles.

In MI sister kinetochores attach to the tubulin filaments from the same spindle pole. This is known as monopolar attachment. When correctly attached the homologues are pulled to opposite poles The chiasmata help the bivalent resist the spindle tension of the maternal and paternal kinetocores. During

programmed segregation cohesion is lost only from the chromosomes arms allowing the homologue separation but keep the sister chromatids tethered at the kinetochore. Protection of centromeric cohesin is dependent on shugoshins. Protection of the cohesin by shugoshin appears to be mediated by tension across the kinetochore. In MI Sgo2 co-localizes with Rec8 but in prometaphase II Sgo2 moves nearer to the kinetochore.

Stages of meiosis – Overview

Meiosis is divided into stages that can be identified cytologically; interphase I, early and late prophase I, metaphase I, anaphase I, telophase I, interphase, prophase II, metaphase II, anaphase II, and telophase II. The duration of meiotic prophase I is longer then mitotic prophase or meiotic prophase II. In organisms as diverse as yeast and mice prophase chromosomes undergo twisting, bending, and folding during continuous movement around the nucleus (Morelli et al., 2008; Scherthan et al., 1994; Scherthan et al., 2007). Meiotic prophase is broken down into separate stages known as leptotene, zygotene, pachytene, diplotene, and diakinesis recognised by chromosome organisation.

In leptotene a tangled mass of thread like chromosomes are observed, however they are ordered on a proteinaceous axial element. During this phase Spo11 catalyses the formation of DNA double stand breaks (DSBs) that stimulate meiotic recombination. At the leptotene to zygotene transition chromosomes form a structure known as the bouquet structure, caused by telomere clustering around the spindle pole body. In zygotene chromosomes are more condensed. Also axial pairing becomes distinct at DSB sites that will form crossovers. A tripartite proteinaceous structure the synaptonemal complex (SC) forms between the homologues which are now observably adjacent to each other (Fig 1.3). During pachytene, chromosomes are tightly associated, sister chromatids develop a single kinetochore and the bouquet structure is no longer visible as telomeres are dispersed. Recombination is completed during pachytene and the homologues are completely joined by a mature SC that is fixed in the nuclear envelope at each end. In the SK1 yeast strain commonly used for the study of meiosis, pachytene lasts for one hour. The SC is completely lost rapidly during diplotene in yeast and is undetectable in cells that have assembled the metaphase I spindle; therefore homologues are only joined by chiasmata (site of chromosome axes exchange and limited parting of sisters; Padmore et al., 1991).

In contrast to yeast worms SC disassembly is asymmetric, after crossover maturation SC is lost from the long axis of the crossover and persists on the short axis. When viewed using fluorescent microscopy the distribution of the Aurora-like kinase (AIR-2) associated with the loss of cohesion closely resemble the distribution of SC components during the transition from pachytene to diplotene in wild type cells; but not in recombination deficient cells such as *spo11Δ*. The asymmetric loss of SC provides a link between crossover maturation and limited cohesion loss during anaphase I (Nabeshima et al., 2005). Worms unlike yeast do not have a localised centromere that is protected during cohesion loss consequently worms require a mechanism that links cohesion loss with SC dissociation allowing a controlled cohesion loss in the absence of a localised centromere. During diakinesis the sisters are still fully aligned, however the homologues appear to repulse each other (Fig 1.3). Diakinesis is the final stage in prophase I, chromosomes in this phase can be seen to dramatically condense (reviewed in (Zickler and Kleckner, 1998); Fig 1.3).

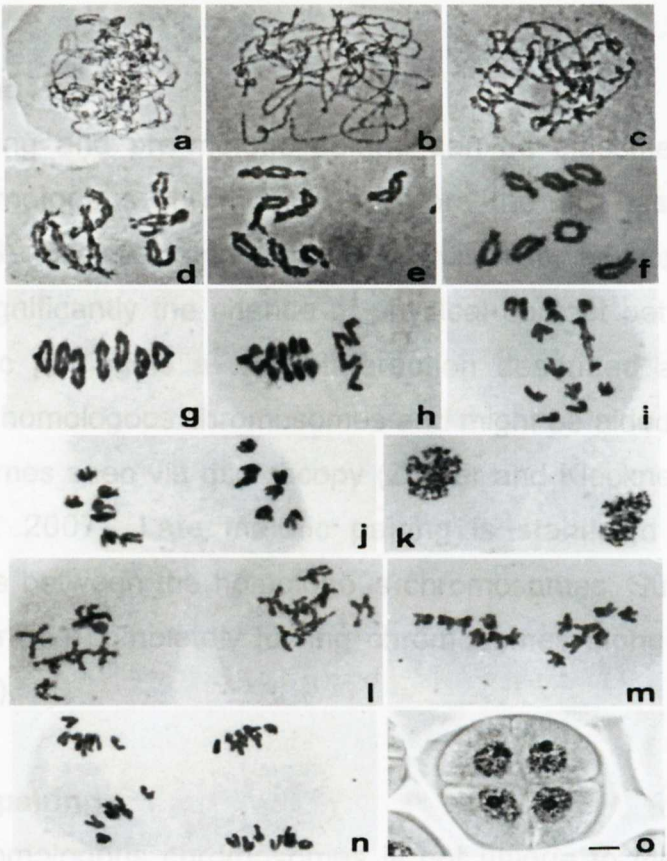


Figure 1.3 Meiotic divisions I and II in the rye *Secale cereale* microsporocytes.

(a) In leptotene chromosomes are thin and individualised. (a,b) In late leptotene/ early zygotene chromosomes are condensed and chromosomes can be seen in a knot or in the bouquet formation. A tripartite proteinaceous structure the synaptonemal complex (SC) forms between the homologues. (c-d) early to late Pachytene chromosomes are associated, sister chromatids develop a shared kinetochore .The bouquet formation is not visible,recombination is complete, homologues are completely joined by a mature SC. (e) Diplotene. The SC is completely lost and homologues are only joined by chiasmata. The homologues appear to repulse each other. (f) Diakinesis is the final stage in prophase, chromosomes appear condensed. (g) Metaphase I The homologues fully align on the spindle. (h,i, j) Anaphase cohesion is lost from the arms of the chromosomes but persists at the at the centromere. The kinetochores move to opposite poles and the homologous chromosomes disjoin. (k) Telophase I. (l) Prophase II. (m) Metaphase II. (n) Anaphase II chromatid disjunction (o) Four haploid pollen cells (Bar = 5µ) (Figure taken from Zickler and Kleckner 1998).

Stages of meiosis – Detail

Chromosome Pairing

Meiotic pairing and pre-meiotic pairing can be classified as associations between homologous chromosomes before the SC has been established (Zickler 2006). Correct pairing is a pre-requisite for accurate disjunction as it increases significantly the chance of physical contact between homologues. Early meiotic pairing is a weak interaction described as the presynaptic alignment of homologous chromosomes and might be aided by stirring motions of chromosomes seen via microscopy (Zickler and Kleckner 1998; Scherthan, Wang et al. 2007). Late meiotic pairing is stabilised by recombination intermediates between the homologous chromosomes. Subsequent to paring the SC is formed completely joining chromosomes along their entire length (Zickler 2006).

Pre-meiotic pairing

Pairing of homologous chromosomes is not unique to meiosis, non-random homologue associations are well documented in vegetative cells of *Schizosaccharomyces pombe* and somatic cells of *Drosophila melanogaster* cells (reviewed in McKee, 2004; Scherthan et al., 1994; Zickler, 2006). Pre-meiotic pairing is the close non random association of homologues before meiotic S-phase and may be a result of mitotic events (Zickler, 2006). Unlike late meiotic pairing pre-meiotic pairing is DSB and can be detected by FISH in recombination deficient strains such as *spo11*Δ, *hop1*Δ and *mer1*Δ diploids (Burgess et al., 1999; Weiner and Kleckner, 1994). Pre-meiotic pairing could be a precursor to homologue pairing aiding homologue recognition and alignment after replication (Weiner and Kleckner, 1994). Interestingly the frequency of unstable pre-meiotic pairing sites detected by fluorescence *in situ* hybridisation (FISH) is similar to the frequency of recombination events, (one per ~ 65 kb) suggesting pre-meiotic pairing may occur at future DSB sites (Weiner and Kleckner, 1994). Although several studies of yeast have observed non-random homologue pairing in pre-sporulation media using ectopic recombination

assays, FISH and PCR assays with cross-linking, the existence and significance of pre-meiotic pairing remains controversial in budding yeast (Burgess and Kleckner, 1999; Burgess et al., 1999; Chen et al., 2004; Dekker et al., 2002; Jin et al., 1998; Loidl et al., 1994; Peoples et al., 2002; Weiner and Kleckner, 1994). It is hypothesised by some that pre-meiotic associations are disrupted or weakened during S phase possibly to allow the replication fork to pass (Burgess et al., 1999; Loidl et al., 1994; Weiner and Kleckner, 1994). In leptotene, homologue associations are formed again followed by the chiasmata and SC formation (Loidl et al., 1994; Weiner and Kleckner, 1994).

Chromosome architecture in the meiotic nucleus is also influenced by factors independent of meiosis. In vegetative growth centromeres cluster before being loaded onto the spindle. In anaphase the centromeres disjoin then move to opposite poles followed by the telomeres. Consequently in the anaphase daughter cells telomeres and centromeres exhibit a polarised arrangement known as the Rabl-orientation (Jin et al., 1998). Vegetative pairing is suggested to be a result of Rabl organisation where chromosomes of similar sizes would share the same topological constraints and so share similar space (Jin, Trelles-Sticken et al. 1998; Lorenz, Fuchs et al. 2003). Loidl *et.,al* detected pre-meiotic pairing by FISH but attributed it to residual mitotic chromosome organisation (Loidl et al., 1994). One influence on chromosome positioning might be replication which is associated with a complex nuclear organisation of chromosomes seen in flies, yeast and higher Eukaryotes (reviewed in Cimbora and Groudine, 2001; Gasser, 2001). During replication chromosomes are organised into territories and localisation this is mediated by telomere attachment to the nuclear envelope. This organisation has been suggested to be involved in silencing (Cimbora and Groudine, 2001; Gasser, 2001). These chromosomal arrangements may force homologous chromosomes to be juxtaposed during vegetative growth (discussed in Lorenz et al., 2003). This organisation may still be present after pre-meiotic replication, before entry meiotic S-phase and be seen as pre-meiotic pairing.

Early meiotic pairing

After replication has disrupted pre-meiotic pairing, homologous chromosomes become closely juxtaposed by weak interactions (Zickler and Kleckner, 1998). Early meiotic pairing like pre-meiotic pairing occurs by multiple weak transient interactions that align homologous chromosomes independently of synapsis and recombination; and can be detected by FISH in recombination deficient *spo11* and *rad50S* cells. The mechanism by which homologous chromosomes recognise each other and become aligned during early meiotic pairing is unknown, but is suggested to be the same mechanism used during pre-meiotic pairing. Although pre-meiotic pairing is suggested to be a result of vegetative nuclear architecture this has not been proven. Close homologue interactions during pre-meiotic and early meiotic pairing are suggested to be dependent on DNA homology sensed in accessible, nucleosome free, regions of DNA (Keeney and Kleckner, 1996). Pre-meiotic pairing, late meiotic pairing and Spo11 DSBs are associated with open chromatin suggesting pre-meiotic pairing may ensure the existence of homology before entering meiosis and committing to recombination (Keeney and Kleckner, 1996; Weiner and Kleckner, 1994). Kleckner proposed a model in which homologue contacts in both vegetative and early meiotic/pre meiotic yeast cells might be aided by mechanical forces like expansion and contraction of chromosomes (Kleckner et al., 2004). Changes in chromosomes structure such as expansion and contraction could result from histone modifications. In this model periods of expansion allow intermingling of chromosomes aiding initial homologue pairing and contraction facilitates full homologue pairing (Kleckner et al., 2004; Loidl et al., 1994). Another suggested mechanism for early meiotic pairing is telomere clustering, during meiosis telomeres attach to the nuclear envelope, then congregate at one specific area normally proximal to the spindle pole body (SPD). This creates a highly conserved structure known as the bouquet (de La Roche Saint-Andre, 2008; Trelles-Sticken et al., 2000). During pachytene the telomeres are distributed evenly and the structure is no longer observable. The specific function of the bouquet is unknown however the associated telomere movement is implicated in untangling the chromosomes, influencing crossover formation and regulating recombination at the telomeres. Telomere clustering and dispersion requires

extensive and rapid chromosomes movement; the chromosome movement is so vigorous in yeast that the NE becomes deformed and nuclear protrusions are seen, caused by maverick/orphan chromosomes that move away from the main chromosome mass (Koszul et al., 2008; Scherthan et al., 2007). Analysis using live cell imaging suggests that chromosome movement also depends upon telomeres passively associating with nucleus hugging actin cables during zygotene and pachytene (Koszul et al., 2008). Chromosome movement is also depends upon telomeres associating with the NE mediated by Ndj1 and Csm4. In the absence of these proteins movement is reduced (Conrad et al., 2007). The movement associated with the bouquet is suggested to increase homologous chromosome interaction, aiding pairing. This is implied because homologue pairing is delayed in the absence of Ndj1, which is a meiosis specific telomere protein required for association of the telomeres with the NE (de La Roche Saint-Andre, 2008; Trelles-Sticken et al., 2000).

DSB dependent pairing

In contrast to *Caenorhabditis elegans* and *Drosophila* males, complete late meiotic paring in yeast and mammals is dependent on DSBs and homologous recombination (Dernburg et al., 1998; McKee, 2004). Homologous recombination is stimulated in meiosis by the programmed formation of DSBs by Spo11. Repair of the DSBs requires the homologues to be close and aligned. Once Spo11 is removed an early stage of repair requires resection to expose 3′ ssDNA. The ssDNA invades the undamaged duplex (known as single-end invasion events; SEI) bringing the duplexes closer together strengthening early weak homologue interactions. In meiosis a proportion of DSBs are destined to form crossovers that bring homologues into extremely close local proximity. Burgess *et al* 1999 assayed Cre / *loxP* recombination and gene conversion at linked and unlinked sites. The group observed that the frequency of Cre-mediated recombination events increased near recombination events destined to become COs (Mell et al., 2008). This evidence supports the hypothesis that CO events bring together nearby sites physically stabilising early pairing. The dependence of pairing on recombination intermediates is

illustrated by the absence of full homologue juxtaposition in DSB deficient mutants strains such as *spo11Y135F* diploids. However pairing is observed in *ndt80Δ* cells that form DSB and initiate recombination but are unable to resolve crossover intermediates (Neale et al., 2002; Peoples et al., 2002).

SEI events are an early step in recombination and are mediated by two highly conserved strand invasion proteins Rad51 and Dmc1 (Rockmill et al., 1995; Sheridan et al., 2008). SEI events are involved in homology searching and are required for full meiotic pairing. The importance of SEI events is demonstrated by function of Hop2, which promotes synapsis between homologous chromosomes and Mnd1 that is required for DSB repair. In yeast *hop2Δ* cells arrest in pachytene; are defective in chromosome pairing, crossover formation and joint molecule formation; and form SCs between non-homologous chromosomes (Leu et al., 1998). *mnd1Δ* cells also arrest during pachytene, accumulate Rad51 foci, display ineffective pairing and mature SC are absent (Tsubouchi and Roeder, 2002; Zierhut et al., 2004). Hop2 and Mnd1 form an elongated heterodimer (H2M1) and are involved in recombination mediated homologue pairing in both yeast and mice by aiding strand invasion mediated by Rad51 and Dmc1 (Chen et al., 2004; Petukhova et al., 2005; Sheridan et al., 2008; Tsubouchi and Roeder, 2002; Zierhut et al., 2004). In yeast and mice H2M1 has been shown to bind Dmc1 and both H2M orthologues improve the efficiency of D-loop formation although the effect is more pronounced in mouse than yeast H2M (36- and 3-fold stimulation leading to 70% and 3% reaction efficiency for mammalian and yeast proteins respectively; Chen et al., 2004; Chi et al., 2007; Henry et al., 2006; Petukhova et al., 2005; Tsubouchi and Roeder, 2003). The stimulation of Dmc1 and Rad51 mediated SEI events by H2M is considered to be highly conserved feature of yeast because organisms in which Dmc1 is absent, Hop2 and Mnd1 are also nonexistent (Loidl et al., 1994). Research *in vitro* using mouse H2M and human Dmc1 suggests the H2M stabilises Rad51 and Dmc1 ssDNA filament and promotes dsDNA recruitment (Chen et al., 2004; Chi et al., 2007; Petukhova et al., 2005). Promoting D-loop formation is a potential precursor to crossover formation that aids full pairing. Importantly H2M also promotes homology searching by capturing of potential

template, strengthening the interactions between homologous chromosomes possibly inhibiting non-homologous synapsis seen in *hop2Δ* cells (Pezza et al., 2007).

DSB independent pairing

Late pairing can be independent of both recombination and synapsis, during non-exchange chromosome segregation (achiasmate segregation/distributive segregation) homologous chromosomes are paired and segregated independently of recombination called distributive segregation. Distributive segregation is routine in *Drosophila* males and for the fourth chromosome pair in *Drosophila* females (McKee, 2004). In yeast distributive segregation has been observed but has a much lower fidelity than in *Drosophila*. It is certainly secondary to homology dependent segregation, and, recombination has to be present in the cell. Distributive segregation in yeast might be a mechanism to segregate rare chromosomes with failed chiasmata. In yeast members of the spindle checkpoint Mad1 and Mad2 have been shown to help aid segregation of nonexchange chromosomes (Cheslock, Kemp et al. 2005). The exact mechanism by which Mad1 and Mad2 facilitate distributive segregation is unknown. The spindle checkpoint might provide extra time for the homologues to pair or capture the microtubule (Cheslock, Kemp et al. 2005). Achiasmate segregation could have evolved to separate one pair of non-homologous chromosomes, which cannot be segregated using recombination dependent segregation. In a normal meiotic division achiasmate segregation of one chromosome pair unable to undergo recombination could be a consequence of structures formed by normal meiotic division including pairing or SC complexes. In non-exchange segregation the formation of SC complex between non-homologous chromosomes would strengthen weak interactions. Such segregation might be aided by pre-meiotic pairing that is recombination independent or as a secondary result of spindle shape (Weiner and Kleckner, 1994).

Synapsis

The synaptonemal complex (SC) is a ribbon-like tripartite proteinaceous structure, that forms between the entire lengths of paired homologues (Roeder, 1997; Fig 1.4). Failure to form a complete SC results in polycomplexes, which are SC aggregates distinct from the chromatids and is rarely seen in wild type cells (Chua and Roeder, 1998; Loidl et al., 1994). The proteins of the SC include Zip1, Zip2, Zip3 and Zip4. The SC as a structure is highly conserved from yeast to mammals (Baier, Alsheimer et al. 2007).

In worms SC formation can occur independently of recombination. Also *Schizosaccharomyces pombe, Aspergillus nidulans* and male *Drosophila* do not exhibit SC. In yeast SC forms asynchronously and precursors are visible at zygotene, mature SCs become visible at pachytene and are absent at the start of metaphase (Padmore et al., 1991). The SC is presumed to hold the homologues together along their entire lengths while some DSBs form crossover products to ensure a correct reductional segregation of the bivalents (Roeder, 1997). In prophase a proteinaceous core, referred to as the axial element, forms between sister chromatids (Rockmill et al., 1995). In mature SC the axial elements are known as lateral elements (Rockmill and Roeder, 1990; Roeder, 1997). Regions of homologous chromosomes become coupled when a central element (CE) is formed between the axial elements. This is termed synapsis and occurs before the axial elements are complete. The CE is elongated in both directions until the homologues are joined along their enter length. The CE runs parallel from and equidistant to the lateral elements holding them in opposition (Fig 1.4). The space between the lateral elements also contains transverse filaments that lie perpendicular to the long axis of the complex (Fig 1.4). Zip1 forms dimers that are components of the transverse filaments. Zip2 and Zip3 are members of the synaptonemal initiation complex (SIC; as termed in Fung et al., 2004) that localises to axial associations and subsequently polymerise Zip1 in both directions, initiating zippering up of the SC (Agarwal and Roeder, 2000; Chua and Roeder, 1998; Padmore et al., 1991; Tsubouchi et al., 2006). Zip1, Zip2, Zip3, Zip4, Msh4 and Msh5 are collectively known as ZMM.

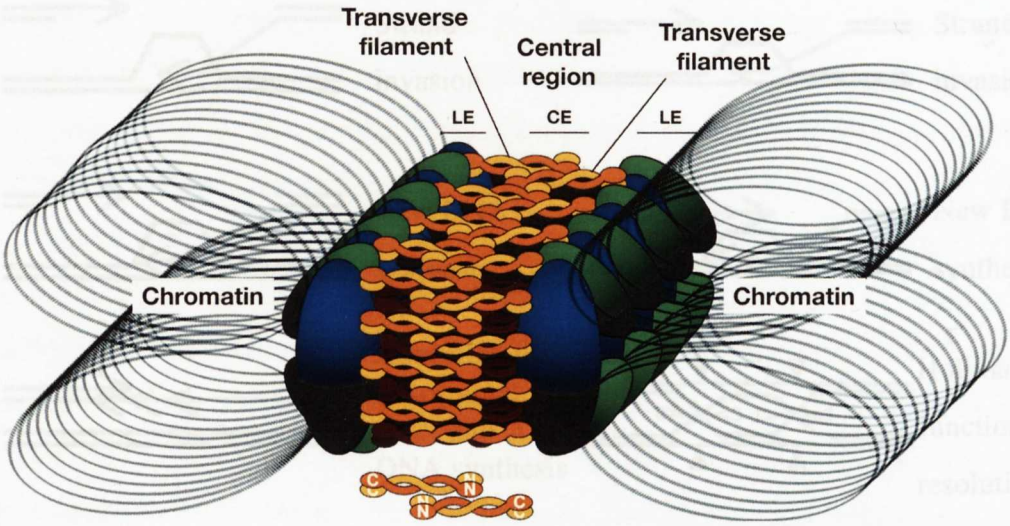


Figure 1.4 The synaptonemal complex (SC). In prophase a protenaceous core develops between the sister chromatids known as an axial element. The axial elements are known as lateral elements in a full SC. The chromatin loops of each sister are attached to the lateral elements. Lateral elements become full joined by the central elemant (CE) physically joining the homologous chromosomes. The CE is comprised of transverse filaments which perpendicular to the lateral elements. Zip1 is a component of the transverse filaments that span the CE from one lateral element to another or from one lateral element to the CE. Synapsis is the polymerisation of Zip1.

(Figure taken from Page and Hawley, 2004)

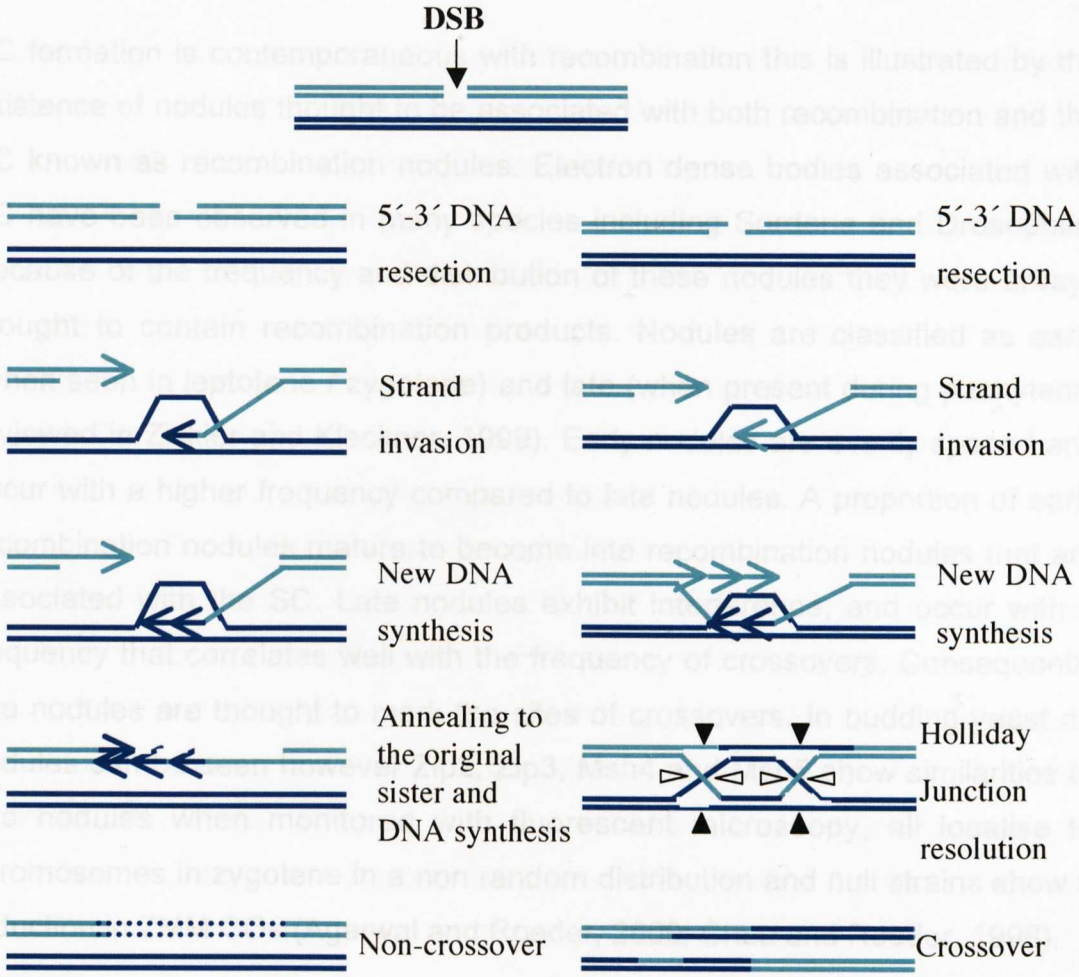


Fig 1.5 Double strand repair pathways. In meiosis a DSB can be repaired by the synthesis dependent strand-annealing pathway (SDSA). In SDSA the 5′ ends of the DSB are resected exposing 3′ single stranded DNA. Subsequently single strand invasion of the donor duplex occurs and is followed by extension and displacement of the invading strand. This repair mechanism results in a non-crossover event. Alternatively repair can occur by the canonical DSBR, in this pathway invasion of the donor duplex by ssDNA is also followed by replication. Unlike SDSA a second strand invasion event occurs creating two Holliday junctions. DNA synthesis occurs in both the broken duplex and the donor duplex creating a joint molecule (JM). Resolution of the JM can results in a crossover. Both pathways require a single strand invasion event that is dependent upon Rad51 or Dmc1, however in the SDSA pathway the SEI event is unstable. White arrows represent 3' extension, arrows indicate cleavage.

SC formation is contemporaneous with recombination this is illustrated by the existence of nodules thought to be associated with both recombination and the SC known as recombination nodules. Electron dense bodies associated with SC have been observed in many species including Sordaria and Drosophila. Because of the frequency and distribution of these nodules they were always thought to contain recombination products. Nodules are classified as early (when seen in leptotene / zygotene) and late (when present during pachytene; reviewed in Zickler and Kleckner, 1999). Early nodules are evenly spaced and occur with a higher frequency compared to late nodules. A proportion of early recombination nodules mature to become late recombination nodules that are associated with the SC. Late nodules exhibit interference, and occur with a frequency that correlates well with the frequency of crossovers. Consequently late nodules are thought to mark the sites of crossovers. In budding yeast no nodules can be seen however Zip2, Zip3, Msh4 and Msh5 show similarities to late nodules when monitored with fluorescent microscopy, all localise to chromosomes in zygotene in a non random distribution and null strains show a reduction in ZMM COs (Agarwal and Roeder, 2000; Chua and Roeder, 1998).

The evidence indicates that SC formation is not only contemporaneous with, but also dependent upon, early steps of recombination and Spo11 DSBs (Agarwal and Roeder, 2000; Borner et al., 2004; Chua and Roeder, 1998; Henderson and Keeney, 2004). In the absence of a functional Spo11 mature SC formation is undetectable or occurs at very low level (~ 10) (Loidl, Klein et al. 1994; Bhuiyan and Schmekel 2004; Henderson and Keeney 2004). In *rad50S* cells, which are unable to process DSBs, Zip2 and Zip3 localises to DSB sites as seen by co-localisation of Zip2, Zip3 and Mre11 (a member of the MRX complex required for recombination) however mature SC is not formed (Agarwal and Roeder, 2000; Chua and Roeder, 1998). Strains unable to stabilise DSB repair intermediates (*hop2*Δ and *mnd1*Δ) do not form full SC between homologous chromosomes (Leu et al., 1998; Zierhut et al., 2004). Also Zip3 interacts with Rad57 that simulates Rad51 binding and Msh4 that is required for normal levels of crossovers in yeast two-hybrid screen (Agarwal and Roeder, 2000; Chua and Roeder, 1998). The localisation of Zip2 also strongly supports the relationship

between recombination and SC formation. CO interference is the reduced chance that a CO is formed in region where CO already exists resulting in non-random distribution of COs that guarantees each chromosome receives at lest one CO (Fung et al., 2004; Tsubouchi et al., 2006; Zickler and Kleckner, 1998). The distribution of Zip2 (a member of the SIC) foci on chromosome III, IV and XV suggests that, when SC formation is initiated, the likelihood of another complex being created nearby is reduced (Fung et al., 2004). The observation that the SIC displays interference suggests that interference is imposed before formation of the SC; this is important because COs, but not NCOs, display interference. Therefore Zip2 localises to CO recombination intermediates not NCO recombination intermediates. However recently a high resolution map can detect interference not only between COs but also between Msh4 dependent interference between COs and NCOs, although the group did not detect interference between NCOs and NCOs (Mancera et al., 2008). The connection between the SC and crossover formation between the homologues could be indicative of a regulation mechanism by ensuring mature synapsis can only occur between chromosomes capable of initiating recombination via strand invasion.

Although the SC formation is dependent upon stable recombination intermediates such as JMs, CO intermediates are formed after the SC is fully formed and appear to be significantly dependent upon the formation of a mature SC. This hypothesis is supported because there is a strong correlation between the frequency of crossover events and SC formation, COs are reduced in *zip1Δ, zip2Δ, zip3Δ, zip4Δ mer3Δ* and *msh5* diploid cells at 23°C 30°C and 33°C (collectively known as ZMM proteins) (Agarwal and Roeder, 2000; Chua and Roeder, 1998; Dong and Roeder, 2000; (Borner, Kleckner et al. 2004) Tsubouchi et al., 2006). At 30°C and 23°C the frequency of NCOs are unaffected. However at 33°C NCO repair products are increased although the number of DSBs is normal (Borner, Kleckner et al. 2004). Therefore the CO/NCO decision is made prior to SC maturation because Zip2, Zip3 and Zip4 are required for mature SC (Borner et al., 2004). How does the SC regulate the frequency of COs in the cell? One likely protein is Pch2, Pch2 is required for the

pachytene checkpoint which is absent in organisms that lack the SC and is suggested to specifically monitor formation of the SC (Mitra and Roeder, 2007). In strains that have a none null Zip1 mutation known as *zip1-4LA,* mature SC appears to be formed, the frequency of crossover formation is high and the cells arrest at pachytene as detected by fluorescent microscopy and Southern blot analysis (Mitra and Roeder, 2007). In *zip1-4LA pch2*Δ cells the arrest is alleviated and percent of crossovers is more than double compared to *pch2*Δ, suggesting that crossovers can be formed in the *zip1-4LA* mutant. However *zip1-LA* cells trigger the Pch2 checkpoint before recombination is completed and crossovers are formed (Mitra and Roeder, 2007). The results indicate that the *zip1-4LA* cell undergoes a Pch2 mediated arrest, halting the formation of crossovers. The Pch2 arrest might be caused by an inability to disassemble the SC or an aberrant SC that appears normal under the microscope. A Pch2 mediated arrest in *zmm* mutants would provide an explanation for the lack of crossovers in SC defective mutants; and why Zip2 foci display normal interference in *zip1*Δ cells which do not form mature SC (Mitra and Roeder, 2007, Fung et al., 2004).

The SC also potentially regulates recombination by preventing recombination intermediates from being dismantled by proteins such as the helicase Sgs1 that is known to negatively regulate COs (Jessop et al., 2006). *zmm* mutants display reduced CO and a very low frequency of axial associations (AA). However, when single *zmm* mutants (*msh4*Δ, *mer3*Δ, *zip1*Δ and *zip2*Δ) are combined with *sgs1* mutant alleles the CO frequency and AA are increased, suggesting that ZMM are promoting CO formation and preventing Sgs1 from dismantling recombination intermediates destined to become COs.

Meiotic Recombination

Spo11 is a highly conserved DNA endonuclease that forms approximately 200 double strand breaks via a topoisomerase-like transesterification (Keeney et al., 1997). Spo11 is type II topoisomerase, these proteins are required for the catalysis of programmed DNA DSB by breaking the phosophodiester bond and forming a covalent bond with the now broken duplex. Mutating tyrosine 135 in yeast Spo11 prevents catalysis of DSBs. This tyrosine is common to

topoisomerase VI suggesting Spo11 might be a new group of topoisomerase II proteins. DSB formation occurs approximately one hour after replication and is regulated by the catalytic subunit of the main cell cyclin dependent kinase Cdc28 (Henderson et al., 2006; Keeney et al., 1997; Padmore et al., 1991). Regulation of Spo11-DSB formation by Cdc28 is suggested because Spo11-DSB are undetectable in *mer2(S271A)* in which Mer2 (a protein required for Spo11-DSB formation) can not be phosphorylated by Cdc28 (Henderson et al., 2006). After Spo11-DSBs are catalysed the evidence strongly suggests that Spo11 is removed asymmetrically from the break by single stranded nicks that release Spo11 bound to a single stranded oligonucleotide (Liu, Wu et al. 1995; Neale, Pan et al. 2005). Although the proteins responsible for this action are unknown successive removal is dependent upon Sae2 and Rad50 (Keeney and Kleckner 1995). Controlled timing of DSB creation potentially prevents toxic intermediates from being formed at undesirable stages of meiosis and prevents the formation of DSBs before replication. The next step in repair is resection of the DSB ends in 5′ to 3′ direction, leaving 3′ single stranded DSB ends. The ssDNA is rapidly bound by RPA, Dmc1 and Rad51 which mediate repair. The time at which Spo11 breaks disappear (after 5 h of meiosis) by Southern analysis is always constant even if the time of appearance is variable (Padmore et al., 1991).

In yeast Spo11 DSBs are not distributed randomly throughout the genome, areas that rarely receive Spo11 DSBs (cold spots) have been detected in telomeric regions and selected centromeric regions (Gerton, DeRisi et al. 2000; Borde, Lin et al. 2004; Buhler, Borde et al. 2007). In yeast leptotene the meiosis specific Spo11 catalyses the formation of DSB in open chromatin (Keeney et al., 1997). Open chromatin is hypersensitive to DNase I and micrococcal nuclease as a result of nucleosome disruption, this usually is associated with transcriptionally active regions of DNA; sensitivity has also been detected before meiotic DSB formation. Histone modification is associated with DSB repair in mitosis suggesting histone modification might also be required during repair of meiotic Spo11-DSBs (Costelloe et al., 2006; Maleki and Keeney, 2004). H2B phosphorylation in yeast, H3 phosphorylation in mice and

sumolyation of H4 in human males has been detected in meiosis by western blotting and double immunostaining respectively (Ahn et al., 2005; Metzler-Guillemain et al., 2008; Swain et al., 2007). The lack of H2B ubiquitination and H3 methylation reduce the frequency of Spo11-DSB detected by Southern analysis in yeast (Sollier et al., 2004; Yamashita et al., 2004). The exact function of histone modification during meiosis is unknown. However in *Drosophila* barrier to autointegration factor (BAF) binds DNA and the nuclear envelope. After successful meiotic recombination nucleosomal histone kinase-1 (NHK-1) phosphorylates BAF resulting in release of both the DNA and the envelope allowing formation of the Karyosome. In yeast it has been shown that H3K4 trimethylation is increased and the amount of H3 is decreased in DSB sites compared to cold spots (Borde, Robine et al. 2008). During prophase I chromosomes condense, chromosome compaction and expansion has been implicated in chromosome pairing and homology searching; a lack of histone modification that impairs chromosome compaction and expansion might impair pairing and recombination. Another possibility is that histone modification results in histone eviction allowing the Spo11/recombination machinery access to the DNA this can explain the reduction in Spo11-DSB frequency detected in mutant strains unable to ubiquitinate H2B, or methylate H3. Although Spo11-DSBs are created in open chromatin the chromatin state does not guarantee a Spo11-DSB will be created. When the *ARG4* promoter, a known hot spot, is moved the chromatin can be DNAse sensitive but not receive a Spo11-DSB, suggesting the state of chromatin is not the only factor that determines if a region will receive a DSB break (Wu and Lichten, 1995).

All Spo11 DSBs are repaired by a high fidelity double strand break repair mechanism called homologous recombination. Homologous recombination repair copies homologous DNA from an unbroken template sequence to the damaged duplex (recombination) changing linkage of the alleles therefore creating genetic diversity. HR is used to repair DSB during meiosis, mitosis, immunoglobulin rearrangement and during mating type switching in *S. cerevisiae*. In the mitotic cell cycle, conservation of the damaged sequence is desired therefore the template strand is normally the sister chromatid. In

meiosis the template sequence can theoretically originate from the sister chromatid, a homologous chromosome or an ectopic repeat locus. In yeast and mammals meiotic DSB homologous repair using the homologue is an absolute requirement for successful alignment and homologue segregation (Schwacha and Kleckner, 1994). The use of the homologous template is ensured not only by Dmc1 mediated strand invasion of the homologous chromosome but also by active inhibition of using the sister as a template by creating a barrier to sister chromatid repair (BSCR). In this pathway Hop1 and Red1 bind to unbroken duplexes detected by fluorescent microscopy during meiosis. When Spo11-DSB are created Mek1 is recruited to hyperphosphorylated Red1 which promotes Mek1 dimerisation needed for Mek1 autophosphorylation. Phosphorylation of Mek1 is required for an efficient BSCR, which has been demonstrated by using Mek1 mutants that are unable to be phosphorylated (Niu et al., 2007; Niu et al., 2005; Wan et al., 2004). Recently Mec1/Tel1 phosphorylation of Hop1 has been described, this might be the link between DSB damage and the Mek1 dependent BSCR (Carballo et al., 2008). In mutants deficient in BSCR DSBs are repaired in *dmc1*Δ mutants and meiosis is completed however the spores are dead. Although Mek1, Red1 and Hop1 are known to be required for a barrier to sister chromatid repair the exact mechanism is not known

HR repair of a Spo11 DSB can create two types of product, a non-reciprocal exchange (gene conversion/non crossover events) or a reciprocal exchange (crossover). Crossovers result in chiasmata between the homologues that facilitate chromosome segregation during Telophase I, preventing first meiotic aneuploidy. Mature crossovers arise late in prophase, (at the end of pachytene but before telophase I). They are SC dependent and are non-randomly distributed throughout the genome (Agarwal and Roeder, 2000; Chua and Roeder, 1998; Mitra and Roeder, 2007; Padmore et al., 1991). Approximately 200 DSBs are generated in the yeast genome each chromosome receives a high number of crossovers (Mancera et al., 2008). Once a crossover is created the chance that another will be formed in the same region is reduced. Therefore the repair product of subsequent DSBs created in that region will be a non-crossover event. This is known as positive crossover interference. The number

of crossovers is maintained even when the frequency of Spo11 DSBs formation is reduced; this is known as crossover homeostasis (Henderson and Keeney, 2004; Martini et al., 2006). However crossover homeostasis cannot be maintained when the frequency of Spo11-DSB formation is below ~ 30 % of wild-type (Martini, Diaz et al. 2006). Crossover interference guarantees at least one crossover chromosome occurs regardless of chromosome size. This assurance prevents achiasmate segregation that increases the chance of non-disjunction. Crossover control also prevents crossovers such as centromeric crossovers which might also result in aneuploidy (Rockmill et al., 2006). (Although recombination is generated during meiosis excessive recombination can be harmful, in mammals hyper recombination causes genome instability seen in bloom syndrome that results in a high male sterility rate therefore recombination has to be tightly controlled). The interference seen in COs can also be observed in the formation of SC. Members of the SIC exhibit interference and do not form as frequently at the centromeres further suggesting that the sites of recombination initiation are also sites of SC formation (Fung, Rockmill et al. 2004).

A potential mechanism for crossover homeostasis is that subsets of DSB intermediates are directed towards a crossover event achieving the appropriate number of crossovers. The remaining DSBs automatically resolve to become non crossovers (Martini et al., 2006). A potential crossover interference model is that the homologues align linked by recombination complexes. Once a crossover is generated a signal is dispersed preventing other intermediates maturing into crossovers (Borner et al., 2004). This was observed when Burgess *et al* 1999 assayed Cre-mediated recombination and gene conversion reporter cassette at linked and unlinked sites. Cre-medicated recombination was reduced when a CO occurred distant sites, indicating a repression of recombination in the region distal to the CO (Mell et al., 2008). How the surrounding DSBs receive the interference signal is unknown. Mechanical stress generated by chromosome expansion is one candidate. The stress is relieved by crossover formation, once the stress is released any DSBs in the area will be repaired by gene conversion (Kleckner et al., 2004). Another

mechanism suggested is the counting method in which non crossovers are failed attempts to generate a crossover (Foss et al., 1993; Stahl et al., 2004). In this model each successful crossover is separated by a fixed number of non crossovers (Stahl et al., 2004). Importantly the SC complex is has been implicated in regulating crossover maturation and appears to be dependent on interference. The SC is thought to mediate crossovers by facilitating the transition from DSB into an SEI event which is then potentially processed to become crossover (Fig 1.5; Borner et al., 2004; de los Santos et al., 2003). Interestingly Zip1 the interference signal might be transmitted by mechanical forces acting on the chromosome axis and promote Zip1 polymerisation (Borner et al., 2004; Kleckner et al., 2004). This is suggested because Zip2 foci detected by immunostaining display interference in *zip1*Δ cells; interference is independent of Zip1 (Borner et al., 2004).

In yeast there are at least two crossover pathways, the *MUS81/MMS4* and the *MSH4/MSH5* mediated recombination pathway. In budding yeast *MSH4/MSH5* mediated repair appears to be the primary crossover-generating pathway. This pathway produces approximately 85% of the meiotic crossovers which display interference (Borner et al., 2004; Getz et al., 2008; Stahl et al., 2004). The *MUS81/MMS4* pathway possibly exists as a backup. These crossovers occur at a regular number per Kb and are distributed randomly (Hollingsworth, Ponte et al. 1995; Pochart, Woltering et al. 1997; de los Santos, Hunter et al. 2003). There is speculation that a third pathway also exists (Argueso et al., 2004; de los Santos et al., 2003; Stahl et al., 2004; Tsubouchi et al., 2006). SIC formation displays a similar CO level and interference pattern to *MSH5/MSH4* COs which is altered in *zip4*Δ and *zip1*Δ strains (Fung et al., 2004; Tsubouchi et al., 2006). However the SC might play a part in both the *MUS81/MMS4* and *MSH4/MSH5* pathways therefore would mediate all meiotic crossovers (Tsubouchi et al., 2006).

Until recently both crossovers and non-crossovers were suggested to have the same molecular precursor therefore occurred in equal amounts at the same time. However crossover precursors do not form until non-crossovers have

been fully formed. The temporal difference between crossover/non-crossover formation is reflected in the presence of early and late recombination nodules, supporting the idea that early nodules mark the sites of strand exchange and late nodules are associated with crossovers (reviewed in Zickler, 2006). The temporal difference in product formation indicates the crossover non-crossover decision is made early in meiosis prior to the SEI (Allers and Lichten, 2001a; Allers and Lichten, 2001b). Another implication is that at least two DSB repair mechanisms function in meiosis. The first model is the canonical double strand break repair model; the second is known as synthesis dependent strand annealing (SDSA; Allers and Lichten, 2001b; Holliday, 1974; McMahill et al., 2007; Merker et al., 2003; Paques and Haber, 1999; Fig 15).

In the canonical DSB repair model once Spo11 is removed resection exposes 3′ single stranded DSB ends (Paques and Haber, 1999). The homologous regions align and the ssDNA tail invades and base pairs with the donor duplex. The invading strand is extended using the unbroken DNA as a template. This creates a stable Holliday junction containing heteroduplex DNA (hDNA; reviewed in Paques and Haber, 1999). Intermediate repair structures can be detected that contain hDNA flanked by two Holliday junctions (Allers and Lichten, 2001b). Resolution potentially occurs via two variations of one pathway, generating a crossover or a non-crossover. If both Holliday junctions are cleaved in the same plane a gene conversion event occurs; cleavage in different planes results in crossover (Fig 1.5). In yeast meiosis this pathway is thought to be responsible for the majority of crossovers rather than non-crossovers.

SDSA is conserved and has been proven to exist in *Drosophila, E.coli,* mammals and yeast (reviewed in Paques and Haber, 1999). In this pathway a single strand invasion occurs via the same mechanism as in canonical DSB repair, forming hDNA with the template strand. 3′ synthesis elongates the invading single strand that is subsequently displaced. Once displaced the newly synthesised strand is able to anneal with DNA in the original duplex. Further synthesis of both broken stands allows full repair of the DSB (Fig 1.5). In this

pathway synthesised DNA is only present in the broken duplex (reviewed in Paques and Haber, 1999). To justify the lack of crossover events during mitosis, SDSA was originally thought confined to mitosis. There is now clear evidence that SDSA functions during meiosis and to be responsible for the majority of non-crossover events (Allers and Lichten, 2001a). SDSA has recently been shown to process 3′ ssDNA ejected from a JM (McMahill et al., 2007).

Resection

The MRX complex functions in both meiosis and mitosis and is formed by three highly conserved proteins Mre11, Rad50 and Xrs2 (MRX). Loss of a functional MRX complex results in chromosome instability. Mutations in the human MRX orthologues are manifested as an increase in cancer typified in Nijmegen breakage syndrome (NBS) and ataxia telangiectasia-like disorder. In yeast meiosis the complex is required for both the creation and repair of Spo11 DSBs. ATP has been suggested to regulate the function of the MRX complex, because ATP stimulates the endonuclease activity of Mre11 bound to Rad50 and MRX DNA topology activity is ATP dependent. Although Rad50 is required for Spo11-DSB removal the role of the MRX complex DSB repair is unknown. The requirement for the MRX complex members in DSB formation may reflect the need for prompt repair. MRX formation and presence at the DSB site has been suggested to ensure efficient DSB repair. The complex would not have to be recruited to the site if already bound during formation of the break.

Mre11 does not exhibit the expected nuclease polarity required in meiotic DSB repair (discussed in (Borde 2007)). Several theories have been suggested that tie the nuclease activity of Mre11 to the formation of Spo11 DSB. One possible hypothesis is that the polarity of the endonuclease activity is reversed. This phenomenon is evident in *Escherichia coli* where the RecBCD nuclease exhibits reversed polarity after interacting with the Chi site (Hagemann and Rosenberg 1991). The Rad50 hook-mediated bridging may stabilize the chromatin structure during transition from closed to open allowing Spo11 access to the DSB site (Lichten 2005; Wiltzius, Hohl et al. 2005; Cahill and Carney 2007). Another theory suggests that after binding of Spo11 to the DSB Mre11 could cleave in

region a region adjacent to the DSB creating a 3′ overhang the cleaved duplex is then unwound and further resection occurs via Mre11 or Exo1 (discussed in (Neale, Pan et al. 2005; Borde 2007)). The 3′ ssDNA could be unwound allowing further resection of the 5′ ssDNA end via Mre11. The complex could tether DNA, physically ensuring close physical proximity of two DNA strands. Mammalian Mre11 and Rad50 form a structure capable of tethering linear DNA molecules, supporting the theory (Wiltzius, Hohl et al. 2005).

EXO1

WT Spo11-DSB repair generates several intermediate structures including D-loop formation (duplex DNA which had been invaded by single stranded DNA forms a D-loop) and joint molecules (DSB flanked by two Holliday junctions; Fig 1.5). An early step in meiotic DSB repair is the resection of 5′ ends of the meiotic DSB exposing 3′, ssDNA the intact template duplex is then invaded by the 3′ ssDNA. (Fig 1.5). At HO breaks it has been suggested that resection need only expose 20-30 nt for successful gene conversion (Paques and Haber 1997). However in meiosis the exposed ssDNA has to be able to invade the homologous chromosome which is further away then the template used during HO repair, therefore resection has to be more extensive. Therefore an important factor in DSB repair is resection which cells with insufficient resection might be unable to successfully invade the template duplex. To expose 3′ ssDNA a 5′ – 3′ nuclease is required, although Mre11 is required for resection the polarity of Mre11 is incorrect. One possible model is that Mre11 is required for early repair for example Spo11 removal but another nuclease is required for later long tract resection.

Exo1 is a good candidate for a meiotic nuclease because Exo1 exhibits a 5′ – 3′ exonuclease and a 5′– flap endonuclease activity. In yeast *exo1*Δ diploid cells have a reduced crossover frequency (1.5- to 2 fold in four intervals), a reduced spore viability (80 % compared to 98 %), and an increased frequency of nondisjunction during meiosis I (Tran, Erdeniz et al. 2004). Exo1 might be the nuclease that exposes the 3′ ssDNA at Spo11-DSBs because in *dmc1*Δ *exo1*Δ

cells DSB bands seen during Southern analysis appear to be more discrete than in *dmc1*Δ cells (Tsubouchi and Ogawa 2000). Normally discrete DSB bands are indicative of a lack of resection intermediates that appear as a smear in wild type. The nondisjunction seen in *exo1*Δ cells might occur if the length of resection is insufficient and strand invasion of the homologue fails, preventing crossover formation resulting in nondisjunction and spore death. This hypothesis is supported by the observation that transcription of Exo1 is increased during meiosis. The role of Exo1 is highly conserved from yeast to mice because both male and female mice deficient in Exo1 are sterile (Wei et al., 2003).

TRM2

Genes that are involved in HR during mitotic DSB repair are often also involved in meiotic DSB repair such as Mre11, Exo1, Rad50, Xrs2, Rad51, Rad52 and Sgs1. Trm2 is an endo-exonuclease that exhibits a 5′ to 3′ nuclease activity expected to be required for DSB processing during HR, consequently Exo1 is a good candidate for a nuclease involved in processing mitotic and meiotic DSBs (Choudhury et al., 2007). In mitosis *trm2*Δ cells show an increased sensitivity to MMS and IR (Asefa et al., 1998). Overexpression of yeast Trm2 in mouse fibroblasts has been shown to increase the frequency of recombination suggesting that the role of Trm2 is conserved in organisms as diverse as yeast and mice (Semionov et al., 1999).

The role of Trm2 during meiosis has been tested by an assay where the HO endonuclease is persistently induced. HR is normally used to repair the HO DSB, in the absence of a functional HR pathway repair can occur via the NHEJ pathway that results in sterile spores. In this assay *trm2*Δ cells show an impaired cell viability and increased sterility when compared to wild type cells. The *trm2*Δ phenotype in this assay suggests the cell was unable to repair via HR therefore had to repair by NHEJ or die (Asefa et al., 1998; Choudhury et al., 2007). A reduced amount of ssDNA was detected in *trm2*Δ cells by slot blot during the HO assay. This suggests that in the absence of Trm2 insufficient resection prevents HR repair therefore repair has to occur via NHEJ

(Choudhury et al., 2007). Exo1 and Trm2 are suggested to complement each other during mitotic DNA damage repair. This relationship is indicated because NHEJ at the HO break results in insertions and deletions that can be detected with PCR both the single mutants had similar insertion and deletion at the breaksite in sterile survivors (Asefa et al., 1998; Choudhury et al., 2007). Also higher sensitivity to MMS and IR is seen in the double mutant compared to the single mutants (Asefa et al., 1998). Also a higher sterility rate was seen in the *trm2*Δ *exo1*Δ double mutant than seen in either single mutant.

Initial aims.

- To analyse processing of meiotic DSBs in three candidate mutants that are thought to influence resection or the timing of meiotic DSB repair.

- To further investigate the function of proteins that do have a role in meiotic DSB repair.

Chapter Two – Materials and Methods

Table 2.1 Haploid Strain List

hAG219	*MATa ho::LYS2 lys2 arg4-nsp,bgl ura3::URA3-[arg4-vde] nuc1Δ::LEU2 ade2 spo11(Y135F)-HA3His6::KanMX*	M. J. Neale.
hAG251	*MATα ho::lys2 lys2 ura3 nuc1D::LEU2 ura3::URA3[arg4-bgl] TFP1::VDE1*	M. J. Neale.
hAG317	*MATa ho::lys2 lys2 ura3 leu2::hisG arg4-bgl rad54Δ*	Prof. D. Bishop
hAG337	*MATα ho::lys2 lys2 ura3::URA3-[arg4-VDE] nuc1D::LEU2 spo11(Y135F)-HA3His6::KanMX arg4-nsp,bgl sae2::KanMX*	M. J. Neale.
hAG339	*MATa ho::lys2 lys2 ura3::URA3-[arg4-bgl] TFP1::VDE1 nuc1D::LEU2 arg4-nsp,bgl sae2::KanMX*	M. J. Neale.
hAG419	*MATα ho::LYS2 lys2 ura3 arg4-nsp,bgl nuc1Δ::LEU2 Δade2 spo11(Y135F)-HA3His6::KanMX*	M. J. Neale.
hAG684	*MATa ura3 lys2 ho::LYS2 trp1::hisG ade2::URA3-[arg4-VDE, ura3]*	A.E.Bisho p-Bailey
hAG803	*Mata lys2 ho::lys2 arg4-nsp,bgl ura3::URA3-[arg4-vde] nuc1Δ::LEU2 spo11-Y135F-HA3His6::KanMX ade2Δ trp1::hisG tel1Δ::ADE2*	M. J. Neale.
hAG804	*Matα lys2 ho::lys2 arg4-nsp,bgl ura3::URA3-[arg4-bgl] nuc1Δ::LEU2 SPO11+ ade2Δ TFP1::VDE tel1Δ::ADE2*	M. J. Neale.
hAG805	*Matα lys2 ho::lys2 arg4-nsp,bgl ura3::URA3-[arg4-bgl] nuc1Δ::LEU2 spo11-Y135F-HA3His6::KanMX ade2Δ TFP1::VDE tel1Δ::ADE2*	M. J. Neale.
hAG126	*MATa ho::LYS2 lys2 arg4-nsp,bgl ura3::URA3-[arg4-*	This

2	*vde] nuc1Δ::LEU2 ade2 spo11(Y135F)-HA3His6::KanMX rad6::hpMX*	study
hAG126 6	*MATα ho::lys2 lys2 ura3 nuc1D::LEU2 ura3::URA3[arg4-bgl] TFP1::VDE1 rad6::hphMX*	This study
hAG150 0	*MATα ura3 lys2 ho::LYS2 leu2 (Xho1-Cla1) trp1::hisG srs2-101*	This study

dAG744	*lys2 ho::LYS2 arg4-nsp,bgl ura3::URA3-[arg4-vde] lys2 ho::LYS2 arg4-nsp,bgl ura3::URA3-[arg4-bgl] nuc1Δ::LEU2 spo11-Y135F-HA3His6::KanMX Δ ade2 TFP1::VDE TRP1 tel1Δ::ADE2 ade2Δ TFP1 trp1::hisG tel1Δ::ADE2*	M. J. Neale, unpublished
dAG1312	*lys2 ho::LYS2 arg4-nsp,bgl ura3-URA3-[arg4-bgl] lys2 ho::LYS2 arg4-nsp,bgl ura3::URA3-[arg4-vde] SPO11 Δ TRP tel1Δ::ADE2 dmc1::ADE spo11(Y135F) trp1::hisG tel1Δ::ADE2 dmc1::ADE ade2Δ TFP1::VDE nuc1Δ::LEU2 ade2Δ TFP nuc1Δ::LEU2*	This Study
dAG1313	*lys2 ho::LYS2 arg4-nsp,bgl ura3::URA3-[arg4-bgl] lys2 ho::LYS2 arg4-nsp,bgl ura3::URA3-[arg4-vde] nuc1Δ::LEU2 rad6::hphMX ade2Δ nuc1Δ::LEU2 rad6::hphMX ADE spo11(Y135F)-HA3His6::KanMX TFP1 SPO11 TFP1::VDE1*	This Study
dAG1393	*ho::lys2 LYS2 ura3::URA3-[arg4-bgl] tel1Δ::ADE2 ho::lys2 LYS2 ura3::URA3-[arg4-vde] tel1::ADE2 leu2Δ SPO11 leu2Δ spo11-Y135FHA3His6::KanMX TFP1::VDE sae2::KanMX TFP1 sae2::KanMX*	This Study

dAG1395	*lys2 ho::LYS2 arg4-nsp,bgl ura3::URA3-[arg4-bgl] lys2 ho::LYS2 arg4-nsp,bgl ura3::URA3-[arg4-vde] sae2Δ:: KanMX::SAE2::LEU TFP1::VDE sae2Δ:: KanMX::SAE2::LEU TFP1*	This Study
dAG1396	*lys2 ho::LYS2 arg4-nsp,bgl ura3::URA3-[arg4-bgl] lys2 ho::LYS2 arg4-nsp,bgl ura3::URA3-[arg4-vde] sae2Δ:: KanMX::SAE215::LEU2 TFP1::VDE sae2Δ:: KanMX::SAE215::LEU2 TFP1*	This Study
dAG1397	*lys2 ho::LYS2 arg4-nsp,bgl ura3::URA3-[arg4-bgl] lys2 ho::LYS2 arg4-nsp,bgl ura3::URA3-[arg4-vde] sae2Δ:: KanMX::sae269::LEU2 TFP1::VDE sae2Δ:: KanMX::sae269::LEU2 TFP1*	This Study
dAG1398	*lys2 ho::LYS2 arg4-nsp,bgl ura3::URA3-[arg4-bgl] lys2 ho::LYS2 arg4-nsp,bgl ura3::URA3-[arg4-vde] sae2Δ::KanMX::sae225689LEU2 TFP1::VDE sae2Δ::KanMX::sae225689LEU2 TFP1*	This Study
dAG1441	*lys2 ho::LYS2 arg4-nsp,bgl ura3::URA3-[arg4-bgl] lys2 ho::LYS2 arg4-nsp,bgl ura3::URA3-[arg4-vde] leu2Δ spo11-Y135F- HA3His6::KanMX leu2 spo11-Y135F-HA3His6::KanMX ade2Δ tel1Δ::ADE2 TFP1::VDE trp1::hisG ade2Δ tel1Δ::ADE2 TFP1 TRP nuc1Δ::LEU2 nuc1Δ::LEU2*	This Study
dAG1473	*lys2 ho:: LYS2 arg4-nsp,bgl ura3::URA3-[arg4-vde] lys2 ho::LYS2 arg4-nsp,bgl ura3::URA3-[arg4-bgl] nuc1Δ::LEU2 rad6::hphMX sae2Δ::KanMX ade2Δ nuc1Δ::LEU2 rad6::hphMX sae2Δ::KanMX ADE spo11(Y135F)-HA3His6::KanMX TFP1 SPO11 TFP1::VDE1*	This Study
dAG1475	*ho::LYS2 lys2 arg4-nsp,bgl ura3::URA3-[arg4-vde]*	This

	ho::LYS2lys2 arg4-nsp,bgl ura3::URA3[arg4-bgl] spo11(Y135F)-HA3His6::KanMX SRS2 SPO11 srs2-101 nuc1Δ::LEU2 ADE2 nuc1Δ::LEU2 ade2Δ	Study
dAG1476	*ho::LYS2 lys2 ARG ura3::URA3-[arg4-vde] ho::LYS2 lys2 arg4-nsp,bgl ura3::URA3[arg4-bgl] spo11(Y135F)-HA3His6::KanMX srs2-101 SPO11 srs2-101 nuc1Δ::LEU2 ADE2 TFP1::VDE1 nuc1Δ::LEU2 ade2Δ TFP1*	This Study
dAG1488	*ho::LYS2 lys2 arg4-nsp,bgl ura3::URA3-[arg4-vde] ho::LYS2 lys2 arg4-nsp,bgl ura3::URA3[arg4-bgl] spo11(Y135F)-HA3His6::KanMX srs2-101 SPO11 srs2-101 nuc1Δ::LEU2 ADE2 TFP1::VDE1 sae2Δ::KanMX nuc1Δ::LEU2 ade2ΔTFP1 sae2Δ::KanMX*	This Study
dAG1493	*ho::LYS2 lys2 arg4-nsp,bgl ura3::URA3-[arg4-vde] ho::LYS2 lys2 arg4-nsp,bgl ura3::URA3[arg4-bgl] spo11(Y135F)-HA3His6::KanMX srs2-101 SPO11 srs2-101 nuc1Δ::LEU2 ADE2 TFP1::VDE1 nuc1Δ::LEU2 ade2Δ TFP1*	This Study
dAG1501	*ho::LYS2 lys2 arg4-nsp,bgl ura3::URA3-[arg4-vde] ho::LYS2 lys2 ARG ura3 leu2-K ade2Δ TFP1::VDE1*	This Study

	leu2::URA3-[arg4-bgl] ADE2 TFP1 *srs2-101* *srs2-101*	
dAG1502	*ho::LYS2 ura3 leu2(Xho1-Cla1) srs2-101* *ho::LYS2 ura3 leu2::hisG SRS2* *ARG4 RAD54 TRP* *arg4-bgl rad54::hisG trp1::hisG*	This Study
dAG1507	*ho::LYS2 ade2::URA3 –[arg4-VDE, ura3]* *trp1::hisG* *ho::LYS2 Δade2(EcoRV-StuI) TRP1* *ARG4 LEU2 TFP1* *arg4-nsp,bgl leu2-R TFP1::VDE* *rad6::hphMX* *rad6::hphMX*	This Study
dAG206	*lys2 ho::LYS2 leu2-K arg4-nsp,bgl* *l ys2 ho::LYS2 leu2-R arg4-nsp,bgl* *ura3::URA3–[arg4–bgl] nuc1::LEU2 TFP1::VDE* *ura3::URA3–[arg4–vde] nuc1::LEU2 TFP1* *SPO11* *spo11–Y135F–HA3-His6::KanMX4*	M. J. Neale.
dAG205	*lys2 ho::LYS2 leu2-K arg4-nsp,bgl* *lys2 ho::LYS2 leu2-R arg4-nsp,bgl* *ura3::URA3–[arg4–bgl] nuc1::LEU2 TFP1::VDE* *ura3::URA3–[arg4–vde] nuc1::LEU2 TFP1* *spo11–Y135F–HA3-His6::KanMX4* *spo11–Y135F–HA3-His6::KanMX4*	M. J. Neale.

Primers

Name	Primer size	Sequence
Sae2::KanMX6 F	20	GCC AGT AAT TGA CGA TGC GG
Sae2::KanMX6 R	21	GAC CTT CAG TGA GAG AAT GCG
Rad6-471 Primer 1	24	GGGCACATCAAATATGAAACTCCC
Rad6+574 Primer 2	23	GGA AGA GCA TAG ATC CAT TCA GC
Hyg3'+Rad6 Primer 3	45	GCG TCAA TCG TAT GTG AAT GCT GTC TTA ATG ATG AAT GCC GAG CCG
Hyg5'+Rad6 Primer 4	42	GGG TAT TCT GGG CCT CCA TGT CAG CTG GTG TGG ACA TGA CGC
Probe forward		ATCGAAAAACTAGCTGAAAAATGTGATGTGCTAACG
Probe Reverse		CCTTGGGGCAATTTCGTTAATAAGCAATTCCCCTG
THR4-2-F (ARE1-DSB-2-F)	24	CCAAGGCCACGGTGAAGACTACGG
THR4-2-R (ARE1-DSB-2-R)	26	GGCTCTTGCTCAAATGCAATGCGCCC
THR4-3-F (ARE1-DSB-3-F)	24	GGTAACACAGACCAATCCGGTCCC
THR4-3-R (ARE1-DSB-3-R)	23	GCTTCACTTCACCCAATTCGGGC
THR4-4-F (ARE1-DSB-4-F)	22	CTCAGAATTCTCGTTCCCAGGG
THR4-4-R (ARE1-DSB-4-R)	22	GAGAAGGGCAGAAACTATCTGG
drugpromotor TEF promoter	20	*CCTTGACAGTCTTGACGTGC*
drugterminator TEF terminator	20	*CAGATGCGAAGTTAAGTGCG*
Forward srs2 101		CATGCTAGGGTAACGAGACGC
Reverse srs2 101		ATCACCTACGATGGTCATCCC
MN11		AAAGGAACTATCCAATACCTCGCC
MN12		AAGGATCCCCACCTATGGGC
CYS3-F		GTCGCAGTCAACT ACCCAGG
CYS3-R		CCAGGATGATC TCCGCACCTTGAACG
Zip1F (Slava)	20	TGCTATAAGCCGTGTTGTCC
Zip1R (Slava)	21	CCTAAGTCGTGCACCAAGATC
CA probe F		TAAACTGGATAATGTAGGGCC
CA Probe R		CGCCAGCAAGACGTAGCCCAGCGC
CLB2SRS2 F	59	atgatagcgccgcttaaaacatgct agggtaacgagacgcgaattcgagc tcgtttaaa
CLB2SRS2 R	87	ccgctgctctctgttgagtatttaa ctgggatactaaatgcaaccaaaga tcattgttcgacgacatgcactgag cagcgtaatctg
HIS4 F	23	ATTCTAGCCCCACCAAACCATGC
HIS4 R	23	AGGAATGAAATCTGGATCAAGGG
hed1F	60	cta cat gtc aga gac gaa cga aag aga gaa atc aag acg aca tgg agg ccc aga ata ccc
hed1R	60	cac cga act ctt ttt caa acg ttc tcc tct ttg aac tta gca ttc aca tac gat tga cgc
hed1 check f	26	ACT ATT TAT TAA GAT AGC CGC CCA GG

All oligonucleotides used in PCR were synthesised by MWG Biotech or biomers.net with high-purity salt free (HPSF) purification.

General Techniques

Saccharomyces cerevisiae permanent storage of yeast clones

5 ml cultures were incubated at 30^{0}C overnight then centrifuged at 3000 g for 5 min. The pelleted cells were resuspended in 2 ml of sterile 50 % glycerol and aliquoted into sterile screw capped vials then stored at –80^{0}C.

Saccharomyces cerevisiae growth conditions

Diploid SK1 cells sporulate rapidly therefore all strains were streaked freshly for each experiment from –80°C glycerol stocks onto solid media and incubated at 30^{0}C.

Haploid cell mating

Fresh single colonies of opposite mating types (a/α) were mixed on a YEPAD plate and incubated at 30°C overnight. Then a proportion of the cells were streaked for single colonies after 48 hr these were streaked again and tested for diploidisation.

Diploid testing

Potential diploid strains were tested by complementation test with hAG55 (MATa) and hAG56 (MATα), all strains used in the study are *URA2,* hAG55 and hAG56 are WT except for *ura2* mutation. Prospective diploids were mated with hAG55 and hAG56 overnight then replica plated onto minimal media. Diploid strains would be unable to mate with hAG55 and hAG56 therefore would not grow; however haploids would be able to mate and consequently would grow.

Possible diploids were also patched onto YEPAD and incubated 30°C overnight then replica plated onto solid potassium acetate. After a second 30°C incubation for 1-2 nights the presence of tetrads was confirmed with light microscopy.

Tetrad Dissection

Diploids were sporulated asynchronously on solid potassium acetate and incubated at 30°C for two to three days. The resulting tetrads were incubated in 20 µl of ß-glucuronidase (9.45U/µl) to break down the asci. 300 µl of water was added to the cell suspension and plated to a small area on a flat plate and allowed to dry. The tetrads were dissected using a micro manipulator then incubated 30°C for 48 hr then replica plated onto selective media and YPEAD then incubated at 30°C overnight. Most non-wild type genes were marked with genes required for synthesis of amino acids essential for vegetative growth, and could be identified by growth on SC- plates lacking the appropriate supplement. Haploid strains containing genes marked with hphMX or *KanMX* were selected for by growth on YPD with Hygromycin B or G418 plates respectively. Where no prototrophic or antibiotic resistance phenotype was present for a particular gene, PCR and digestion was used. When the same marker allele was required

more than once in the same strain parental ditype (PD)s and nonparental ditype (NPD)s were scored to find spores that had a 2:2 or 4:0 segregation. To create a double homozygous mutant several rounds of mating and dissection were required to produce experimental diploid strains. The haploid strain list contains only the strains from which the alleles in the final experimental diploids originate. The diploid list contains all diploids used in experiments.

Chemical transformation

A 5 ml overnight culture was used to inoculate 20 mls of YEPAD to a real OD_{600} of 0.1 and 0.2, this was incubated at 30°C until a real OD_{600} of 0.6 to 0.8 was reached. The culture was centrifuged 3000g for 5 min, harvested cells were washed in 10 ml H_2O and resuspended in 1 ml of 100mM lithium acetate then spun at 3000 g 15 s. The cell pellet was resuspended in 150 µl of 100 mM lithium acetate, 50 µl aliquots were spun at 3000 g 15 s. 240 µl of PEG was layered over the pellet, followed by 36 µl 1M lithium acetate, 10 µl boiled salmon sperm DNA, transformation DNA, the mixture was made up to 360 µl final volume and vortexed. The cell suspension was incubated at 30°C for 30 min then transferred to 42°C for another 30 min. After the successive incubations the cell suspension was centrifuged at 3000g 15 s the liquid removed and the pellet resuspended in 400 µl of sterile water. For prototrophic selection the cells were plated directly onto solid media, however for selection by antibiotic resistance the cells were added to 5 ml YEPAD and allowed to complete two cell divisions at 30°C then plated onto YEPAD with antibiotic.

Electroporation

A 5 ml overnight culture was spun at 3000 g for 5 min the pellet was washed in 1.2 M sorbitol three times then placed on ice for a minimum of 5 min. 40 µl of cell suspension was added to chilled DNA with heat denatured salmon sperm DNA and mixed well. The transformation mix was added to a 2 mm chamber, electroporated using the settings for yeast transformation 1.5kV, 200 Ohms, 25 µF for a 5 msec pulse. 400 µl of cold 1.2 M sorbitol was immediately added to the cells then incubated on ice for 5 min and plated onto solid dropout media

with 1M sorbitol for prototrophic selection. Cells were plated onto YEAPD then replica plated onto YEAPD with Hygromycin B for hphMX selection.

Return to growth

0.5 ml of cells from synchronously sporulating cultures were diluted with sterile H_2O in 10-fold increments. The cell suspension was plated onto duplicate YEPAD and Arg dropout media then incubated at 30°C for 2 days, slow growing strains required 3 days. To calculate the viability of the strain and the proportion of arginine prototrophs the number of colony forming units was divided by the volume plated out then multiplied by the dilution factor.

Synchronous sporulation of *S. cerevisiae*

Three 5 ml YPD overnight cultures were inoculated from fresh single diploid colonies and incubated at 30°C overnight. The cultures were used to inoculate multiple dilutions of 300 ml PSP2 in 2 l flasks which were incubated at 30°C, 300 rpm for 24 hr. Typically PSP2 dilutions were 1:100 and 1:250, for slow growing strain a 1:50 dilution was used.

The PSP2 culture density was measured with OD_{600} of 2-fold dilution, an OD_{600} within 1.4-2.0 were selected, except for extremely slow growing strains when the range 0.5-1.0 was acceptable. For slow growing strain synchrony was tested by DAPI or SC formation and the meiotic progression was comparable to wild type. The cells were rapidly harvested at 3000 g in a Beckman centrifuge at 30°C 4500 rpm for 2 min then washed in 300 ml of 1% potassium acetate and centrifuged a second time. Cells were resuspended in 300 ml potassium acetate plus supplements, and transferred to a 37°C pre-warmed 2.8 l baffled flask and incubated at 30°C and 300 rpm. Time point T=0 was taken immediately following the flask being placed in the incubator.

Harvesting cells for DNA extraction

25 ml of synchronously sporulating cells from time course culture were removed at each time point and added to 6 ml ice-cold 50% glycerol and 300 µl 10% sodium azide. Cells were harvested by centrifugation at 3000 rpm for 5 min and

resuspended in 6 ml 20% glycerol spheroplasting solution, then centrifuged again for 5 min. The cell pellets were frozen in liquid nitrogen and stored at –80°C.

DAPI staining of cells to monitor nuclear divisions

500 µl of synchronously sporulating cells were fixed in 1 ml of 100% ethanol, and stored at –20°C. The cells were prepared by centifugation 17,000 g for 1 min then resuspended in 1 ml of H_2O with 1 µl of 0.5 mg/ml DAPI and incubated in the dark on the bench for 1 min. Cells were harvested by centrifugation and resuspended in 20 µl of 50% glycerol then visualised using a fuorescence microscope (model DMLB – Leica) with a standard DAPI filter. 200 cells from each time point were scored for the number of discrete DAPI-stained bodies present in each cell.

DNA restriction digests

DNA was digested according to instructions supplied by the enzyme manufacturer, the majority of restriction enzymes used were supplied by New England Biolabs (NEB). Digest volumes were made up with mqH_2O. All digest incubations took place in water baths. Digests of yeast genomic DNA for Southern analysis were incubated for 3-5 hr.

Culture of *E. coli* DH5α

DH5α bacterial cells were streaked from –80°C stocks onto 2TY medium and incubated overnight at 37°C. A fresh single colony was used to inoculate 100 ml of pre-warmed, pre-aerated 2TY media and grown at 37°C with vigorous aeration to an OD_{550} of 0.5. Cells were harvested at 4°C, resuspended in 25 ml TFBI (100mM RbCl, 50mM $MnCl_2$, 30mM K-acetate, 10mM $CaCl_2$, 15% glycerol, pH 5.8), and incubated on ice for 15 min. Cells were reharvested at 4°C, resuspended in 4 ml TFBII (10mM MOPS, 10mM RbCl, 75mM $CaCl_2$, 15% glycerol, pH 6.8), and incubated on ice for 15 min. 100 µl aliquots were snap-frozen in liquid nitrogen and stored at –80°C.

Transformation of chemically-competent DH5α *E. coli*

100 µl aliquots of frozen cells were thawed on ice then spilt into 50 µl aliquots and incubated with 1-2 ng of plasmid DNA. Cells were heat-shocked at 42°C for 90 s then 450 µl of liquid 2TY was added, the cell suspension was then placed on ice for 2 min. Subsequently cells were incubated for 90 min at 37°C then plated to 2TY plus ampicillin plates which were incubated for 24 hr at 37°C.

Large-scale isolation of DNA from *E. coli* (midiprep)

Bacterial cells were struck from –80°C stocks onto 2TY media and incubated overnight at 37°C a single colony was cultured for 6 hr in 5 ml 2TY (50µg/ml ampicillin) at 37°C. 1 ml of this culture was diluted into 100 ml 2TY (50µg/ml ampicillin) and incubated at 37°C for 18 hr. DNA was extracted from cells in this culture using the Wizard Plus Midiprep kit (Promega), following the instructions supplied.

Ethanol precipitation of DNA

3 M sodium acetate was added at one tenth the volume of DNA solution and mixed. Twice the total volume of ice-cold 100 % ethanol was layered on top and mixed by gentle inversion. DNA was then precipitated for 1 hr at –80°C or 20°C for 24 hr. Purified DNA was isolated by centrifugation at 14,000 rpm for between 5 min and 30 min. The DNA pellet was then washed in 70 % ethanol and air-dryed DNA was resuspended in 30-50 µl of 1×TE.

Gel purification of DNA fragments

DNA was isolated from contaminating template DNA by gel electrophoresis using appropriate percentage agarose gel (Bio-gene) with 1×TAE and ethidium bromide. The DNA to be isolated was cut from the gel, keeping UV exposure to a minimum. DNA was then purified from the agarose block using the QIAquick Gel Extraction Kit (Qiagen), following supplied instructions.

Polymerase chain reaction (PCR)

Routine PCR

Routine diagnostic extensions, 2.0m M $MgCl_2$, 200 µM dNTPs, 5-50 units/ml *Taq* (NEB or BIOLINE), 0.1 µM forward and reverse primers, 100-1000ng of yeast genomic DNA template made up to 20 µl final volume with autoclaved Millipore water.

Example of routine PCR

Short range

Denature 94°C for 2 min, 1 cycle

Denature 94°C for 30 s,
Annealing *x*°C for 30 s, 25-30 cycles of:
Extension 72°C for *y* s,

Final extension 72°C for 5min.

Long range

Denature 94°C for 2 min, 1 cycle

Denature 94°C for 30 s,
Annealing 50°C for 30 s, 5 cycles of:
Extension 72°C for *y* s,

Denature 94°C for 30 s,
Annealing *x*°C for 30 s, 25-30 cycles of:
Extension 72°C for *y* s,

Final extension 72°C for 5 min.

where *x* is the primer-specific annealing temperatures and *y* is proportional to the expected size of the product to be obtained (1 min per kb).

Colony PCR.

DNA for PCR was extracted from fresh colonies, a small section of a single colony was incubated in 10 µl of spheroplasting solution with zymolyase solution (5 mg/ ml 20T zymolyase; MP Biomedicals) for 15 min at 37°C, 1 µl was added to the PCR reaction.

Alternatively a small section of very fresh yeast colony was boiled in 40 µl of water and 5 µl was added to PCR reaction.

Measurement of DNA concentration in solution

DNA concentrations was measured using a DyNA Quant 200 fluorometer (Hoefer), using the DAPI fluorophore. Samples were measured in a quartz cuvette, in filter-sterilised 1×TNE, 1µg/ml DAPI. The fluorometer was calibrated to 100 ng of λ*Bst*EII DNA (New England Biolabs).

Alternatively DNA was run on an agarose gel and compared to a ladder with known concentration typically Bioline hyperladder.

Native DNA electrophoresis

DNA was fractionated in suitable percentage agarose gels for the expected product size in 1×TAE 10 µg/l Ethidium (10 mg/ml BioRad). 250 ml 25 cm × 15 cm 0.5 % agarose gels were used to fractionate 0.5-1µg digested yeast genomic DNA to be southern blotted. These were run at 70 V for 10-14 hr with circulating buffer. For all native electrophoresis. Ethidium bromide was mixed with the running buffer prior to running.

Southern blots

Once fractionated stained DNA was visualised with minimal UV, the agarose gels were rinsed in 1 litre dH_2O for 5 min to removed ethidum bromide. To depurinate the DNA the gels were agitated in 1 litre 0.25 M HCl for 45 min a 15 min rinse in 1 litre dH_2O removed HCl, then agitated in 0.4M NaOH for 45 min to denature DNA. ssDNA was blotted to either Zetaprobe GT (Bio-Rad) or Biotrans (+) (MP Biomedicals) nylon membrane via a VacugeneXL blotter

(Amersham) at 50-100 mbar for 1.5 hr in 0.4M NaOH. The membrane was rinsed in 200 ml 2×SSPE. The transferred DNA was covalently linked to the membrane using a UV crosslinker (model XL-1500 - Spectronics).

Generating ^{32}P –labelled DNA probe

The product of four PCR reactions was purified by gel extraction and subsequently used as the template for second round of PCR. The products from the second round was ethanol precipitated then diluted to around 0.05 µg. 50-100 ng of probe and 0.2 ng of λ*Bst*EII were denatured at 100°C then cooled on ice, 4 µl dCTP High Prime random priming labelling kit (Roche) was added. 5 µl of ^{32}P–dCTP (MP Biomedicals and Amersham) was used as the radioactive substrate the solution was incubated at 37°C for 20 min. Unincorporated bases were removed using a G30 Biospin column (Bio-Rad). Salmon sperm DNA boiled for 5 min was added to the probe then boiled for 4 min at 100°C the ssDNA probe was then added to hybridisation solution.

Southern hybridisation

Nylon membranes were placed into glass hybridisation tubes that were washed, coated with 1 ml ethanol, 1 ml sigmacote, 1 ml ethanol then rinsed with water and pre warmed in 65°C oven. The membranes were pre-hybridised for a minimum of 3 hr at 65°C in 40 ml of pre-hybridisation solution then subsequently hybridised at 65°C for a minimum of 6 hr in 20 ml hybridisation solution. To remove nonspecific bound probe membranes were agitated in three washing solutions for 15 min each at room temperature. After washing the membranes were blotted dry, wrapped in cling-film and exposed to a blanked phosphor screen (K-screen, Kodak).

Scanning densitometry

To quantify the amount of DNA the probe bound to Quantity One software (Bio-Rad) was used. Kodak phosphor screens were exposed to radioactive hybridised filters these were scanned (In the dark) using a Personal FX phosphoimager (Bio-Rad). The background levels were calculated and removed

boundaries were placed around the upper and lower limits of each band to be measured.

Yeast genomic prep

1.5 ml YPD overnight culture was pelleted at 14000 rpm for 1 min the supernatant removed and cells resuspended in 200 µl of genomic TENS (10 mM Tris.HCl pH7.8, 1 mM EDTA, 100 mM NaCl, 1 % SDS). The cells were vortexed with sterile glass beads for 1 min, 100 µl of phenol : chloroform : isoamylalcohol (25 : 24 : 1) was added followed by another 1 min vortex and 2 min 14000 rpm centrifugation. The aqueous top layer was transferred into a fresh tube then 200 µl of phenol was added followed by an additional 1 min vortex and 2 min 14000 rpm centrifugation. The aqueous layer transferred to a fresh tube and DNA was extracted by ethanol precipitation.

CTAB yeast genomic DNA extraction

For quantitative Southern analysis a modified CTAB protocol was employed (Allers and Lichten, 2000). Thawed cell pellets from meiotic culture were washed in 1.5 ml of ice-cold spheroplasting solution then pelleted at 4000 rpm for 1 min. The pellet was resuspended in 100 µl of spheroplasting solution with 0.5 mg/ml 100T zymolyase (MP Biomedicals), and 1% β-mercaptoethanol. The solution was then incubated at 37°C for 6 mins the tubes were inverted every three min. Post incubation 200 µl of CTAB extraction solution was added and mixed by pipetting subsequently 0.5 µl of 10 mg/ml RNase and 5 µl of 20mg/ml Proteinase K was then added and tubes mixed gently. The cell suspension was then incubated at 37°C for 15 min with gentle vortexing and inversion every 5 mins. 100 µl chloroform : isoamylalcohol (24:1) was added to extract the CTAB-DNA complexes, each tube was vortexed for 20 s, allowed to rest for 2 min, vortexed for 20 s then centrifuged at 14,000 rpm, 18°C for 7 min. The aqueous upper layer was transferred to a new tube and one volume of CTAB dilution was added, after a white precipitate become visible a second volume of dilution solution was added. The interface was disturbed by gentle agitation until the two layers were mixed, leaving a white precipitate in a clear liquid. The solution was

removed and the remaining CTAB-DNA pellet was washed twice in 0.4 M NaCl in 1XTE, and completely resuspended in 300 µl of ice-cold 1.42 M NaCl in 1XTE. DNA was precipitated by the addition of 600 µl of 100% ethanol and washed twice in 600 µl of 70% ethanol then pulse centrifuged. Excess ethanol removed and the pellet was briefly air dried but not allowed to completely dry out and resuspended in 30 µl ice-cold 1×TE.

Media

YPD
2% D-glucose
2% Bacto peptone (Difco)
1% Yeast extract (Difco)
2% Agar (Sold media)
40 µg/ml Adenine
Strains expressing resistance genes were selected for on YEPAD plus antibiotic.
*Kan*MX 200 µg/ml G418 (Melford-Labs)
hphMX 300 µ/ml Hygromycin B (Sigma-Aldrich)
Minimal Medium
2% D-glucose
0.67% yeast nitrogen base w/o amino acids (Difco)

Synthetic complete and dropout medium
2% D-glucose
0.67% yeast nitrogen base w/o amino acids
0.85 g/l dropout mastermix.
1 µl/ml of 2M NaOH

Complete mastermix comprised: 0.8 g adenine, 0.8 g arginine, 4.0 g aspartic acid, 0.8 g histidine, 2.4 g leucine, 1.2 g lysine, 0.8 g methionine, 2.0 g phenylalanine, 8.0 g threonine, 0.8 g tryptophan, 1.2 g tyrosine, 0.8 g uracil. Dropout mastermixes were as complete but lacked the relevant supplement(s).

Used as positive selection media for prototrophic colonies.

K-Ac Liquid

1% potassium acetate (J.T. Baker)

10 µg/ml amino acid supplement(s)

KAc Solid

1% potassium acetate (J.T. Baker)

2% agar (Solid media)

0.1% yeast extract

0.05% D-glucose

10 µ/ml amino acid supplement(s)

PSP2

0.67% yeast nitrogen base w/o amino acids

0.1% yeast extract

1% potassium acetate

1.02% potassium hydrogen phthalate

10 µg/ml amino acid supplement(s)

2TY

1.1% tryptone (Difco)

1% yeast extract

0.5% NaCl

1.5% agar (Solid media)

pH7.4

Bacteria expressing plasmids carrying drug resistant genes were selected for with 2TY plus 50µg/ml Ampicillin (Sigma-Aldrich).

General Solutions

10xTE:

100 mM Tris base, 10 mM EDTA, pH 7.5

10xTNE:

100 mM Tris.HCl, 2 M NaCl, 10 mM EDTA, pH7.4

50xTAE:

2 M Tris base, 100 mM EDTA, 0.95 M acetic acid

20xSSPE:

3.6 M NaCl, 200 mM NaH_2PO_4, 20 mM EDTA, pH7.4

Genomic TENS:

10 mM Tris.HCl pH7.8, 1 mM EDTA, 100 mM NaCl, 1 % SDS

6x Loading Dye:

0.25% Bromophenol-blue, 20% sucrose

Heavy Loading Dye (southern gels):

0.25% Bromophenol blue, 0.25% xylene cynanol, 20% ficol

Proteinase K Solution:

10 mM Tris.HCl pH7.5, 20 mM $CaCl_2$, 50% glycerol, filter sterilised before addition of 20 mg/ml Proteinase K

RNase Solution:

10 mg/ml RnaseA, 10 mM Tris.HCl pH7.5, 22.5 mM NaCl. Incubated at 100°C for 15 min, cooled on bench to room temperature.

Spheroplasting Solution (±20% glycerol):

1 M sorbitol, 50 mM KPO_4, pH7.5, 10 mM EDTA, pH7.5, (20% glycerol)

CTAB Extraction solution:

3% CTAB, 0.1 M Tris-HCl pH7.5, 25 mM EDTA, 2M NaCl, 2% PVP_{40}

CTAB Dilution solution:

1% CTAB, 50 mM Tris-HCl pH7.5, 10 mM EDTA pH8.0, Stored at 37 °C

Prehybridisation solution:

2X SSPE, 1% SDS, 0.5% non-fat dry milk, 5 μg/ml boiled salmon sperm DNA

Hybridisation solution:

2X SSPE, 1% SDS, 0.5% non-fat dry milk, 5% dextran sulphate (Sigma-Aldrich)

Washing buffers (southern hybridisation):

First wash: 2% SSPE, 1% SDS, Second wash: 0.5% SSPE, 1% SDS, Third wash: 0.1% SSPE, 1% SDS

Plasmids

Plasmids were kindly given by Maria Pia Longhese, Ph.D, Dip. Biotecnologie

Bioscienze, Università di Milano-Bicocca and Hannah L Klein Ph.D. Professor of Biochemistry, Medicine and Pathology New York University School of Medicine.

Chapter Three – The influence of *TEL1* on VDE-DSB repair

Brief introduction

Tel1 and Mec1 belong to a conserved family related to the phosphoinositide 3-kinases, that includes the mammalian *TEL1 MEC1* orthologues ataxia-telangiectasia-mutated (ATM) and ataxia-telangiectasia, RAD3 related (ATR; Mallory and Petes, 2000). ATM and ATR respond to DNA damage by inducing a signalling cascade that is relieved when the damage has been repaired. Mutations in ATM results in ataxia telangiectasia (AT). Sufferers have predisposition to cancer, sensitivity to IR, and chromosomal instability (Fritz et al., 2000; Morrow et al., 1995). In yeast Tel1 and Mec1 mutations result in an increased sensitivity to IR and MMS.

One substrate for Tel1 is Mre11, which forms a complex with Xrs2 and Rad50 known as MRX. In meiosis MRX is required for Spo11-DSB formation, DSB repair and removal of bound Spo11 from the break (Cherry et al., 2007; Mantiero et al., 2007; Usui et al., 2001; Usui et al., 1998). In mitosis Mre11 and Xrs2 are phosphorylated by the protein kinase Tel1 in response to DNA damage. This is termed the TM pathway (Usui et al., 2001). In meiosis Spo11 remains covalently bound to the 5′ end of DSBs and has to be removed for successful meiotic HR repair (Prieler et al., 2005). Spo11 removal is dependent upon Sae2 and Rad50 (Prieler et al., 2005). Tel1 dependent phosphorylation of MRX has been shown to occur in both *sae2Δ* and *rad50S* cells (Usui et al., 2001). A plausible hypothesis is that during meiosis, like mitosis, MRX is phosphorylated by Tel1 in response to blocked DSBs (discussed in Usui et al., 2001). In wild type cells Tel1 dependent phosphorylation of MRX is undetectable (Usui et al., 2001). This could be indicative of a transient MRX phosphorylation, because Spo11 bound DSBs are repaired. The observed Tel1 phosphorylation of MRX in *sae2Δ* and *rad50S* strains suggests that in meiosis Tel1 could initiate a signal cascade that instigates DSB repair in response to Spo11 bound DSBs (Usui et al., 2001).

To further analyse the role of Tel1 during meiosis the VDE reporter assay was used. In meiosis the VDE cutsite is cleaved by the VMA1 derived endonuclease therefore the break is site specific. Also the VDE-DSB is formed approximately at the same time as Spo11-DSBs are created. The cassette is heterozygous: on one chromosome the VDE-DSB site is inserted into the *ARG4* open reading frame forming *arg4-VDE*. The arg4-VDE is flanked by *ura3::*ty upstream and URA3 downstream. The homologous chromosome has an *arg4,bgl* allele which can be used as a template for repair of the VDE-DSB by HR. The VDE-DSB can also be repaired by SSA. In this form of repair resection can expose the flanking *URA3* and *ura3::ty* repeats which anneal forming *URA3*.

Results

Tel1 is required for timely VDE-DSB repair

To see if Tel1 is important for timely meiotic DSB repair I assayed the meiosis specific Spo11 independent VDE-DSB reporter cassette (Neale et al., 2002; Fig 3.1). The cassette consists of a VDE cutsite in *ARG4* creating an *arg4-vde* allele that is inserted into *ura3::Ty*. The meiotic break is catalysed by the VMA derived endonuclease, the VDE intein expressed from the TFP1 locus (Fukuda et al., 2003). A template for interhomologue recombination repair of the VDE-DSB is an *arg4-bgl* allele that is inserted at the *ura3::Ty* in the homologous chromosome. Repair of the VDE-DSB by recombination can result in gene conversion forming an *ARG4* or *arg4-bgl* co-converted product. An alternative repair product is created by SSA repair using the flanking *URA3* repeat sequences created by the integrations at the *ura3::Ty* locus. Repair by SSA deletes the sequence between the flanking repeats producing either a *ura3::Ty* or *URA3* repair product. Although SSA can occur at the VDE-DSB Spo11-DSBs do not repair by SSA.

Any differences in the VDE-DSB steady state in *tel1Δ* cells and wild type cells could be caused by differences in the efficiency of cleavage by VDE in the two strains. To assay the rate of cleavage by VDE Southern analysis was used; the assay allows visualisation of unique restriction pattern of each homologue and a loading control (Fig 3.2). The amount of DNA in *arg4-vde* parental band decreases as VDE cleaves the recognition site. Consequently the band disappears as the timecourse progresses. The rate of VDE cleavage can be calculated by normalising the amount of DNA in the *arg4-vde* parental band to the amount of DNA in the loading control. The rate of VDE cleavage in the *tel1Δ* cells is the same as wild type cells (Fig 3.3).

Tel1 dependent phosphorylation of MRX has been shown to occur in both *sae2Δ* and *rad50S* cells after induction of DNA damage (Usui et al., 2001). Consequently Tel1 is implicated in starting a signalling cascade in response to DNA damage (Mantiero et al., 2007; Usui et al., 2001). If Tel1 is involved in sensing and signalling the presence of DNA damage the repair of the VDE-DSB

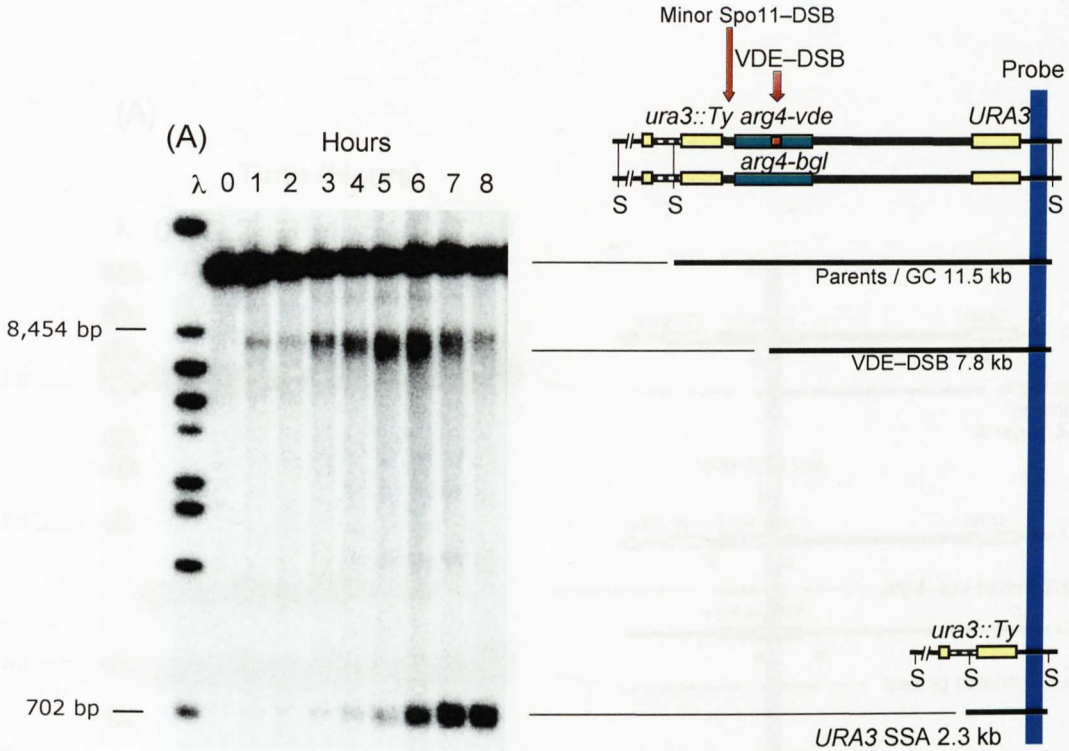


Figure 3.1 Diagram of the the VDE-DSB reporter cassette.

Cells with the VDE reporter cassette have an *arg4-vde* or an *arg4-bgl* allele inserted in to a *ura3::Ty* locus on both chromosomes. The VDE-DSB is created during meiosis at the recognition site in the *arg4-vde* allele. The break can be repaired by recombination using sequence homology from the unbroken *arg4-bgl* allele on the homologue. Recombination repair generates an *ARG4* product or an *arg4-bgl* repair product. Repair can also occur via SSA resulting in a *ura::Ty* product or a *URA3* repair product. After DNA digestion with *Spe*I, fractionation on 0.5 % gel followed by southern blotting and probing the parental fragments and gene conversion products are visible as an 11.5 kb band, the VDE-DSB is identified by a 7.8 kb band and the SSA *URA3* product forms a 2.3 kb band.

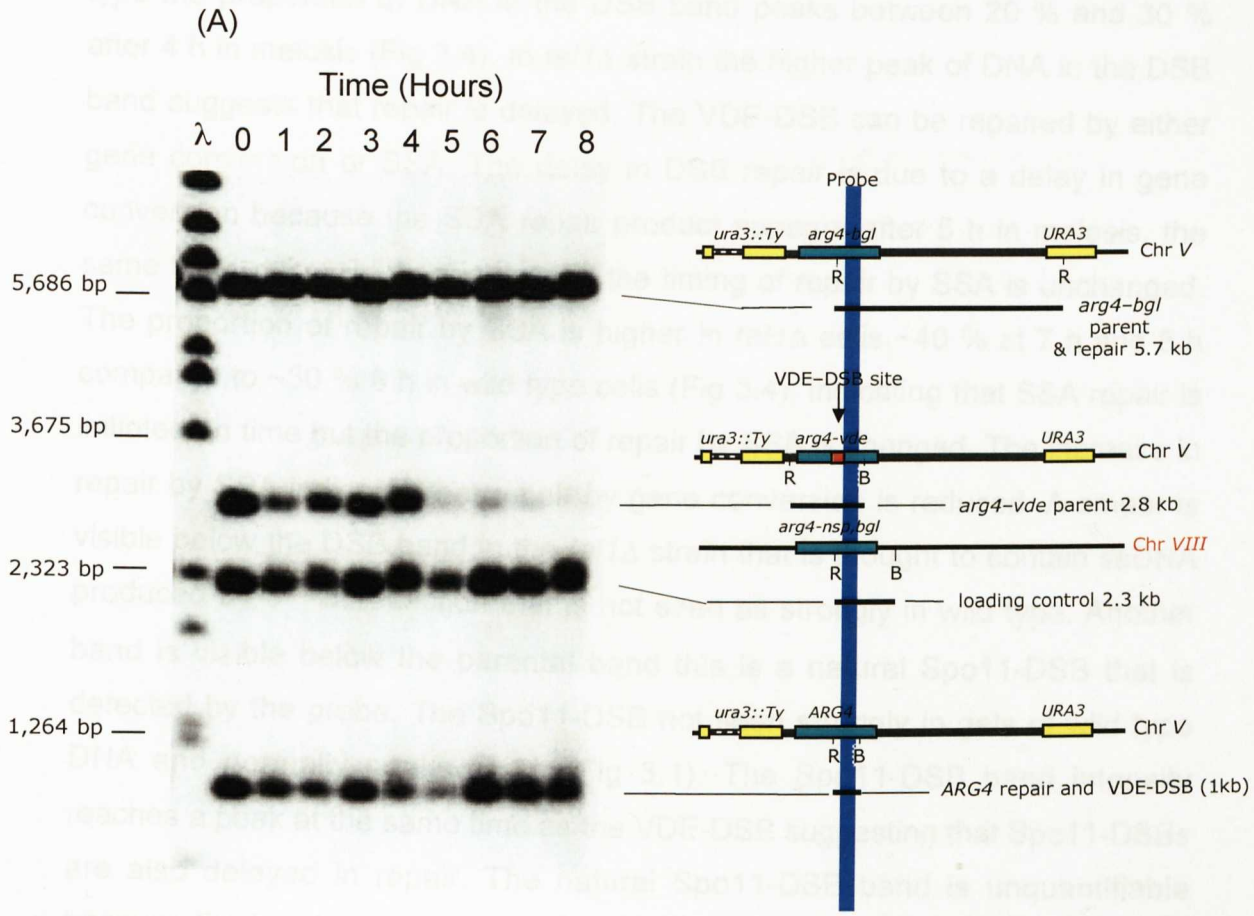


Figure 3.2 Proportion of uncut *arg4-vde* chromatids.

(A) Cells were removed from sporulation medium at hourly intervals and the DNA extracted double digested with *Eco*RV and *Bgl*II. The DNA was was fractionated on a 0.5% gel for 680 mins, southern blotted and probed downstream of the *Eco*R*V*. The digest releases a 2.8 kb *arg4-vde* parental fragment, during the timecourse the percentage of DNA in *arg4-vde* fragment decreases as VDE cleaves the recognition site. The digest also releases a 2.3 kb loading control that contains the *arg4-nsp,bgl* allele at the *ARG4* on chr *VIII*. The DNA is normalised to the loading control then the normalisation is multiplied by 100 and not doubled because only one homologue has the *arg4-nsp,bgl* allele the other carries *arg4-VDE*.

is expected to be slower or faster in a *tel1*Δ strain compared with wild type strain. When the VDE-DSB was assayed in the *tel1*Δ strain the percentage of DNA in the DSB band reached a maximum of 50 % after 5 h in meiosis. In wild type the proportion of DNA in the DSB band peaks between 20 % and 30 % after 4 h in meiosis (Fig 3.4). In *tel1*Δ strain the higher peak of DNA in the DSB band suggests that repair is delayed. The VDE-DSB can be repaired by either gene conversion or SSA. The delay in DSB repair is due to a delay in gene conversion because the SSA repair product appears after 5 h in meiosis, the same time as in wild type indicating the timing of repair by SSA is unchanged. The proportion of repair by SSA is higher in *tel1*Δ cells ~40 % at 7 h and 8 h compared to ~30 % 8 h in wild type cells (Fig 3.4), indicating that SSA repair is initiated on time but the proportion of repair by SSA is changed. The increase in repair by SSA indicates that repair by gene conversion is reduced. A smear is visible below the DSB band in the *tel1*Δ strain that is thought to contain ssDNA produced by 5′ - 3′ resection that is not seen as strongly in wild type. Another band is visible below the parental band this is a natural Spo11-DSB that is detected by the probe. The Spo11-DSB not seen strongly in gels of wild type DNA and normally peaks at 5h (Fig 3.1). The Spo11-DSB band intensity reaches a peak at the same time as the VDE-DSB suggesting that Spo11-DSBs are also delayed in repair. The natural Spo11-DSB band is unquantifiable because the band is too close to the parental band to be accurately quantified (Fig 3.4).

The late VDE-DSB repair in *tel1*Δ cells is not dependent on Spo11-DSBs

If Tel1 initiates a signalling cascade required for timely repair in response to blocked Spo11-DSBs, the delayed VDE-DSB repair seen in *tel1*Δ cells should be dependent upon the presence of Spo11-DSBs. To see if the late VDE-DSB repair seen *tel1*Δ cells is dependent upon Spo11-DSBs a *tel1*Δ *spo11-Y135F* strain was created. In the *spo11-Y135F* mutant a point mutation changes a tyrosine to lysine preventing Spo11-DSB formation, but allowing other roles of Spo11 to be fulfilled. In *spo11-Y135F* cells the VDE-DSB is repaired faster and a higher proportion is repaired by SSA comparison to wild type cells

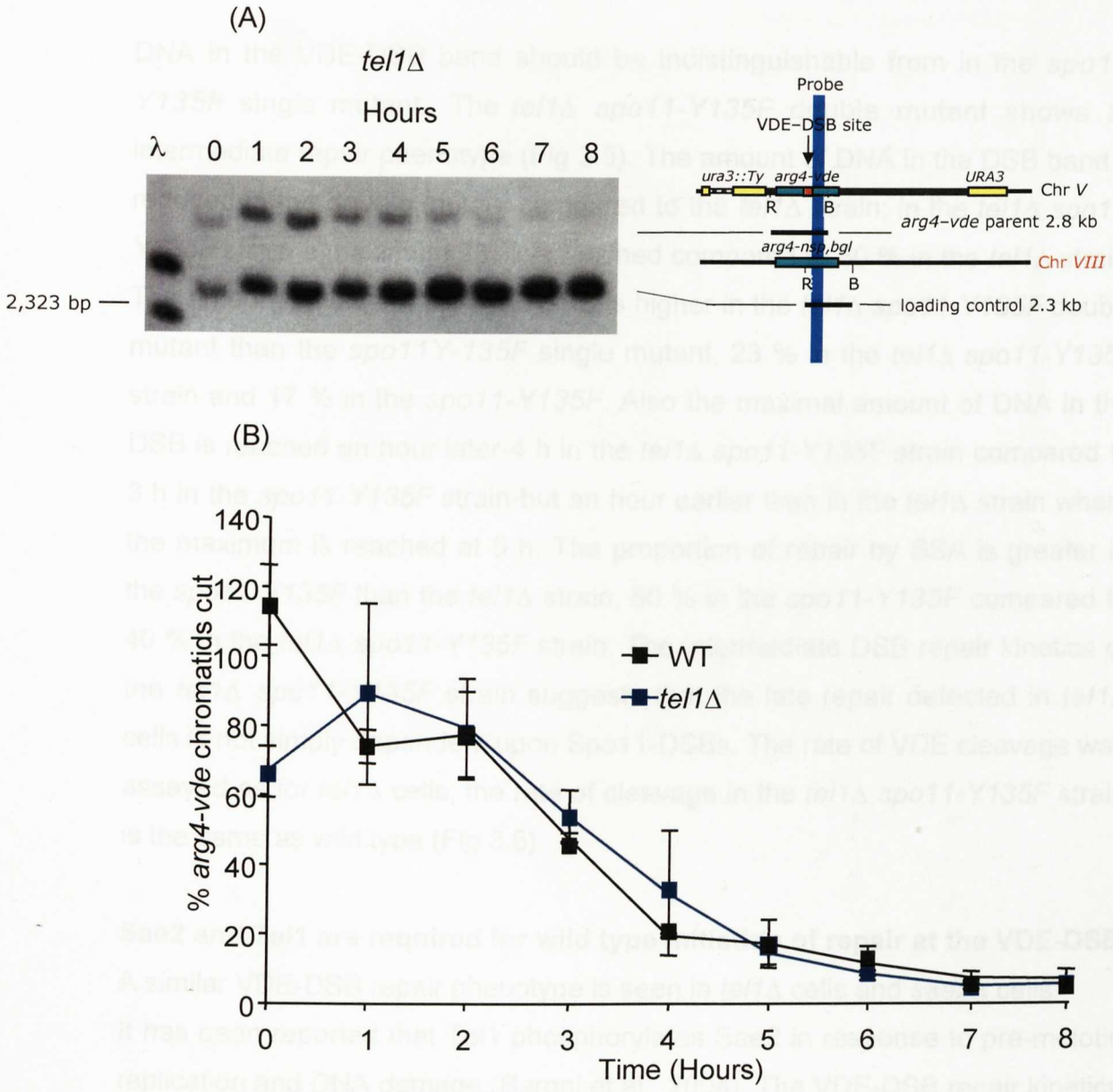


Figure 3.3 Proportion of uncut arg4-VDE chromatids.

(A) *tel1Δ* (dAG744) cells were removed at hourly intervals from meiotic sporulation media, the DNA was extracted then processed as described in Fig 3.3 A) Quantification of the *rad6Δ* blot in (A) and of the wild type blot in Fig 3.3. The rate of VDE cleavage is indistinguishable from wild type in the *tel1Δ* strain.

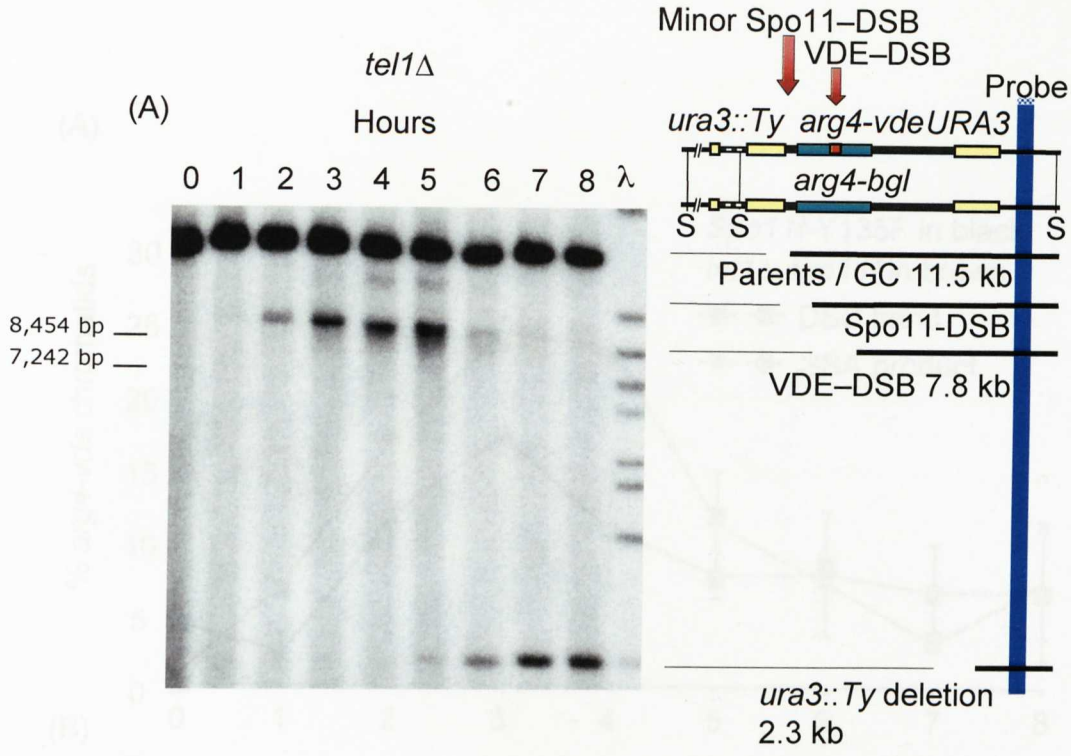
(Neale et al., 2002). If the late repair of VDE-DSB in *tel1Δ* cells is dependent on the presence of Spo11-DSBs, then in a *tel1Δ spo11-Y135F* strain the amount of

DNA in the VDE-DSB band should be indistinguishable from in the *spo11-Y135F* single mutant. The *tel1Δ spo11-Y135F* double mutant shows an intermediate repair phenotype (Fig 3.5). The amount of DNA in the DSB band is reduced in the double mutant compared to the *tel1Δ* strain; in the *tel1Δ spo11-Y135F* strain a maximum 23 % is reached compared to 50 % in the *tel1Δ* strain. The amount of DNA in the VDE-DSB is higher in the *tel1Δ spo11-Y135F* double mutant than the *spo11Y-135F* single mutant, 23 % in the *tel1Δ spo11-Y135F* strain and 17 % in the *spo11-Y135F*. Also the maximal amount of DNA in the DSB is reached an hour later-4 h in the *tel1Δ spo11-Y135F* strain compared to 3 h in the *spo11-Y135F* strain-but an hour earlier than in the *tel1Δ* strain where the maximum is reached at 5 h. The proportion of repair by SSA is greater in the *spo11-Y135F* than the *tel1Δ* strain, 60 % in the *spo11-Y135F* compared to 40 % in the *tel1Δ spo11-Y135F* strain. The intermediate DSB repair kinetics of the *tel1Δ spo11-Y135F* strain suggests that the late repair detected in *tel1Δ* cells is not simply dependent upon Spo11-DSBs. The rate of VDE cleavage was assayed as for *tel1Δ* cells; the rate of cleavage in the *tel1Δ spo11-Y135F* strain is the same as wild type (Fig 3.6).

Sae2 and Tel1 are required for wild type initiation of repair at the VDE-DSB

A similar VDE-DSB repair phenotype is seen in *tel1Δ* cells and *sae2Δ* cells.

It has been reported that Tel1 phosphorylates Sae2 in response to pre-meiotic replication and DNA damage (Baroni et al., 2004). The VDE-DSB repair kinetics in the *sae2Δ* strain resemble a *tel1Δ* strain; in both mutants the amount of DNA in the VDE-DSB reaches a maximum at 5 h (Fig 3.7). In the *tel1Δ* strain the DSB reaches a maximum of 50 %, which is similar, the maximum of 44 % reached in the *sae2Δ* strain. The proportion of repair by SSA is different between the two strains, in *tel1Δ* cells 40 % of the DNA is in the SSA repair product band at 8 h. Conversely ~30 % of the DNA is in the SSA product band at 8 h in *sae2Δ* cells (Fig 3.7). Therefore phosphorylation of Sae2 by Tel1 might ensure correct timing of DSB repair in meiosis.



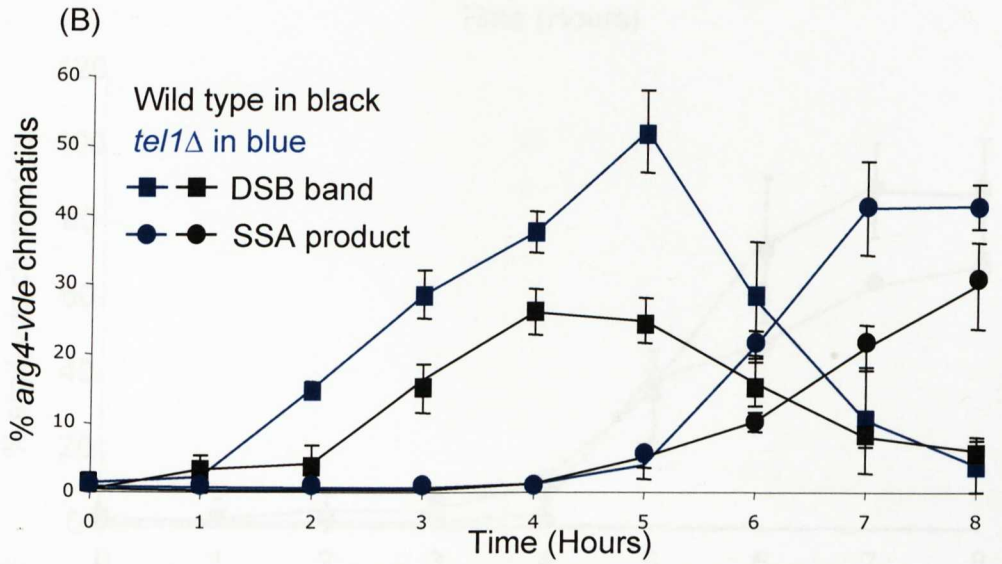
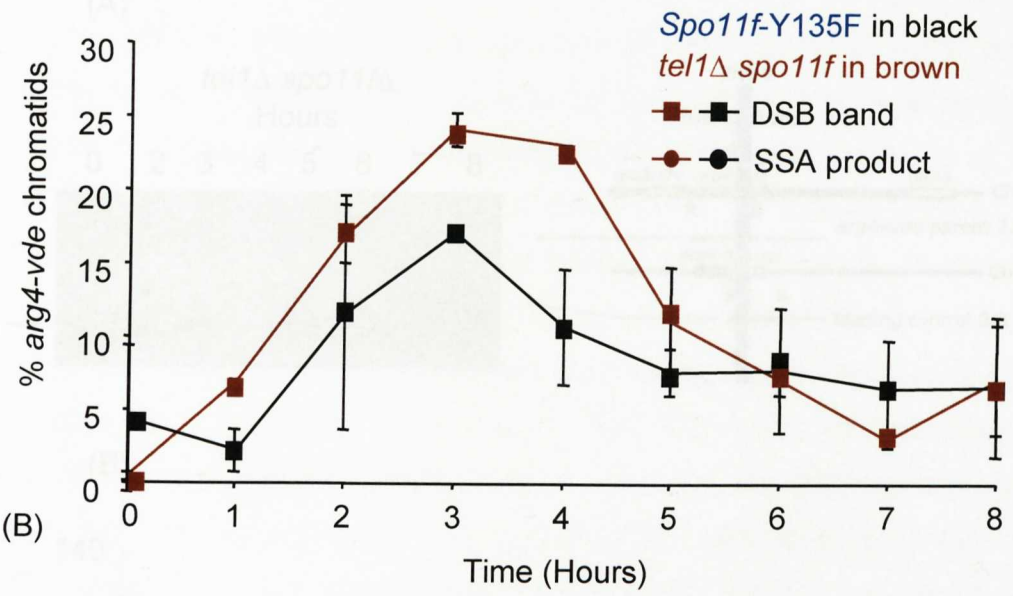


Figure 3.4 In the absence of Tel1, VDE-DSB repair is delayed.

(A) DNA was extracted from meiotic cultures of *tel1Δ* and processed as described in Fig 3.1 DNA extracted from *tel1Δ* cells shows a smeared VDE-DSB band which can also be seen in wild type but is less pronounced. The smear contains DSB intermediates that do not normally accumulate. The band visible between the VDE-DSB band and the parental band is a natural Spo11-DSB detected by the probe. (B) Quantification of gel in (A). Each band was quantified and expressed as a proportion of total DNA in that lane. The error bars show the standard error of at least two Southern gels. In the *tel1Δ* strain the VDE-DSB reaches a maximum of 50 % at 5 h compared to 25 % at 4 h seen in the wild type strain suggesting the VDE-DSB accumulates. Therefore VDE-DSB repair is slower in the *srs2-101/srs2-101* strain compared to wild type cells. The amount of DNA in the deletion band is lower than published in Johnson, Borde et al, 2007.

(A)



(B)

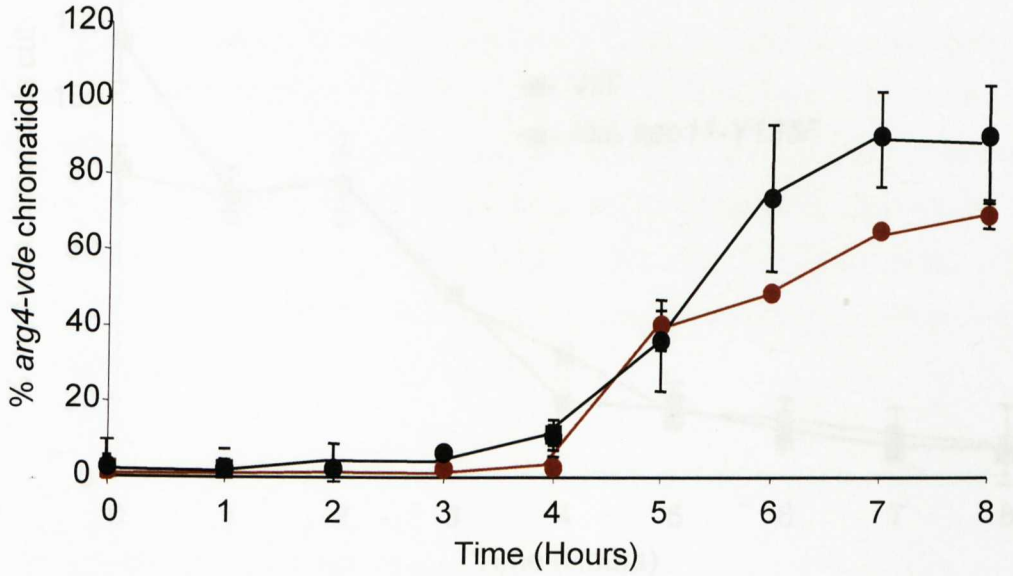


Figure 3.5 The late DSB repair seen in *tel1Δ* cells is not dependent upon the presence of Spo11-DSBs.

DNA was extracted from meiotic cultures of *tel1Δspo11f* digested, fractionated then southern blotted as in Fig 3. 2. (A) In the *tel1Δ spo11-Y135F* strain a higher percent of DNA is in the VDE-DSB band compared to the *spo11-Y135F* mutant; also the percent of DNA in the VDE-DSB band peaks later in the double mutant (4 h) than the *spo11-Y135F* (3 h). After 5 h the amount of DNA in the VDE-DSB appears to be the same in both the *tel1Δ and the* spo11-Y135F. (B) The amount of repair by SSA in *tel1Δ spo11-Y135F* appears to be very similar in both strains.

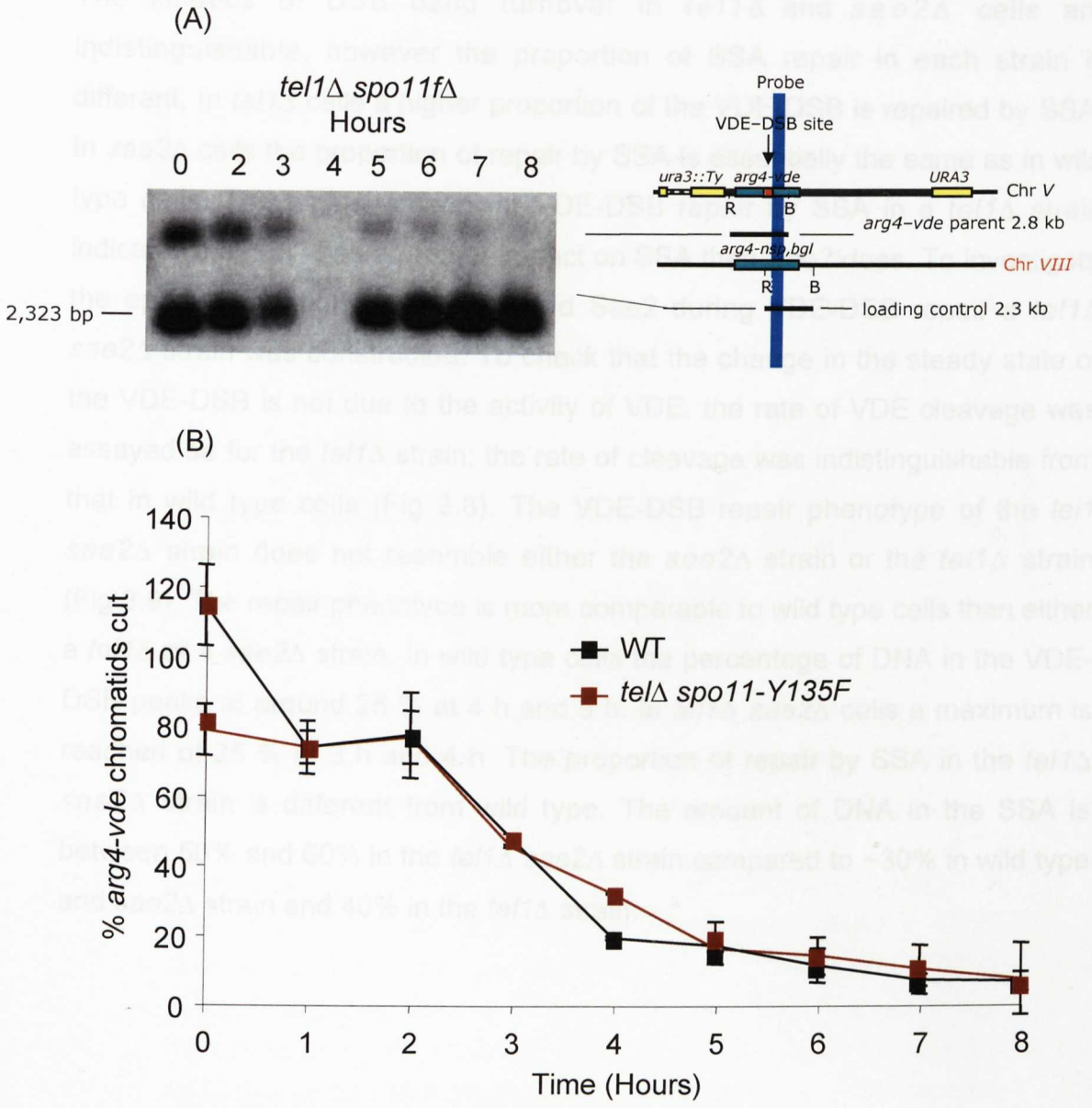


Figure 3.6 Proportion of uncut arg4-VDE chromatids in the *tel1Δ spo11Y135F*.

(A) DNA was extracted from *tel1Δ spo11-Y135F* cells then processed as described in Fig 3.3 (B) Quantification of the blot in (A) The rate of VDE cleavage is similar to wild type in the *tel1Δ spo11-Y135F* strain.

VDE-DSB turnover similar to wild type in a *tel1*Δ*sae2*Δ strain.

The kinetics of DSB band turnover in *tel1*Δ and *sae2*Δ cells are indistinguishable, however the proportion of SSA repair in each strain is different. In *tel1*Δ cells a higher proportion of the VDE-DSB is repaired by SSA. In *sae2*Δ cells the proportion of repair by SSA is essentially the same as in wild type cells. The higher amount of VDE-DSB repair by SSA in a *tel1*Δ strain indicates that Tel1 has a greater impact on SSA then Sae2 does. To investigate the epistatic relationship of Tel1 and Sae2 during VDE-DSB repair a *tel1*Δ *sae2*Δ strain was constructed. To check that the change in the steady state of the VDE-DSB is not due to the activity of VDE, the rate of VDE cleavage was assayed as for the *tel1*Δ strain; the rate of cleavage was indistinguishable from that in wild type cells (Fig 3.8). The VDE-DSB repair phenotype of the *tel1* *sae2*Δ strain does not resemble either the *sae2*Δ strain or the *tel1*Δ strain (Fig 3.9). The repair phenotype is more comparable to wild type cells than either a *tel1*Δ or a *sae2*Δ strain. In wild type cells the percentage of DNA in the VDE-DSB peaks at around 25 % at 4 h and 5 h. In *tel1*Δ *sae2*Δ cells a maximum is reached of 25 % at 3 h and 4 h. The proportion of repair by SSA in the *tel1*Δ *sae2*Δ strain is different from wild type. The amount of DNA in the SSA is between 50% and 60% in the *tel1*Δ *sae2*Δ strain compared to ~30% in wild type and *sae2*Δ strain and 40% in the *tel1*Δ strain.

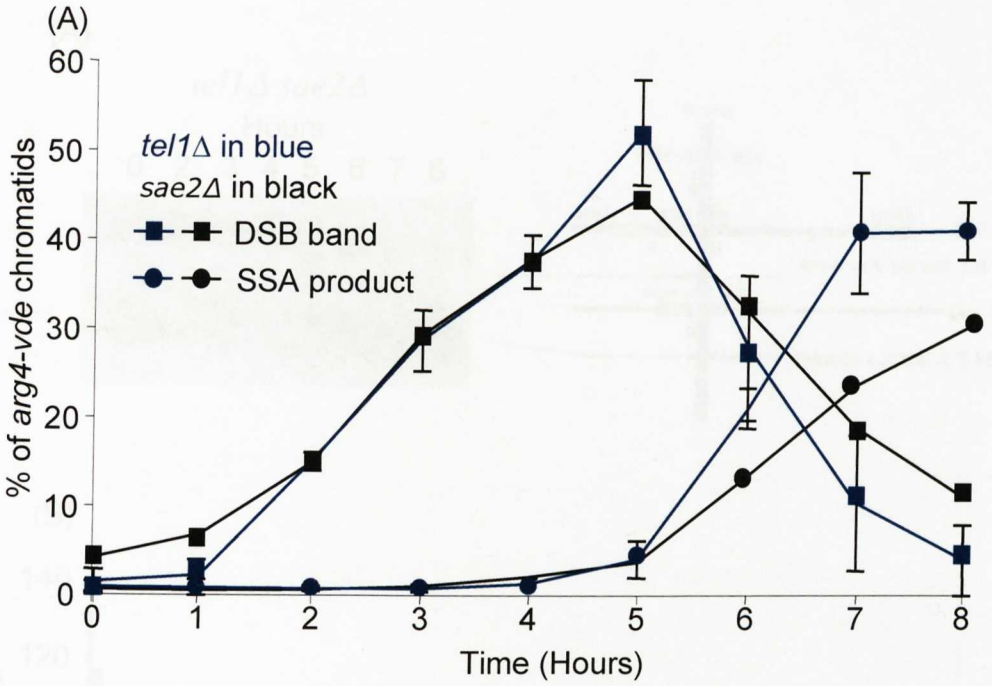


Figure 3.7 VDE-DSB repair is delayed in *sae2*Δ cells and *tel1*Δ cells. The *sae2*Δ cells and DNA were processed by Miss R Johnson.

(A) Comparison of VDE-DSB repair in *sae*Δ *and tel1*Δ cells. In *tel*Δ mutant the VDE-DSB reaches a maximum between 40 % and 50 % at 5 h as does the amount on DNA in the DSB In *sae2*Δ cells. The kinetics of the VDE-DBS band suggests that the timing of repair is the same in both the *sae2*Δ strain and the *tel1*Δ strain. The proportion of VDE-DSB repair by SSA appears to be different in the strains. In *tel1*Δ cells 40 % of the DNA is in the SSA repair product band at 8 h however, in the *sae2*Δ strain 30 % of the DNA is in the band at 8 h.

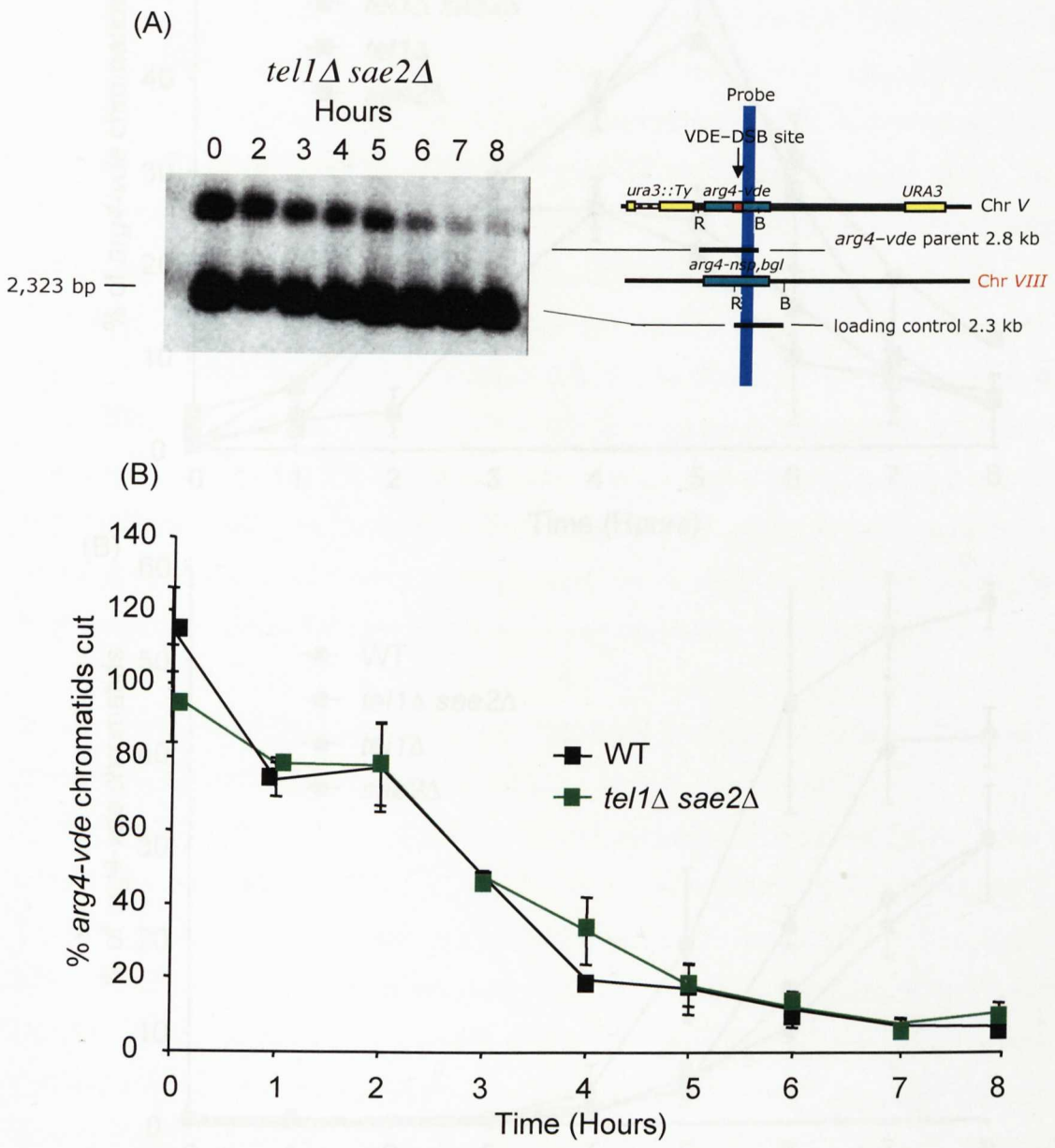


Figure 3.8 Proportion of uncut *arg4-vde* chromatids.

(A) Cells from a *tel1Δ sae2Δ* sporulation culture were removed at hourly intervals and the DNA extracted then processed as described in Fig 3.3 (B) Quantification of the blot in (A) The rate of VDE cleavage is indistinguishable from wild type cells in the *tel1Δ sae2Δ* strain.

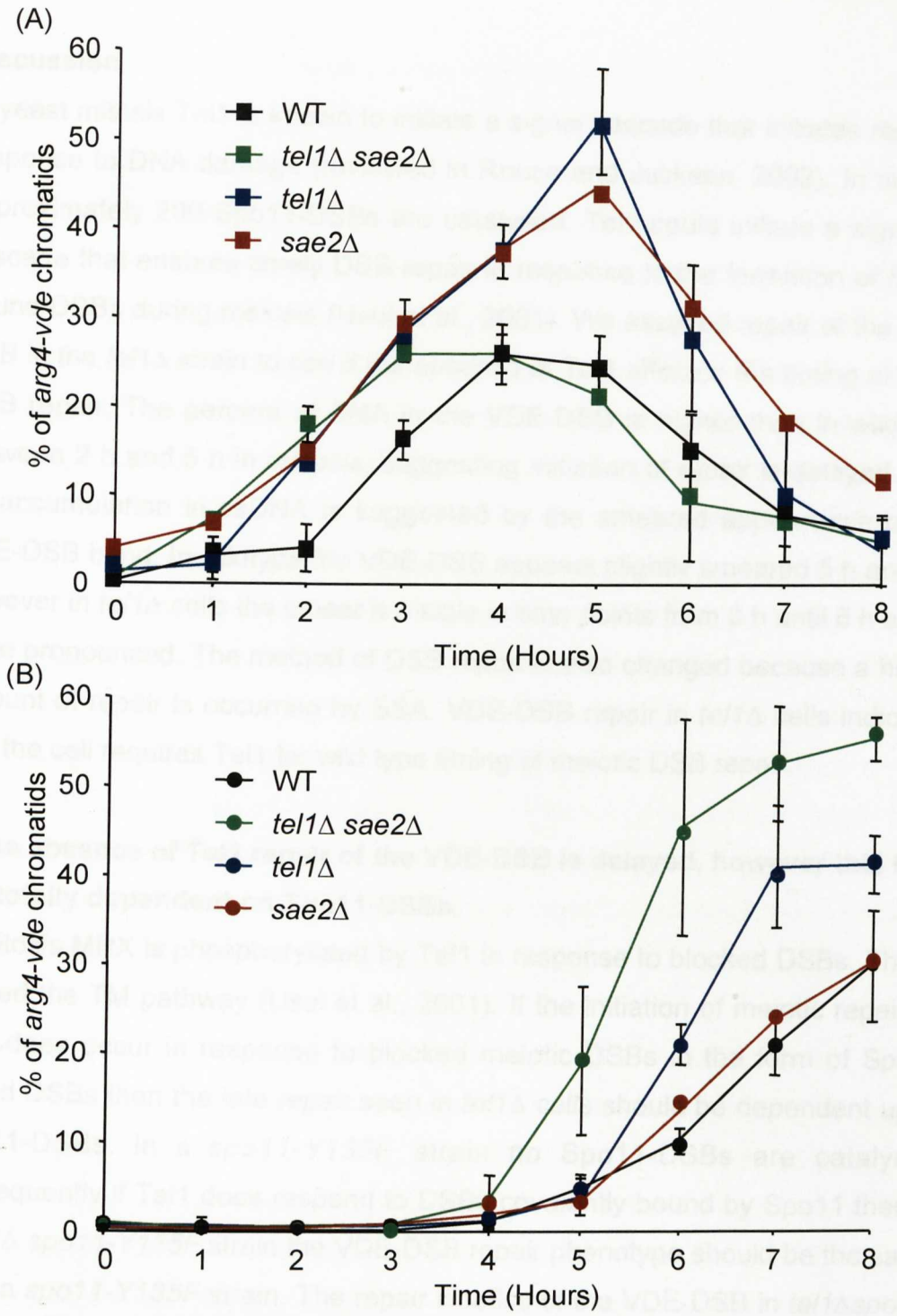


Figure 3.9 VDE-DSB repairs by high proportion of SSA in *tel1Δ sae2Δ* cells but repair is not delayed.

(A) The VDE-DSB band in *tel1Δ sae2Δ*, *tel1Δ*, *sae2Δ* and wild type cells. The percent of DNA in the DSB band peaks at 25 % in both wild type and *tel1Δ sae2Δ*. However, the percent of DNA in the VDE-DSB reaches a peak earlier in the *tel1Δ sae2Δ* strain (between 3 h and 4 h) compared to wild type (4 h). (B) The percent of DNA in the SSA repair product band in *tel1Δ*, *sae2Δ*, *tel1Δ sae2Δ* and wild type cells. The amount of DNA in the SSA repair product band is much higher in the *tel1Δ sae2Δ* 52 % than wild type, *sae2Δ*, or *tel1Δ* cells.

Discussion

In yeast mitosis Tel1 is known to initiate a signal cascade that initiates repair in response to DNA damage (reviewed in Rouse and Jackson, 2002). In meiosis approximately 200 Spo11-DSBs are catalysed. Tel1 could initiate a signalling cascade that ensures timely DSB repair in response to the formation of Spo11 bound DSBs during meiosis (Usui et al., 2001). We assayed repair at the VDE-DSB in the *tel1Δ* strain to see if the absence of Tel1 affected the timing of VDE-DSB repair. The percent of DNA in the VDE-DSB is higher than in wild type between 2 h and 5 h in meiosis, suggesting initiation of repair is delayed. Also an accumulation in ssDNA is suggested by the smeared appearance of the VDE-DSB band. In wildtype the VDE-DSB appears slightly smeared 5 h and 6 h however in *tel1Δ* cells the smear is visible in time points from 3 h until 6 h and is more pronounced. The method of DSB repair is also changed because a higher amount of repair is occurring by SSA. VDE-DSB repair in *tel1Δ* cells indicated that the cell requires Tel1 for wild type timing of meiotic DSB repair.

In the absence of Tel1 repair of the VDE-DSB is delayed, however this is not totally dependent on Spo11-DSBs.

In mitosis MRX is phosphorylated by Tel1 in response to blocked DSBs. This is termed the TM pathway (Usui et al., 2001). If the initiation of meiotic repair by Tel1 does occur in response to blocked meiotic DSBs in the form of Spo11 bound DSBs then the late repair seen in *tel1Δ* cells should be dependent upon Spo11-DSBs. In a *spo11-Y135F* strain no Spo11-DSBs are catalysed consequently if Tel1 does respond to DSBs covalently bound by Spo11 then in a *tel1Δ spo11-Y135F* strain the VDE-DSB repair phenotype should be the same as in a *spo11-Y135F* strain. The repair kinetics of the VDE-DSB in *tel1Δspo11-Y135F* cells does not resemble either a *tel1Δ* or a *spo11-Y135F* strain. The *tel1Δ spo11-Y135F* strain has an intermediate VDE-DSB repair phenotype; that is not expected if the late VDE-DSB repair in *tel1Δ* cells is a result of Tel1 signalling repair in response of blocked DSBs.

In the absence of Sae2 repair of the VDE-DSB is also delayed

Another protein that is reported to be phosphorylated by Tel1 is Sae2, which is a small protein with no known homologues or obvious motifs (Cartagena-Lirola et al., 2006). In *sae2Δ* cells Spo11 remains bound to the DSBs therefore the DSBs are not repaired (Keeney, Giroux et al. 1997). In mitosis *sae2Δ* cells show reduced or absent SSA-dependent recombination at the HO break and a slight delay in resection (Baroni et al., 2004; Clerici et al., 2005; Clerici et al., 2006). Also ctp1, a possible Sae2 ortholog in *S.pombe*, is involved in MRN dependent DSB processing of DSBs (Takeda, Nakamura et al. 2007; Akamatsu, Murayama et al. 2008). Sae2 is phosphorylated by Mec1 and Tel1 during the initiation of premeiotic DNA replication, and is dephosphorylated when Spo11-DSBs are completely repaired (Baroni et al., 2004). Replication is completely independent of Spo11-DSBs. Therefore the late VDE-DSB repair seen in *tel1Δ* cells might be due to a lack of Tel1 dependent Sae2 phosphorylation. The repair kinetics of the VDE-DSB in a *tel1Δ* and a *sae2Δ* strain are similar. Both *tel1Δ* and *sae2Δ* cells show a delay in the initiation of VDE-DSB repair. If the late repair seen *tel1Δ* cells is a result of Tel1 dependent phosphorylation of Sae2 then repair of the double mutant was expected to resemble VDE-DSB repair in *sae2Δ* and *tel1Δ* cells.

Both Tel1 and Sae2 are required for regulation of VDE-DSB repair

Repair of the VDE-DSB in the *tel1Δsae2Δ* strain is remarkably similar to wild type although repair of the VDE-DSB is quicker in the *telΔ1 sae2Δ* strain. This is probably because the proportion of repair by SSA is increased which is faster than gene conversion (Neale et al., 2002). The increased proportion of VDE-DSB repair by SSA indicates that the amount of repair by gene conversion is down, suggesting that the amount of strand invasion is reduced therefore the break has to repair by SSA. An alternative explanation for the increased proportion of SSA is that resection is increased. For the VDE-DSB to repair by SSA repair the flanking *URA3* and *ura3::ty* repeats have to uncovered, which requires more resection than gene conversion (Fig 3.1; Neale et al., 2002).

In both *tel1Δ* cells and *sae2Δ* cells VDE-DSB repair is delayed, however in the *tel1Δ sae2Δ* repair of the VDE-DSB is not delayed. One explanation of this is that Sae2 phosphorylation by Tel1 is required for timely repair. This is consistent with the recent finding that Sae2 has an endonucease activity, because Tel1 phosophorylation of Sae2 might be required for Sae2 endonuclease activilty at the break. However an increase in SSA is only seen in cells that lack Tel1 therefore Tel1 might be required for directing repair of the VDE-DSB towards gene conversion. In *tel1Δ* cells repair by SSA is increased however in *sae2Δ* cells the proportion of repair by SSA is unchanged. In *tel1ΔsaeΔ* cells the amount of repair by SSA is also increased. Therefore the data suggests that Tel1 functions independently of Sae2.

In *sae2Δ* all the Spo11-DSBs are blocked. Consequently, all the resection machinery is available to repair the VDE-DSB and therefore the VDE-DSB would be expected to repair more efficiently (Johnson, Borde et al. 2007). However in *sae2Δ* cells repair of the VDE-DSB is slower than wild type. One possibility is that although there is an excess of repair machinery, repair of the break is tightly controlled. In *tel1Δ* the VDE-DSB is repaired slower then wild type and the proportion of repair by SSA is increased suggesting that repair at the VDE-DSB is less regulated.

In an *sae2Δ tel1Δ* cell repair of the VDE-DSB will be less regulated because Tel1 is absent therefore a higher proportion of the break will be repaired by SSA which is a quick repair pathway. However in an *sae2Δ tel1Δ* cell there is also an excess of DSB repair machinery available because Spo11-DSBs are blocked. Therefore the break can be repaired faster then wild type because the SSA pathway is favoured and there is an excess of repair protein available to the break. This suggests that Tel1 functions independently of Sae2 during repair of VDE-DSB.

Chapter Four - *SRS2* is required for meiotic DSB repair

Brief introduction

Recombination is used by yeast cells during vegetative growth to repair DNA damage, and during meiosis to generate genetic diversity and ensure faithful homologue segregation (reviewed in Zickler and Kleckner, 1998). Uncontrolled or excessive recombination can be harmful to the cells increasing the risk of loss of homozygosity and chromosomal re-arrangements (Foiani, 2003; Le Breton et al., 2008). This chapter investigates the role of one potential regulator of recombination in meiosis Srs2, originally named Hpr5. Srs2 was shown to have a 3′–5′ DNA helicase activity exhibited by Helicase II family members when incubated with linear DNA with duplexed ends of different lengths.

Helicases are required for unwinding DNA and RNA duplexes. They function during DNA replication, repair, transcription, RNA splicing and protein displacement (Lee et al., 1999; Pyle, 2008; Sung and Klein, 2006). Various human disorders are associated with helicase mutants including Bloom syndrome, which is caused by hyper-recombination due to a mutation of BLM helicase (German, 1993). Bloom syndrome is typified by genomic rearrangements and chromosome instability causing premature ageing and a predisposition to cancer (German, 1993; Karow et al., 1997). In men mutations in Bloom syndrome also causes sterility indicating BLM has a role during human meiosis.

There are several potential roles for helicases during meiotic recombination. Possible functions include unwinding the broken duplex to allow recombination machinery access to the DNA. Helicase action is also required for dismantling unwanted recombination intermediates that accumulate in the absence of Sgs1 although the exact mechanism is unknown (Jessop and Lichten 2008; Oh, Lao et al. 2008). Srs2 belongs to the SF-1 superfamily that share ATPase activity, which has been suggested to allow the proteins to translocate on ssDNA (reviewed in Foiani, 2003; Tuteja and Tuteja, 2004). Srs2 is known to negatively regulate recombination in mitosis (Chanet et al., 1996; Milne et al., 1995). The protein exhibits homology to the bacterial UvrD and Rep helicases, and is the

yeast orthologue of the human Hfbh1 protein (Chiolo et al., 2005). Srs2 is a good candidate for a helicase of importance in meiotic recombination, because *srs2* mutant cells exhibit reduced viability, a delayed commitment to meiosis and a significant reduction in both sporulation and spore viability which could be indicative of pre-meiotic S phase problems. Srs2 also interacts with Sgs1, a helicase that is known to function in meiosis (Chiolo et al., 2005; Palladino and Klein, 1992). Helicase action separates two strands of duplexed DNA an event likely to occur during resection of DNA near a DSB therefore Srs2 is possibly involved in resection of meiotic DSBs. There is evidence for helicases having a role in resection. Sgs1/BLM has been implicated in MRN-independent long resection with Exo1 at the HO break which is required for efficient HR repair (Gravel, Chapman et al. 2008; Mimitou and Symington 2008; Zhu, Chung et al. 2008).

In this chapter analyses of meiosis have been undertaken in cells homozygous and heterozygous for the *srs2-101* allele, which is reported to have no helicase function. The mutant allele has a point mutation in the highly conserved ATP binding domain that changes proline 37 to leucine, rendering the protein helicase null by preventing ATP hydrolysis (Rong et al., 1991). The advantage of using this mutant is that any phenotype detected can be attributed to the ATP dependent helicase activity of Srs2.

Results

Spore viability and meiotic progression in *srs2-101* diploids

It has previously been reported that the sporulation efficiency of *srs2-101* homozygous diploids is 75 % of wild type sporulation efficiency. Spore viability is reduced by 60 % when *srs2-101* cells undergo meiosis, and only 16 % of mature tetrads give four viable spores (Palladino and Klein, 1992). The meiotic progression of *srs2-101/srs2-101* diploids is also affected, cells are delayed in entering both the nuclear divisions (Palladino and Klein, 1992). The published studies on *SRS2* mutants in meiosis were undertaken using BR strains. To characterise repair of the VDE-DSB in *srs2-101/srs2-101* diploids in the SK1 background, an *srs2-101/srs2-101* (dAG1493) diploid containing the *arg4-vde* reporter cassette and expressing the VDE endonuclease, was constructed. Following growth on solid medium and transfer to meiosis inducing medium, cells were monitored by light microscopy and no mature tetrads were visible. To establish at which stage of sporulation dAG1493 arrested, cells were DAPI stained to monitor nuclear divisions. In *srs2-101/srs2-101* (dAG1493) the absence of a functional Srs2 results in 50 % of the culture arresting with one DAPI stained body *i.e* before the first meiotic division (Fig 4.1). The remaining 50 % successfully completed both the first division and second divisions (Fig 4.1). This was seen in the majority of *srs2-101/srs2-101* independent transformants with an identical genotype to dAG1493 (Table 4.1). Another *srs2-101/srs2-101* SK1 diploid (dAG1521) was assayed that does not express VDE or contain the *arg4-vde* allele. For dAG1521, after 8 h in liquid meiosis inducing medium 70 % of cells still had only one DAPI stained body indicating that the first meiotic division was delayed (Fig 4.2). After 24 h in meiotic conditions between 80 % and 90 % of cells had four DAPI stained bodies and the strain formed mature tetrads. When dissected dAG1521 spores have reduced viability, 19 % of tetrads formed no viable spores, 10 % formed one viable spore, 20 % formed two viable spores, 22 % formed three viable spores and 29 % of the tetrads were four spore viable, compared with 93 % in wild type (dAG1522; also lacking the VDE system; Fig 4.3). This suggests that the strains expressing VDE have reduced sporulation. This has also been observed in other lab strains with the reporter cassette (unpublished results).

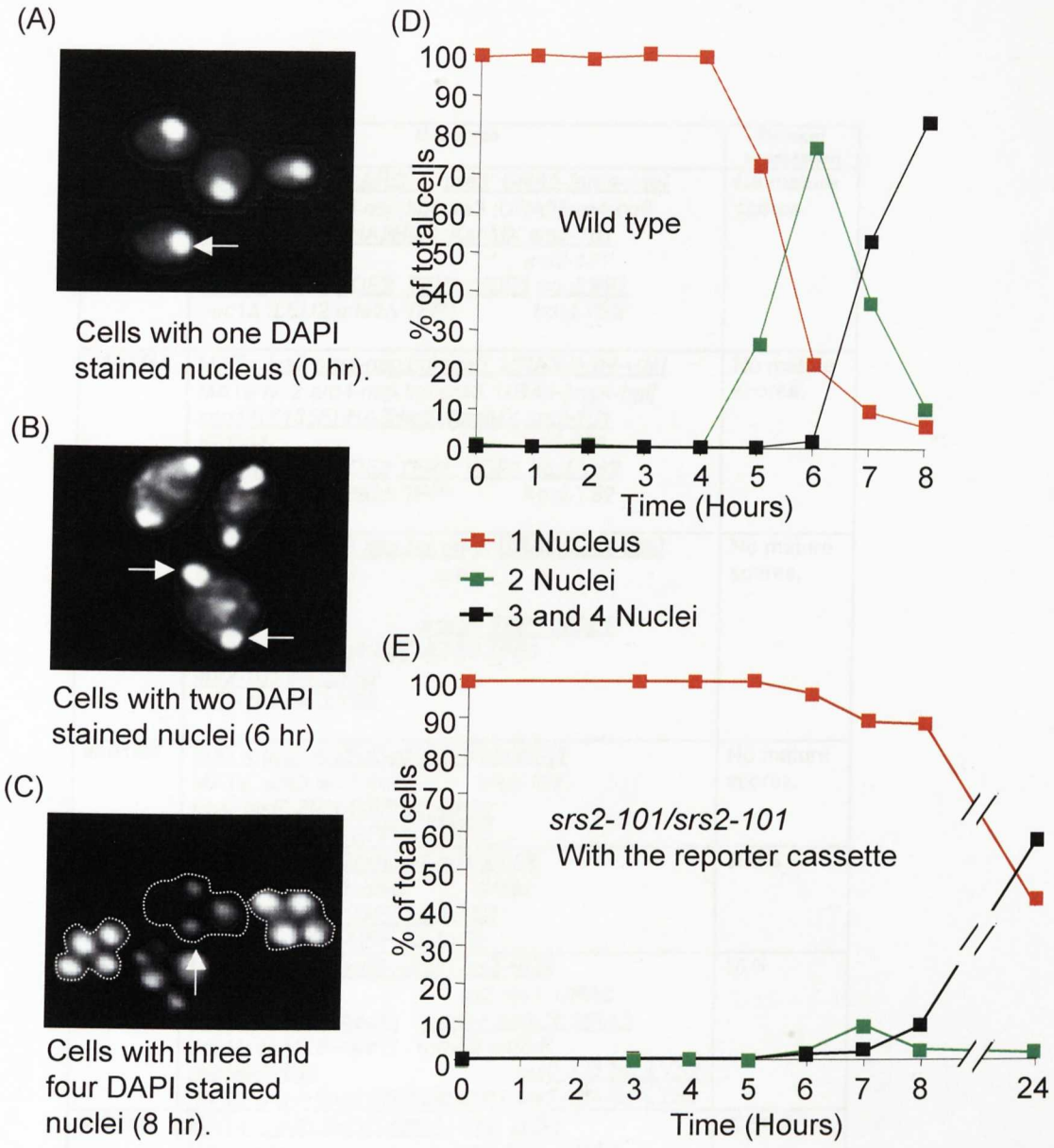


Figure 4.1 Meiotic progression is delayed in *srs2-101/srs2-101* cells with the VDE reporter cassette.

Meiotic progression was determined by scoring nuclear divisions. Cells were harvested and fixed in ethanol, stained with DAPI and visualised using fluorescence microscopy. 200 cells from each timepoint were scored for the number of DAPI–staining bodies (nuclei). Cells containing two nuclei indicate completion of meiosis I. Cells with three or four nuclei indicate the cumulative total of cells that have passed meiosis I. (A-C) Examples of wild type cells at timepoint 0 h, 6 h and 8 h, white arrows indicate the position of DAPI stained nuclei, (C) contains two tetrads with 4 nuclei clearly visible and one tetrad with only 3 visible stained bodies, the other nucleus is in a different focal plane. (D) Wild type meiotic progression. Only cells with 1 nucleus are visible between 1 h and 4 h, cells with 2 nuclei are prevalent at 6 h. At 8 h the majority of cells have 4 nuclei. (E) Meiotic progression of an *srs2-101/srs2-101* culture, at 8 h the majority of cells have only 1 DAPI strained body. After 12 h, only 50% of cells have 4 nuclei.

Strain number	Genotype	Percent sporulation
dAG1476	MATα *lys2 ARG ura3::URA3-[arg4-vde]* MATa *lys2 arg4-nsp,bgl ura3::URA3[arg4-bgl]* *spo11(Y135F)-HA3His6::KanMX srs2-101* *SPO11 srs2-101* *nuc1Δ::LEU2 ADE2 TFP1::VDE1 ho::LYS2* *nuc1Δ::LEU2 ade2Δ TFP1 ho::LYS2*	No mature spores.
dAG1493	MATα *lys2 arg4-nsp,bgl ura3::URA3-[arg4-vde]* MATa *lys2 arg4-nsp,bgl ura3::URA3-[arg4-bgl]* *spo11(Y135F)-HA3His6::KanMX srs2-101* *SPO11 srs2-101* *nuc1Δ::LEU2 ADE2 TFP1::VDE1 ho::LYS2* *nuc1Δ::LEU2 ade2Δ TFP1 ho::LYS2*	No mature spores.
dAG1501	MATα *lys2 arg4-nsp,bgl ura3::URA3-[arg4-vde]* MATa *lys2 ARG ura3* *leu2-K ade2Δ TFP1::VDE1* *leu2::URA3-[arg4-bgl] ADE2 TFP1* *srs2-101 ho::LYS2* *srs2-101 ho::LYS2*	No mature spores.
dAG1537	MATα *ura3 leu2(Xho1-Cla1) srs2-101* MATa *ura3 leu2::hisG srs2-101* *trp1::hisG ZIP1-GFP ho::LYS2* *trp1::hisG ZIP1-GFP ho::LYS2*	No mature spores.
dAG1534	MATα *ura3 leu2(Xho1-Cla1) SRS2* MATa *ura3 leu2::hisG SRS2* *trp1::hisG ZIP1-GFP ho::LYS2* *trp1::hisG ZIP1-GFP ho::LYS*	100%
dAG1521	MATα *ura3Δ (hind3-sma1) lys2 HIS4* MATa *ura3Δ lys2 his4::URA3* *arg4Δ(eco47III-hpa1) cyh2-z leu2-R::URA3* *arg4Δ(eco47III-hpa1) cyh2-z leu2-R* *rev-tel-ARG4 srs2-101 ho::LYS2* *rev-tel-arg4+9pac1(62528)-sph1 srs2-101 ho::LYS2*	16 %
dAG1522	MATα *ura3Δ (hind3-sma1) lys2 HIS4* MATa *ura3Δ lys2 his4::URA3* *arg4Δ(eco47III-hpa1) cyh2-z leu2-R::URA3* *arg4Δ(eco47III-hpa1) cyh2-z leu2-R* *rev-tel-ARG4 ho::LYS2* *rev-tel-arg4+9pac1(62528)-sph1 ho::LYS2*	80-90 %

Table 4.1 Strains used to assay the sporulation efficiency of *srs2-101/srs2-101* diploids.

To assess the sporulation efficiency of *srs2-101/srs2-101* diploids, cells were removed after 24 h in sporulation medium and monitored by light microscopy. 200 cells were scored from each culture for the formation of mature tetrads. The *srs2-101* mutation prevents dAG1476, 1493, 1501 and dAG1537 from forming mature tetrads. dAG1521 is capable of forming mature tetrads however this strain has a reduced sporulation efficiency of 16 % of cells form mature tetrads compared to 80 % - 90 % of cells in the wild type culture (dAG1522).

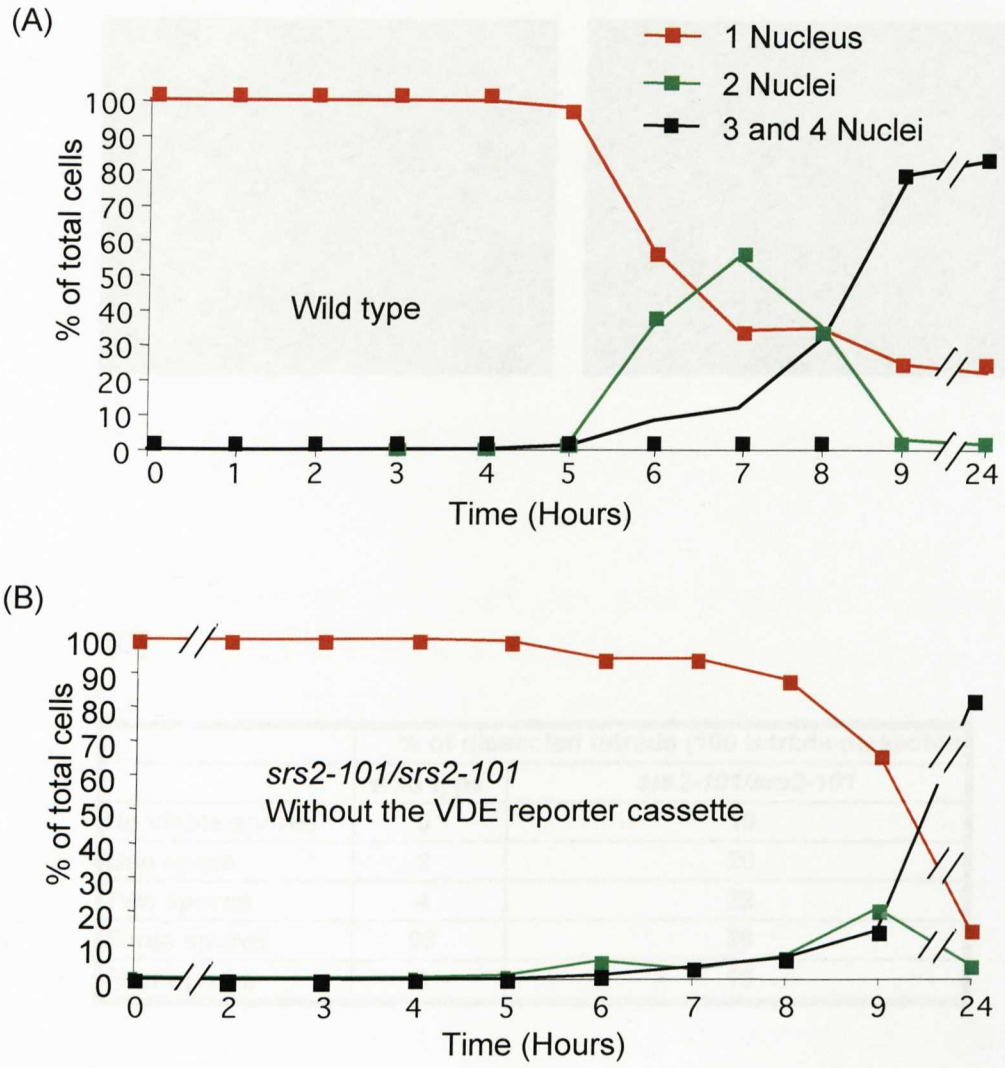
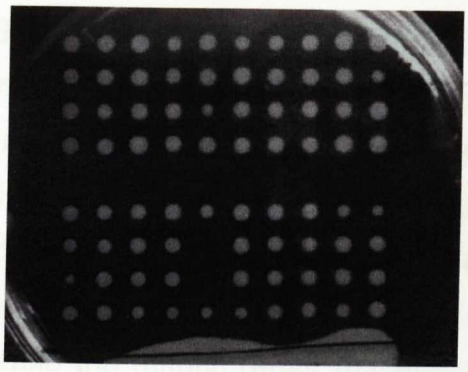


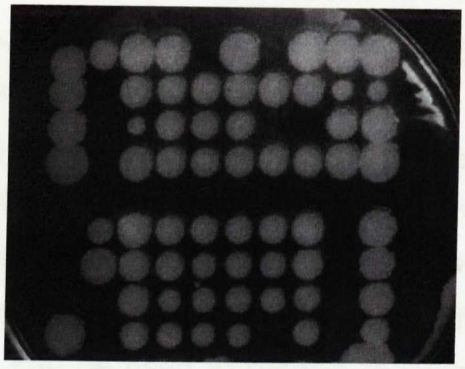
Figure 4.2 Meiotic progression is delayed in *srs2-101/srs2-101* cells without the VDE reporter cassette.

(A) Meiotic progression of wild type cells and (B) *srs2-101/srs2-101* cells. Meiotic progression of the *srs2-101/srs2-101* SK1 cells without the VDE reporter cassette is more successful then with the reporter cassette (the genotype for dAG1521 and dAG1522 is detailed in table 1). However MI is delayed in the *srs2-101/srs2-101* strain compared to wild type. In wild type the majority of cells have three or more DAPI stained bodies at 9 h however in the *srs2-101/srs2-101* culture 70 % of cells have one nucleus.

(A)



(B)



(C)

	% of dissected tetrads (100 tetrads dissected)	
	Wild type	*srs2-101/srs2-101*
No viable spores	0	19
One spore	2	20
Two spores	4	22
Three spores	93	29
Four spores	1	10

Figure 4.3 In the *srs2-101/srs2-101* strain spore viability is reduced.

Wild type cells (dAG1522) and *srs2-101/srs2-101* cells (dAG1521) were incubated on 1 % KAc plates. 100 tetrads were dissected from both strains. Example of (A) wild type dissection, (B) *srs2-101/srs2-101* dissection. (C) The percentage of dissected tetrads that formed one viable spore, two viable spores, three viable spores, four viable spores and no viable spores was calculated for each strain. In the *srs2-101/srs2-101* strain viability is reduced compared to wild type; only 29 % of *srs2-101/srs2-101* tetrads are four spore viable compared to 93 % in wild type.

Following RTG, cell viability is reduced by the *srs2-101* mutation

In a return to growth (RTG) assay to meiotic recombination can be measured (Esposito and Esposito, 1974). The meiotic arrest seen in *srs2-101/srs2-101* (dAG1493; which expresses VDE and contains the *arg4*-vde allele) suggests an error such as a failure to repair DSBs is occurring preventing further meiotic progression. In a return to growth assay cells are removed from starvation medium and introduced to rich medium permitting exit from meiosis and entry into the mitotic cell cycle. This allows cells to be recovered that have committed to heteroallelic recombination but are unable to complete both meiotic divisions (because the mitotic repair machinery can be used to resolve recombination intermediates), and the cell viability at each hour into meiosis can also be calculated.

Cell viability was measured by calculating the colony forming ability at hourly timepoints in meiosis. In *srs2-101/srs2-101* diploids a reduction in cell viability was seen following RTG after 3 h of meiosis (Fig 4.4). This coincides with the beginning of the formation of large numbers of meiotic DSBs, and when Arg+ recombinants start to appear in wild type cells (Fig 4.4). An increased frequency of recombination in *srs2-101/srs2-101* might account for the reduced viability after 3 h in meiosis because hyper recombination is associated with gross chromosomal re-arrangements and precocious sister chromatid separation, all of which result in reduced spore viability. *srs2-101/srs2-101* (dAG 1493) contains the VDE reporter cassette that consists of *arg4* alleles inserted in to the *ura3*::ty locus on both chromosomes. During meiosis the VDE endonuclease creates a DNA DSB at the recognition site in the *arg4-vde* allele that can be repaired by an interchromosomal gene conversion using the unbroken *arg4-bgl* allele on the homologue as a template; forming either an *ARG4* or *arg4-bgl* repair product. In RTG experiments, cells are removed from sporulation medium, washed and plated onto nutrient rich medium and medium lacking arginine. Commitment to meiotic recombination can be measured by determining the frequency of VDE-DSB repair by interchromosomal recombination, which can be assayed during RTG by determining the proportion of cells that are *ARG4*. This assay detects the formation of *ARG4* gene

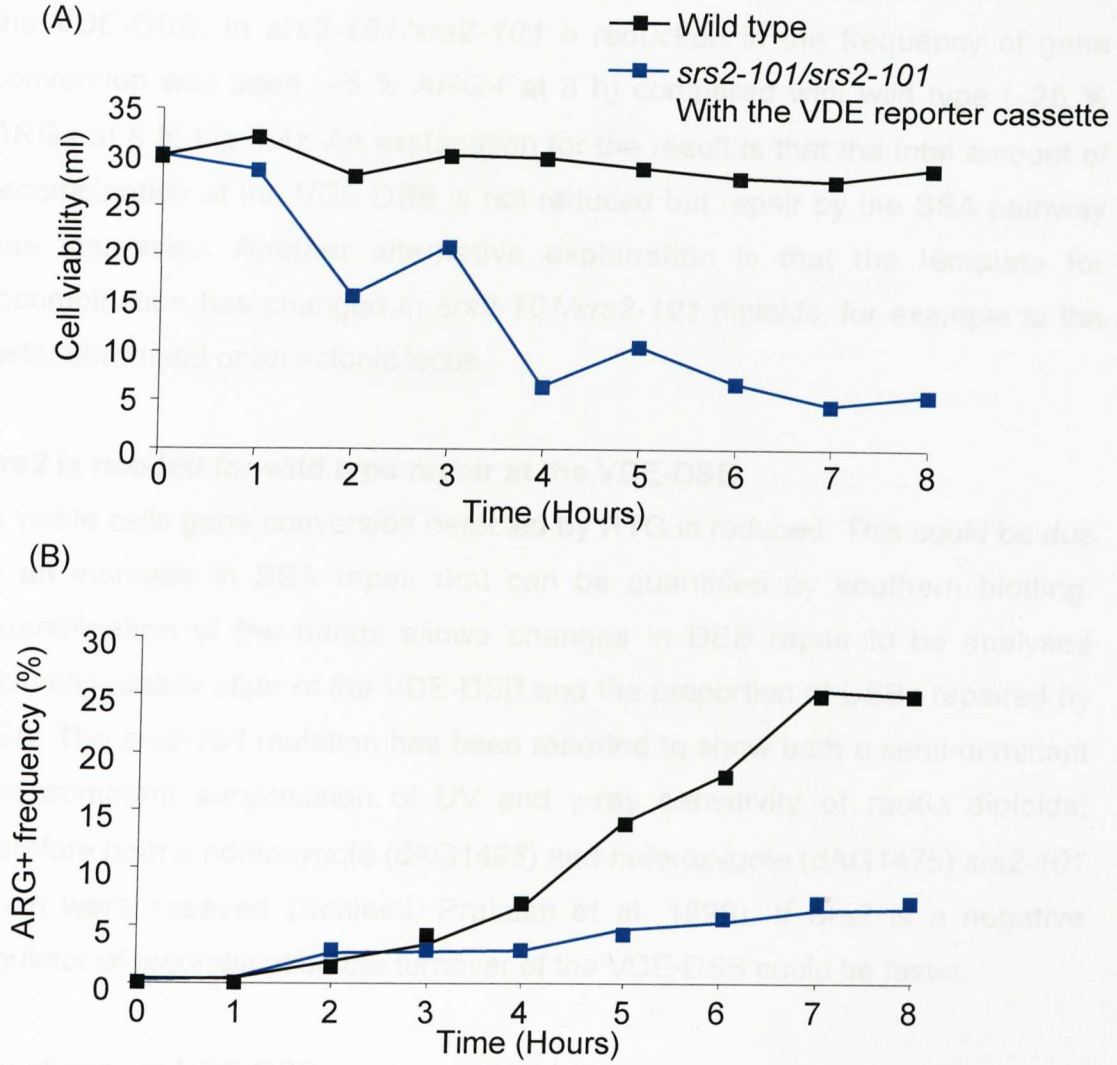


Figure 4.4 Cell viability and gene conversion in *srs2-101/srs2-101* strain.

(A) Wild type and *srs2-101/srs2-101* cells were removed hourly from meiotic starvation media and plated onto YEPAD. Both the strains contain the VDE reporter cassette, once the VDE-DSB is cleaved the break can be repaired by a gene conversion event using the undamaged homologue or by using the flanking homology using SSA. The viability of the strain was determined by calculating the colony forming ability at each hour. The cell viability of the *srs2-101/srs2-101* strain is reduced after 3 h compared to wild type. (B) Frequency of gene conversion events. The number of arginine prototrophs was divided by the total colony forming ability to give the frequency of Arg^+ recombination events. In *srs2-101/srs2-101* diploids the frequency of gene conversion events is reduced (5 %) compared to wild type (25 %).

conversion repair products only, not *arg4-bgl* gene co-conversion repair products. Although the assay only allows a subset of gene conversion events to be calculated the result is indicative of the total frequency of gene conversion at the VDE-DSB. In *srs2-101/srs2-101* a reduction in the frequency of gene conversion was seen (~5 % *ARG4* at 8 h) compared with wild type (~25 % *ARG4* at 8 h; Fig 4.4). An explanation for the result is that the total amount of recombination at the VDE-DSB is not reduced but repair by the SSA pathway has increased. Another alternative explanation is that the template for recombination has changed in *srs2-101/srs2-101* diploids, for example to the sister chromatid or an ectopic locus.

Srs2 is needed for wild type repair at the VDE-DSB

In viable cells gene conversion detected by RTG is reduced. This could be due to an increase in SSA repair that can be quantified by southern blotting. Quantification of the bands allows changes in DSB repair to be analysed including steady state of the VDE-DSB and the proportion of DSBs repaired by SSA. The *srs2-101* mutation has been reported to show both a semi-dominant and dominant suppression of UV and γ-ray sensitivity of *rad6Δ* diploids; therefore both a homozygote (dAG1493) and heterozygote (dAG1475) *srs2-101* strain were assayed (Schiestl, Prakash et al. 1990). If Srs2 is a negative regulator of recombination the turnover of the VDE-DSB could be faster.

Any change in VDE-DSB appearance and disappearance in *srs2-101/srs2-101* might result from a change in kinetics of VDE cleavage. The rate of VDE cleavage can be calculated using southern blots. DNA extracted from *srs2-101/srs2-101* and *srs2-101/SRS2* was digested with *Eco*RV *Bgl*II as described in Chapter 3. The amount of DNA in the uncleaved chromatid band was increased in the *srs2-101/SRS2* cells compared with wild type cells. This indicates that in the *srs2-101/SRS2* timecourses the rate of cleavage is slower than in wild type (dAG206; Fig 4.5). In the *srs2-101/srs2-101* timecouse the rate of VDE cleavage is same as wild type (Fig 4.6). In *srs2-101/SRS2* timecourses the rate of VDE cleavage is consistently slower compared with wild type timecourses. To allow for the different rate of cleavage the amount of

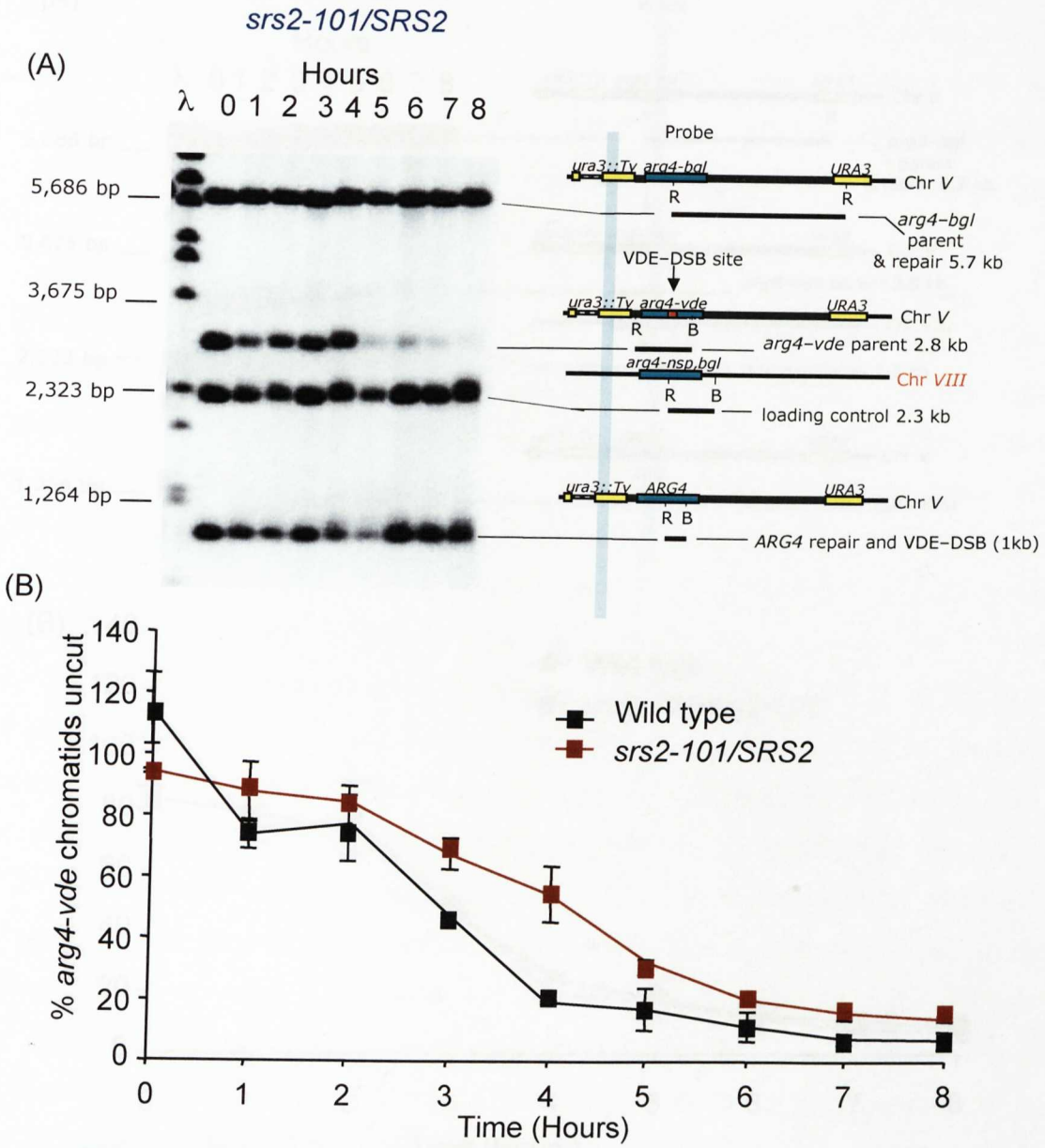


Figure 4.5 Proportion of uncut *arg4-vde* chromatids in a *srs2-101* heterozygote strain.

(A) Cells from a *srs2-101/SRS2* sporulating culture were removed at hourly intervals and the DNA extracted then double digested with *EcoR*V and *Bgl*II. The wild type cultures and the *srs2-101/SRS2* cultures were not done in parallel. DNA was southern blotted and probed downstream of the *EcoR*V. The probe detects the 2.8 kb *arg4-vde* parental fragment and a 2.3 kb loading control that contains the arg4-nsp,bgl allele a the *ARG4* on ch *VIII.* During the timecourse the percentage of DNA in *arg4-vde* fragment decreases at VDE cleaves the recognition site. (B) Quantification of blot in (A) DNA is plotted as percentage of the total amount of probe hybridising to all the bands in each lane. In the *srs2-101/SRS2* timecourse VDE cleavage appears to be slower compared to wild type.

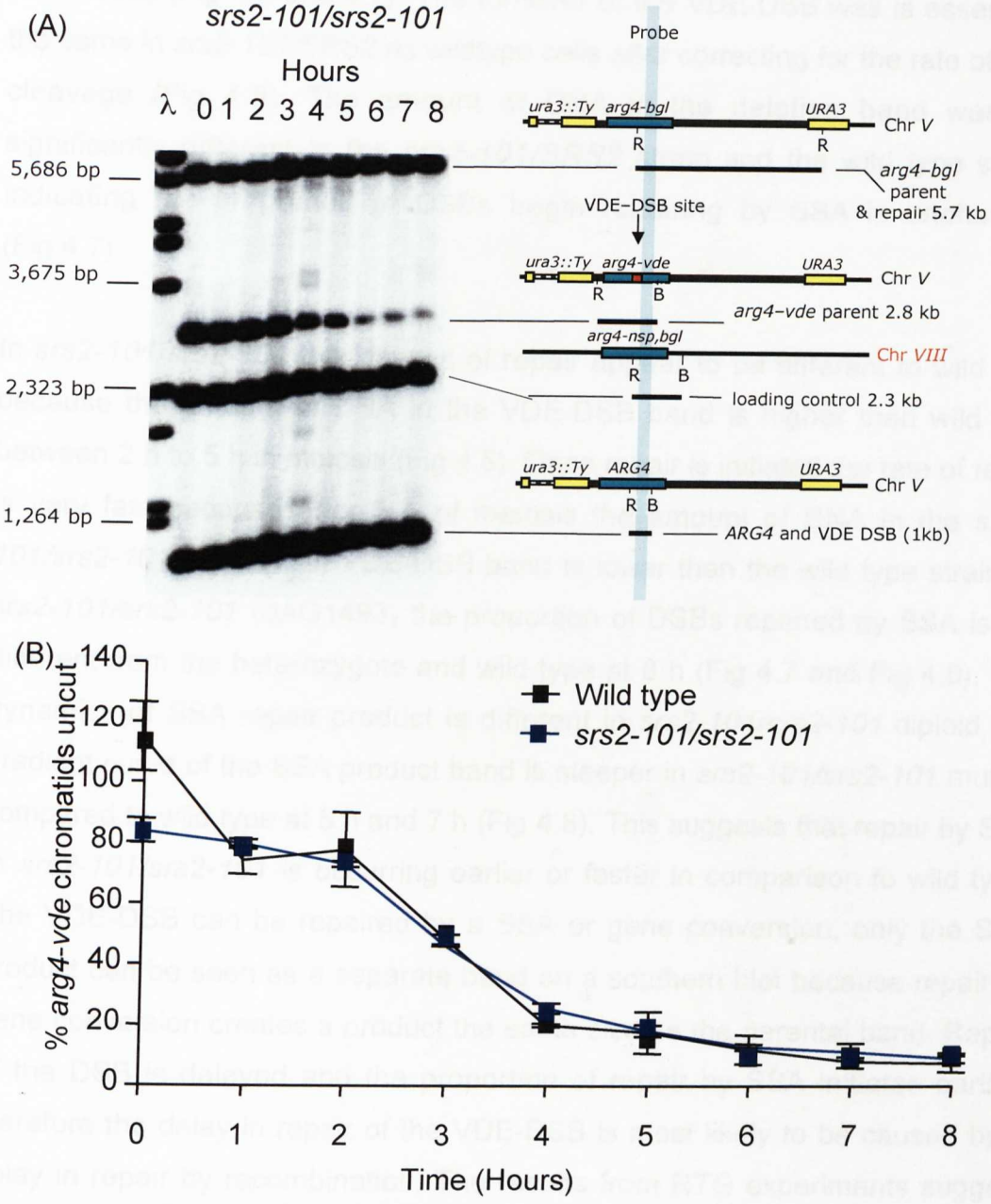


Figure 4.6 Proportion of uncut arg4-VDE chromatids.

(A) Cells from a *srs2-101/srs2-101* sporulation culture were removed at hourly intervals and processed as described in Fig 4.5 (B) The rate of cleavage in *srs2-101/srs2-101* strain is indistinguishable from wild type cells.

srs2-101/SRS2 DNA in the VDE-DSB was expressed as a proportion of cut chromatids (Fig 4.5 and 4.7). The turnover of the VDE-DSB was is essentially the same in *srs2-101/SRS2* as wildtype cells after correcting for the rate of VDE cleavage (Fig 4.8). The amount of DNA in the deletion band was not significantly different in the *srs2-101/SRS2* strain and the wild type strain, indicating the proportion of DSBs begin repairing by SSA is unchanged (Fig 4.7).

In *srs2-101/srs2-101* the kinetics of repair appear to be different to wild type because the amount of DNA in the VDE-DSB band is higher than wild type between 2 h to 5 h in meiosis (Fig 4.8). Once repair is initiated the rate of repair is very fast because after 5 h of meiosis the amount of DNA in the *srs2-101/srs2-101* (dAG1475) VDE-DSB band is lower than the wild type strain. In *srs2-101/srs2-101* (dAG1493) the proportion of DSBs repaired by SSA is not different from the heterozygote and wild type at 8 h (Fig 4.7 and Fig 4.8). The dynamics of SSA repair product is different in *srs2-101/srs2-101* diploid, the gradient curve of the SSA product band is steeper in *srs2-101/srs2-101* mutant compared to wild type at 6 h and 7 h (Fig 4.8). This suggests that repair by SSA in *srs2-101/srs2-101* is occurring earlier or faster in comparison to wild type. The VDE-DSB can be repaired by a SSA or gene conversion, only the SSA product can be seen as a separate band on a southern blot because repair by gene conversion creates a product the same size as the parental band. Repair of the DSB is delayed and the proportion of repair by SSA initiates earlier, therefore the delay in repair of the VDE-DSB is most likely to be caused by a delay in repair by recombination. The results from RTG experiments suggest that repair by gene conversion is impaired because the ARG+ gene conversion frequency is so low.

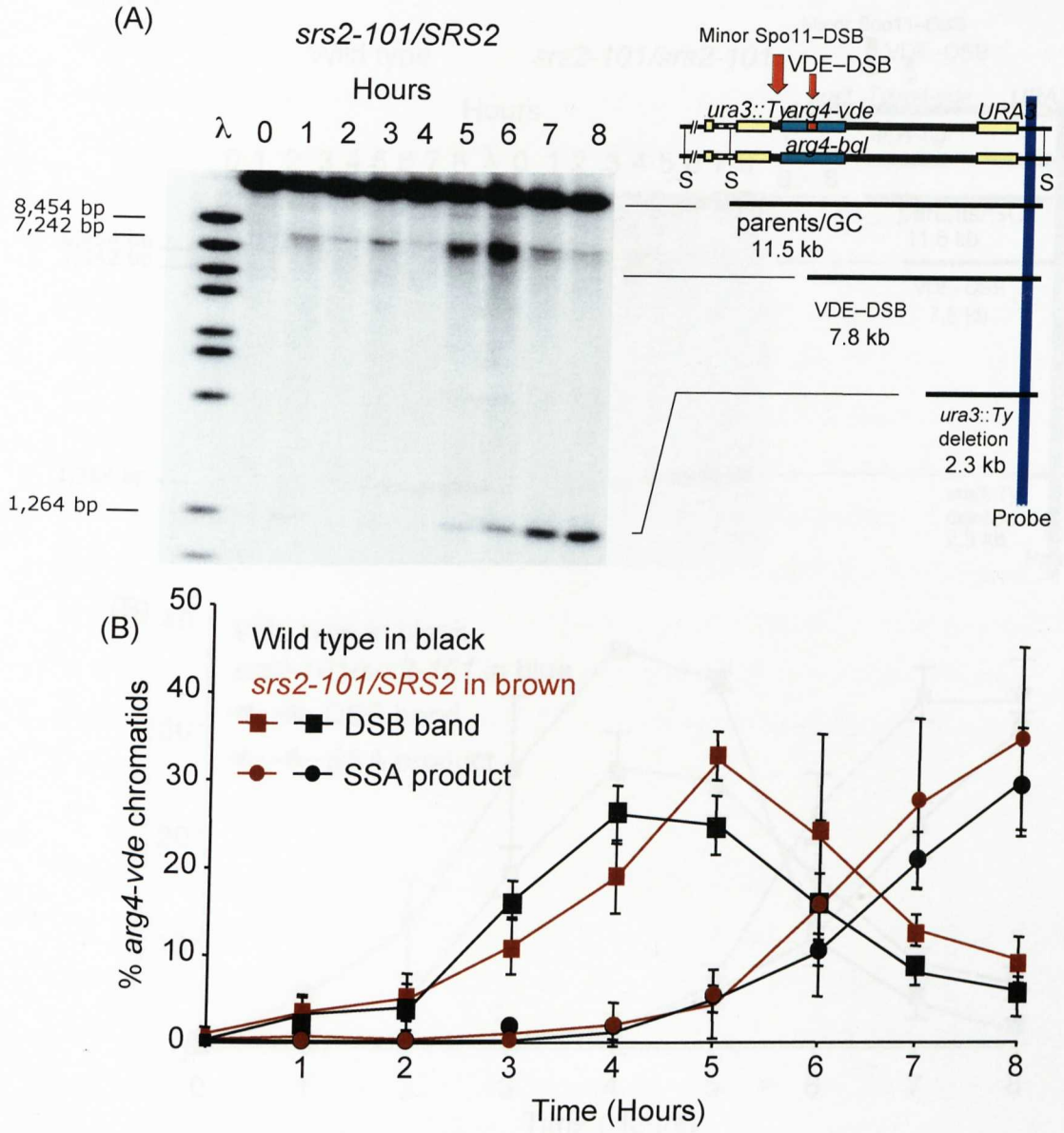


Figure 4.7 Repair of the VDE-DSB in *srs2-101/SRS2* cells.

DNA was extracted from synchronous meiotic cultures of *srs2-101/SRS2* and wild type cells, fractionated, blotted and hybridised with a 1kb probe specific to a chromosome V region. (B) Quantification of wild type and *srs2-101/SRS2* southern blots. The kinetics of VDE-DSB appearance and disappearance and the amount of repair by SSA in *srs2-101/SRS2* diploid cells is indistinguishable from wild type diploids. This data is this graph has been normalised to the rate VDE cleavage. The amount of DNA in the *srs2-101/SRS2* is always greater than wild type at 5 h and 6 h however the difference is not statistically significant.

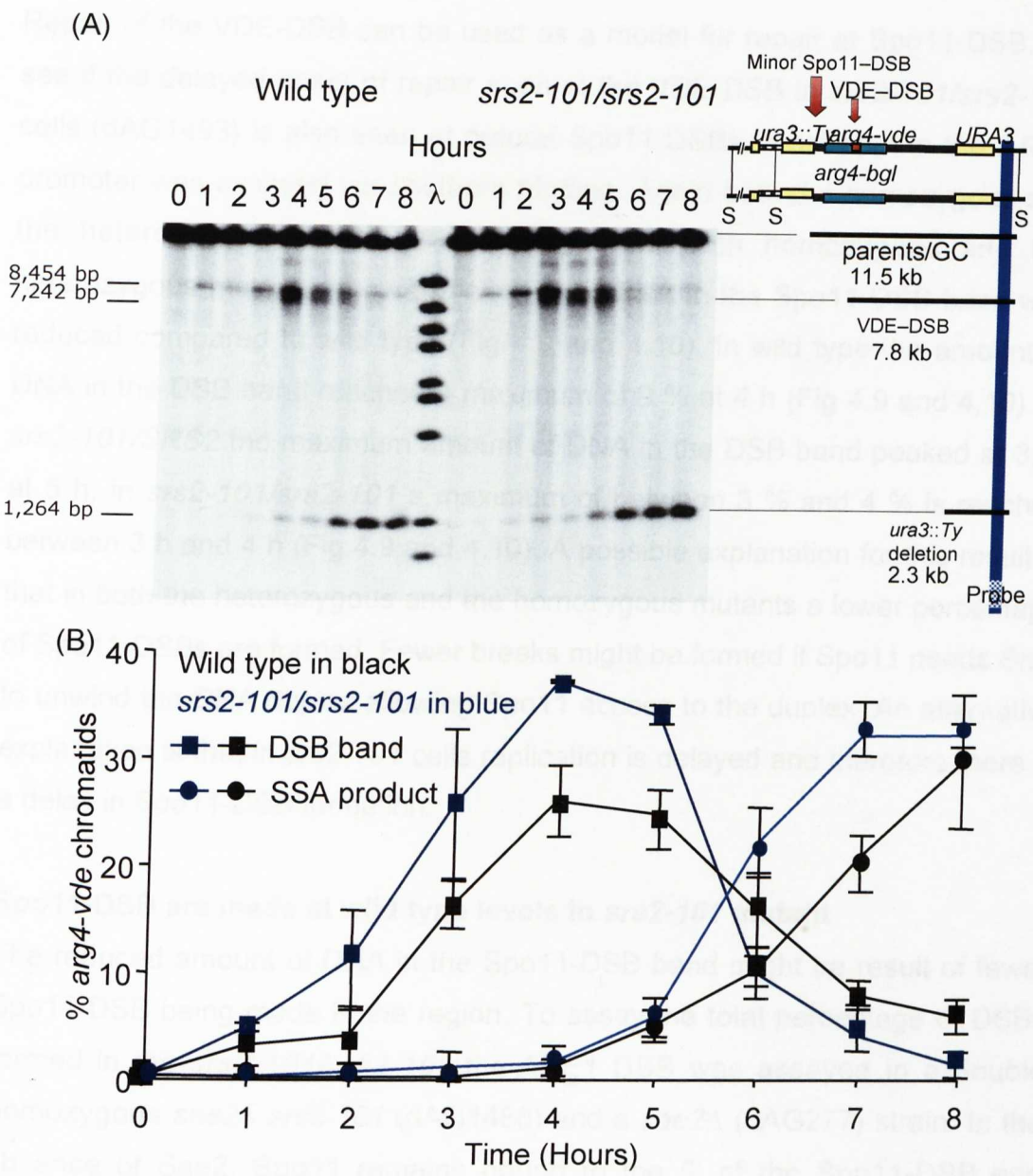


Figure 4.8 *srs2-101/srs2-101* cells exhibit a delay in the initiation of VDE-DSB repair.

(A) DNA was extracted from wild type and *srs2-101/srs2-101* synchronous meiotic cultures and southern blotted. (B) Quantification of gel in (A). In the *srs2-101/srs2-101* strain the amount of DNA in the VDE-DSB accumulates to a higher percentage than in wild type between 2 h and 5 h suggesting a delay in VDE-DSB repair. The percentage of DNA in SSA repair product band is higher then wild type at 6 h and 7 h however is indistinguishable from wild type at 8 h.

Repair at the *ARE1* Spo11-DSB in *srs2-101* cells.

Repair of the VDE-DSB can be used as a model for repair at Spo11-DSB. To see if the delayed onset of repair seen at the VDE-DSB in *srs2-101/srs2-101* cells (dAG1493) is also seen at natural Spo11-DSBs, a hot spot in the *ARE1* promoter was assayed via southern blotting. Again both the homozygous and the heterozygous strains were assayed. In both homozygous and the heterozygous mutant strains the amount of DNA in the Spo11-DSB band was reduced compared to wild type (Fig 4.9 and 4.10). In wild type the amount of DNA in the DSB band reached a maximum of 9 % at 4 h (Fig 4.9 and 4.10). In *srs2-101/SRS2* the maximum amount of DNA in the DSB band peaked at 3 % at 5 h, in *srs2-101/srs2-101* a maximum of between 3 % and 4 % is reached between 3 h and 4 h (Fig 4.9 and 4.10). A possible explanation for this result is that in both the heterozygous and the homozygous mutants a lower percentage of Spo11-DSBs are formed. Fewer breaks might be formed if Spo11 needs Srs2 to unwind the DNA duplex allowing Spo11 access to the duplex. An alternative explanation is that in *srs2-101* cells replication is delayed and therefore there is a delay in Spo11-DSB formation.

Spo11-DSB are made at wild type levels in *srs2-101* mutant

The reduced amount of DNA in the Spo11-DSB band might be result of fewer Spo11-DSB being made in the region. To assay the total percentage of DSBs formed in the *srs2-101/srs2-101* the *ARE1* DSB was assayed in a double homozygous *sae2Δ srs2-101* (dAG1488) and a *sae2Δ* (dAG277) strain. In the absence of Sae2, Spo11 remains bound to the 5′ of the Spo11-DSB end (Keeney et al., 1997). Consequently the Spo11-DSBs are not repaired allowing the total amount Spo11-DSB to be detected. If fewer Spo11-DSBs are formed in *srs2-101/srs2-101* diploids the amount of DNA in the Spo11-DSB band at 8 h will be lower in *srs2-101 sae2Δ* double homozygote than a *sae2Δ*. It was found however that the total amount of Spo11-DSB created in *sae2Δ srs2-101* mutant is the same as the *sae2Δ*. In both strains the maximal amount of DNA in the Spo11-DSB band reached between 12 % and 16 % respectively at 8 h (Fig 4.11). This suggests that in the absence of Srs2 wild type amounts of

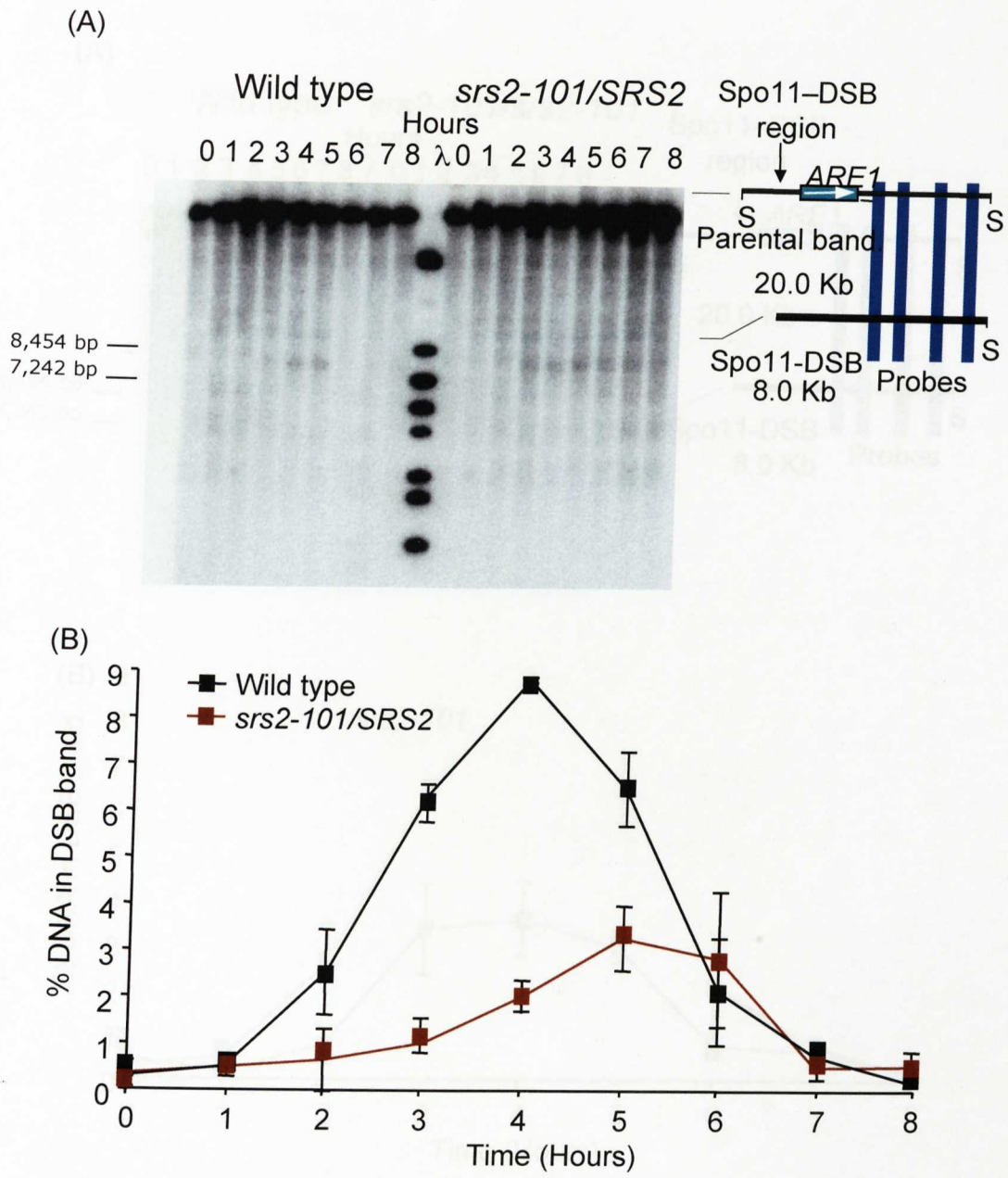


Figure 4.9 The amount of DNA is reduced in *srs2-101/SRS2* cells at the *ARE1* hotspot.

(A) DNA was extracted from wild type and heterozygote *srs2-101* synchronous meiotic cultures and southern blotted. (B) Quantification of southern blot in (A). The maximal amount of DNA in the Spo11-DSB band reaches a maximum of 3 % at 5 h compared to 8.8 % at 4 h in wild type. The lower peak in the DSB band suggests that in the *srs2-101/ SRS2* diploid early repair is much faster, alternatively less Spo11-DSB are being made at the *ARE1* hot spot.

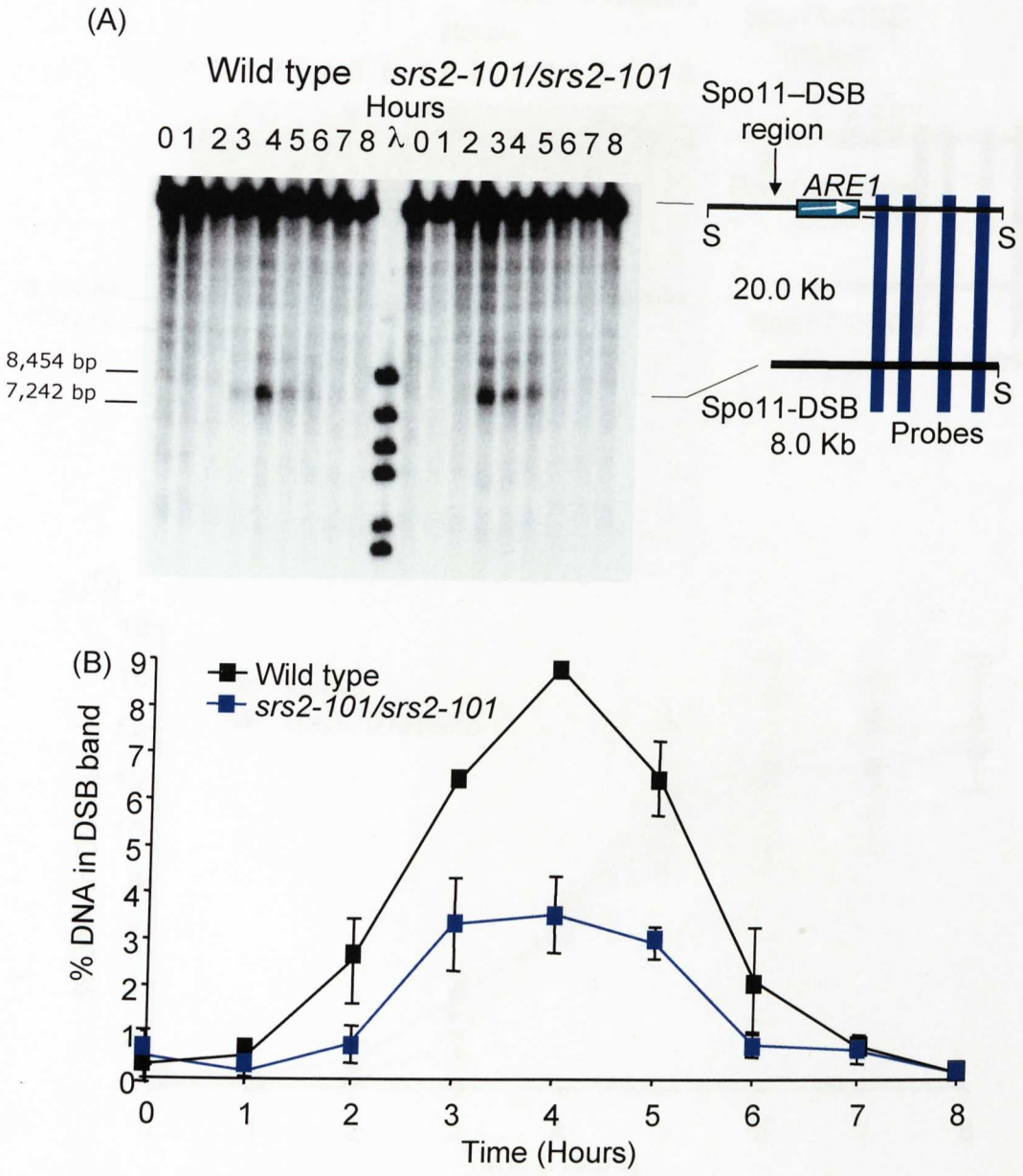


Figure 4.10 The *ARE1* natural hotspot *srs2-101* shows a reduced amount of DNA In DSB band.

(A) DNA extracted from wild type and mutant *srs2-101* homozygote synchronous meiotic cultures digested, fractionated and southern blotted. (B) Quantification of blot plotted as percentage of the total amount of probe hybridising to all the bands in each lane. The amount of DNA in the natural Spo11- DSB band in the *srs2-101* mutant is reduced compared to the wild type.

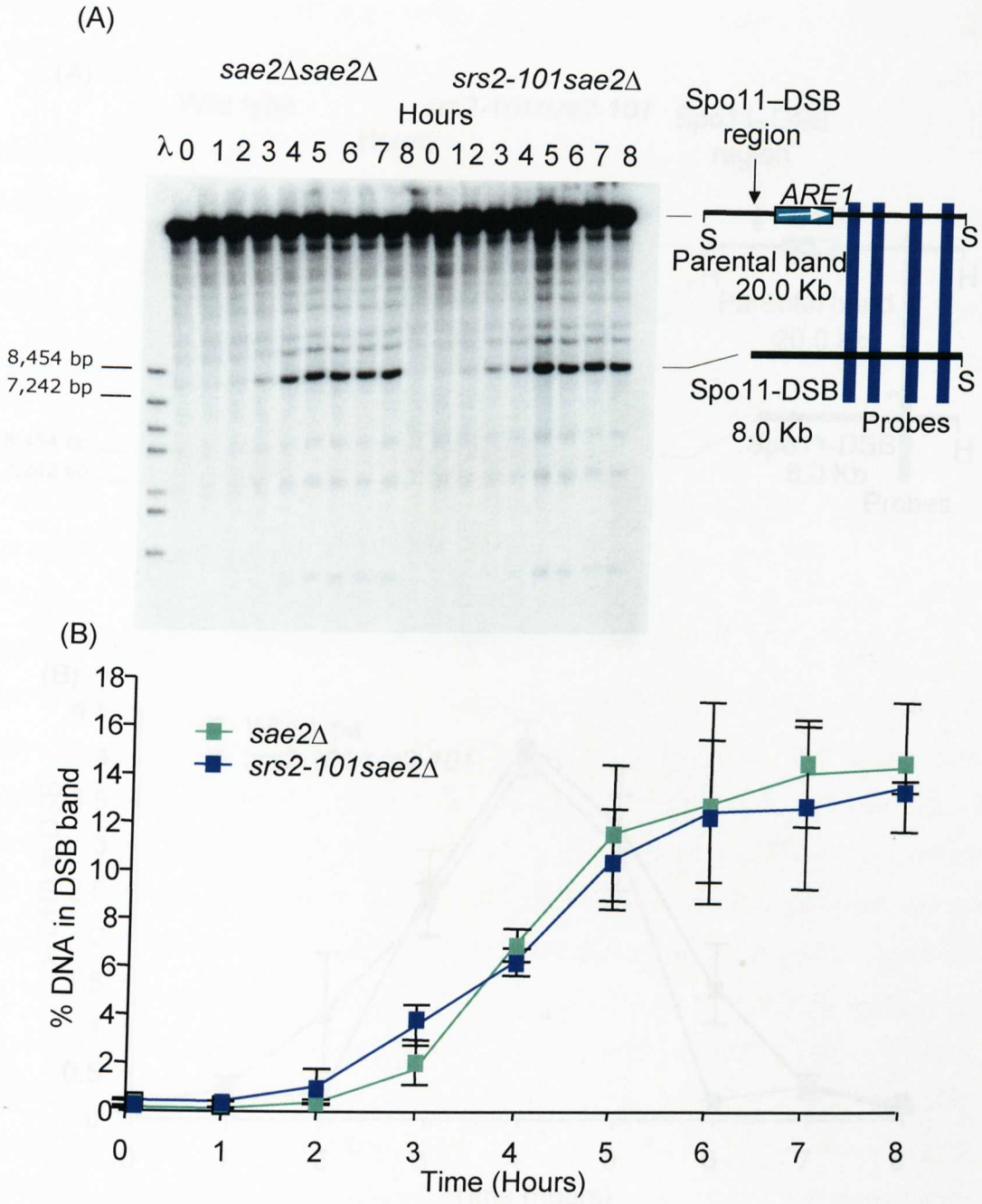


Figure 4.11 Wild type amount of Spo11-DSB are formed at the *ARE1* hotspot.

(A) DNA extracted from *sae2Δ* and *srs2-101 sae2Δ* homozygote synchronous meiotic cultures fractionated and southern blotted. (B) Quantification of southern blot in (A). In the absence of Sae2 Spo11 remains covalently bound to the DSB end preventing resection and subsequent repair. In the *srs2-101 sae2Δ* mutant DNA in the DSB band accumulates to a similar percentage in the *sae2Δ* strain (between 12 % and 16 %), suggesting that Spo11-DSBs are made at the wild type frequency at the *ARE1* hot spot in the *srs2-101* homozygous strain.

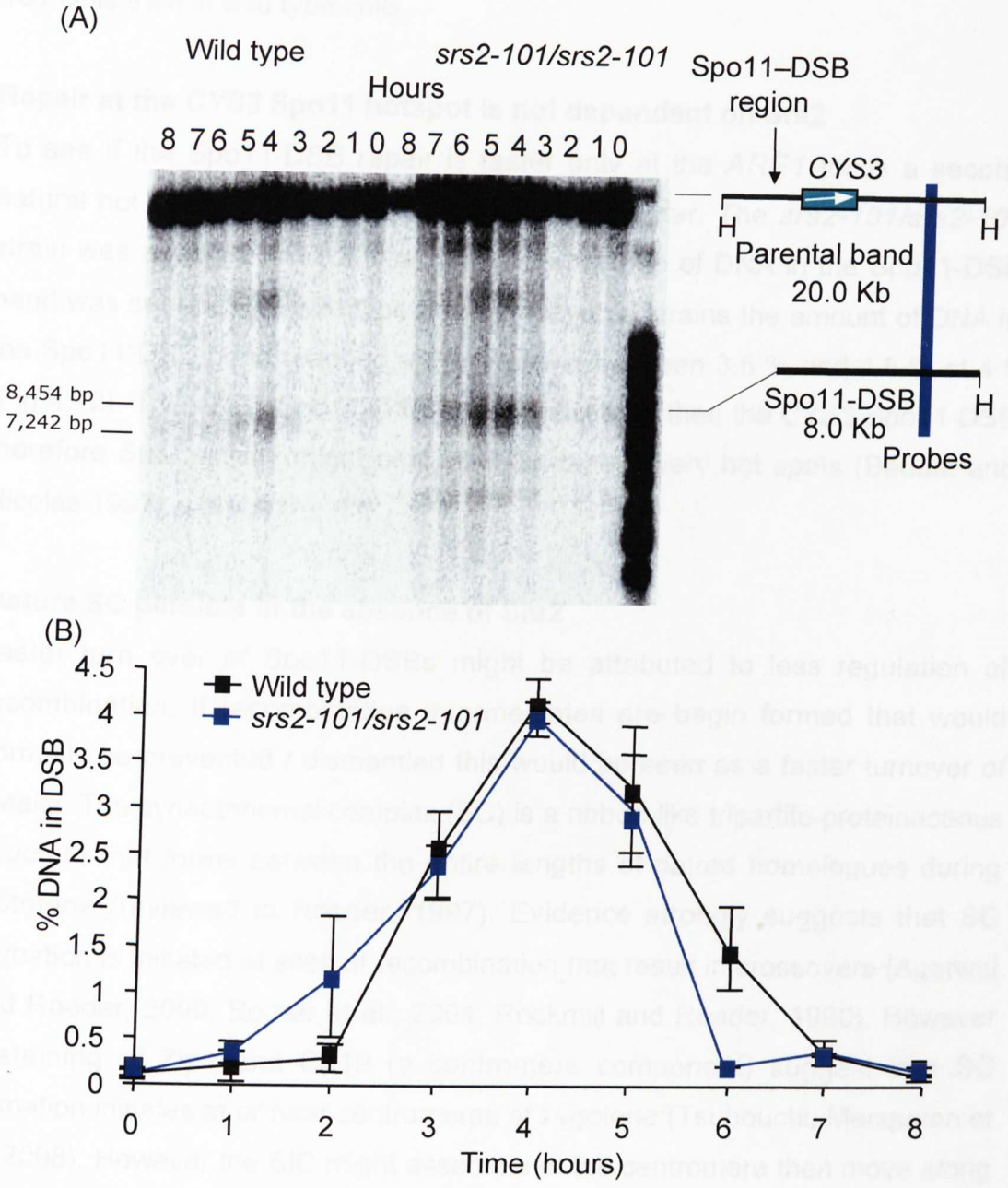


Figure 4.12 Quantification of DNA at the *CYS3* hotspot in *srs2-101* homozygous strain.

(A) DNA was extracted from both wild type and *srs2-101/srs2-101* synchronous meiotic cultures. The DNA was digested with *Hind*II, and southern blotted (B) The repair kinetics of the Spo11- DSB are indistinguishable in both the wild type and the *srs2*-101 homozygous strain.

Spo11-DSBs are created, and repair at the *ARE1* site is faster in *srs2-101/srs2-101* cells than in wild type cells.

Repair at the *CYS3* Spo11 hotspot is not dependent on Srs2

To see if the Spo11-DSB repair is faster only at the *ARE1* locus a second natural hot spot was assayed at the *CYS3* promoter. The *srs2-101/srs2-101* strain was assayed no reduction in the percentage of DNA in the Spo11-DSB band was seen. In the wild type and homozygote strains the amount of DNA in the Spo11-DSB band reached a maximum of between 3.5 % and 4.5 % at 4 h (Fig 4.12). The ARE1 Spo11-DSB is hotter hot spot then the CYS3 Spo11-DSB therefore Srs2 activity might only be important at very hot spots (Baudat and Nicolas 1997).

Mature SC persists in the absence of Srs2

Faster turn over of Spo11-DSBs might be attributed to less regulation of recombination. If recombination intermediates are begin formed that would normally be prevented / dismantled this would be seen as a faster turnover of breaks. The synaptonemal complex (SC) is a ribbon-like tripartite proteinaceous structure that forms between the entire lengths of paired homologues during leptotene (reviewed in Roeder, 1997). Evidence strongly suggests that SC formation is initiated at sites of recombination that result in crossovers (Agarwal and Roeder, 2000; Borner et al., 2004; Rockmill and Roeder, 1990). However costaining of Zip1 and Ctf19 (a centromere component) suggest that SC formation initiates at or near centromeres at zygotene (Tsubouchi, Macqueen et al. 2008). However the SIC might assemble at the centromere then move along the chromosome until they reach a recombination intermediate then stop until SC formation is initiated (Tsubouchi, Macqueen et al. 2008). Like Srs2 the Sgs1 helicase is a known negative regulator of recombination; full chromosome synapsis detected by Zip1 antibody staining is seen earlier in *sgs1*Δ diploids compared to wild type cells (Jessop et al., 2006; Rockmill et al., 2003). An increase in the frequency of recombination in *srs2-101/srs2-101* cells might result in early SC formation. To see if SC formation is altered in *srs2-101/srs2-101* diploids a *ZIP1-GFP* strain was constructed to monitor SC formation

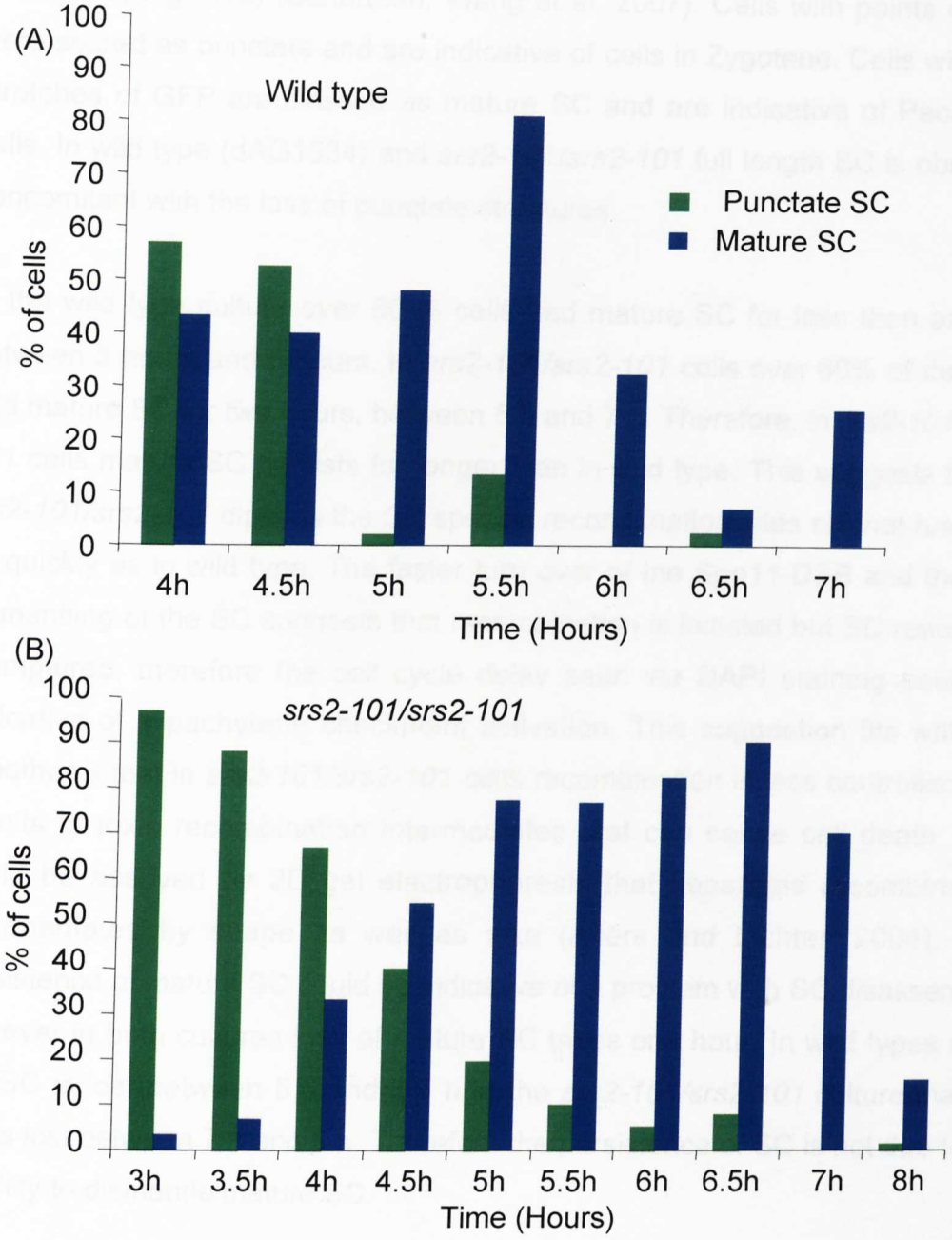


Figure 4.13 visualisation of SC formation.

(A) The *srs2-101 ZIP1::GFP* homozygote and wild type strains were synchronously sporulated and cells were removed in 30 min intervals. Cells were scored for mature fluorescent SC, punctate florescence and diffuse/no visible florescence. Graphs show the percentage of cells that have punctate fluorescence and mature SC in (A) wild type cells and (B) the *srs2-101 ZIP1-GFP* homozygote strain. In the *srs2-101 ZIP1-GFP* homozygote diploid mature SC are visible for longer (two hours) then in wild type cells (one hour).

(dAG1537; Fig 4.13) (Scherthan, Wang et al. 2007). Cells with points of GFP were scored as punctate and are indicative of cells in Zygotene. Cells with long stretches of GFP are classed as mature SC and are indicative of Pachytene cells. In wild type (dAG1534) and *srs2-101/srs2-101* full length SC is observed concomitant with the loss of punctate structures.

In the wild type culture over 60 % cells had mature SC for less then an hour between 5 hours and 6 hours. In *srs2-101/srs2-101* cells over 60% of the cells had mature SC for two hours, between 5 h and 7 h. Therefore, in *srs2-101/srs2-101* cells mature SC persists for longer than in wild type. This suggests that in *srs2-101/srs2-101* diploids the SC specific recombination sites are not resolved as quickly as in wild type. The faster turn over of the Spo11-DSB and the late dismantling of the SC suggests that recombination is initiated but SC resolution is impaired; therefore the cell cycle delay seen via DAPI staining could be indicative of a pachytene checkpoint activation. This suggestion fits with the hypothesis that in *srs2-101/srs2-101* cells recombination is less controlled and results in toxic recombination intermediates that can cause cell death. This could be assayed by 2D gel electrophoresis that separates recombination intermediates by shape as well as size (Allers and Lichten 2001). The persistence of mature SC could be indicative of a problem with SC disassembly, however in both cultures loss of mature SC takes one hour. In wild types cells the SC is lost between 5.5 and 6.5 h in the *srs2-101/srs2-101* culture mature SC is lost between 7 h and 8 h. Therefore the persistence of SC is not due to an inability to dismantle mature SC.

The frequency of ectopic repair is reduced in the absence of Srs2

The faster turn over of the Spo11-DSB might be due to reduced regulation of repair partner choice. In yeast, to ensure a successful meiotic division the template for recombination has to be the homologous chromosome. A reduction in the regulation of crossover formation might result in increased ectopic recombination at the VDE-DSB. To test this hypothesis an ectopic reporter cassette was used (Fig 4.14). In this assay the VDE recognition sequence is inserted at *ura3* on chromosome V and the donor cassette is located at *leu2* on

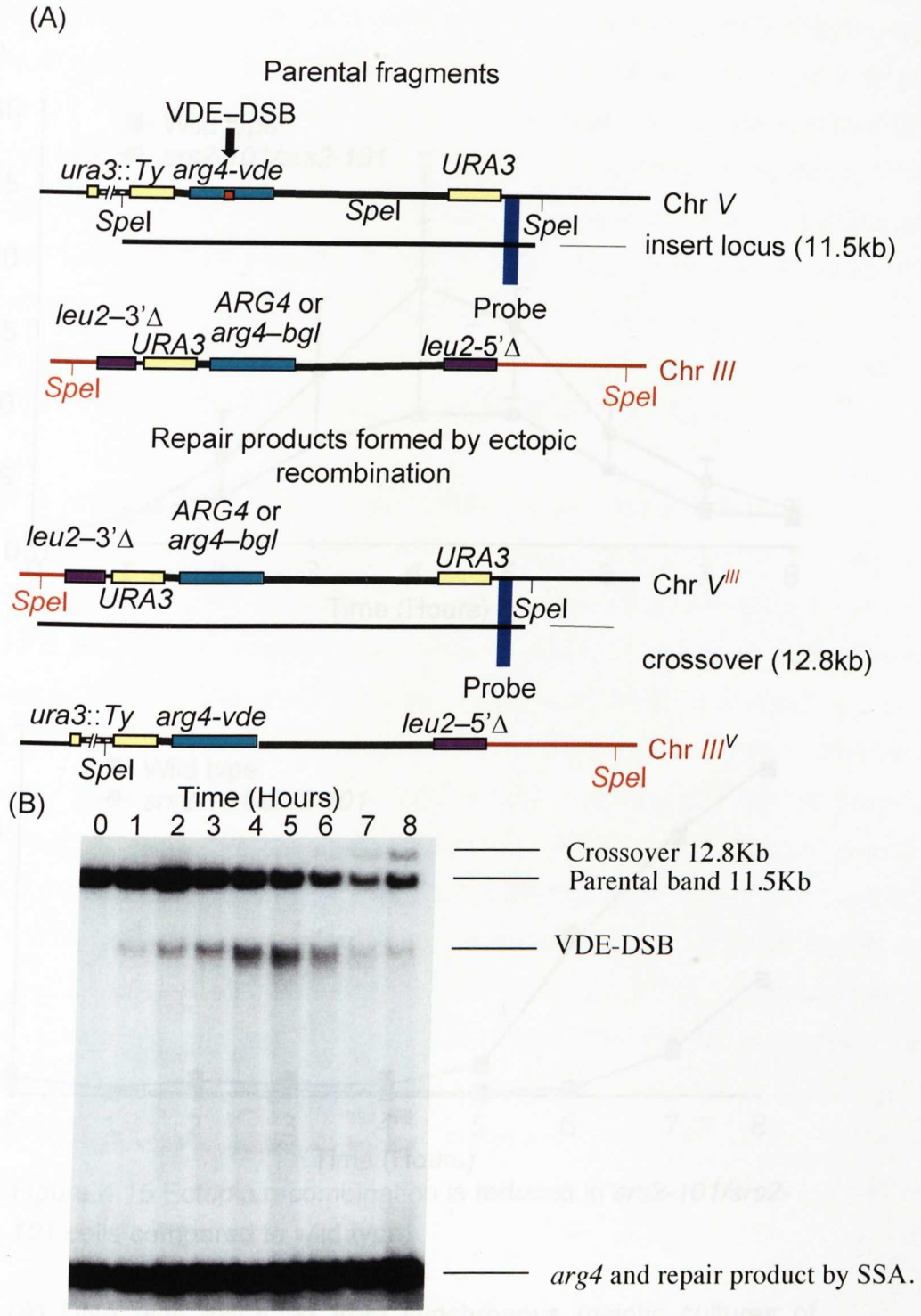


Figure 4.14 Reporter cassette to detect ectopic crossovers.

(A) The VDE-DSB is at *URA3* Chr. *V,* a donor sequence is inserted at *LEU2* Chr. *III.* For successful repair the cell can ether use SSA or ectopic homologous recombination using the template at *LEU2.* b) DNA was digested with *Spe*I and fractionation 0.5% gel blotted onto a nylon membrane and hybridised with a probe specific to Chr. *V. Spe*I digestion releases 11.5 Kb parental band and the VDE-DSB 7.8 Kb band. The crossover product can be seen at 6 h,7 h and 8 h as a 12.8 Kb band above the parental band. A fourth band contains *arg4* and the SSA repair product at 2.3 Kb.

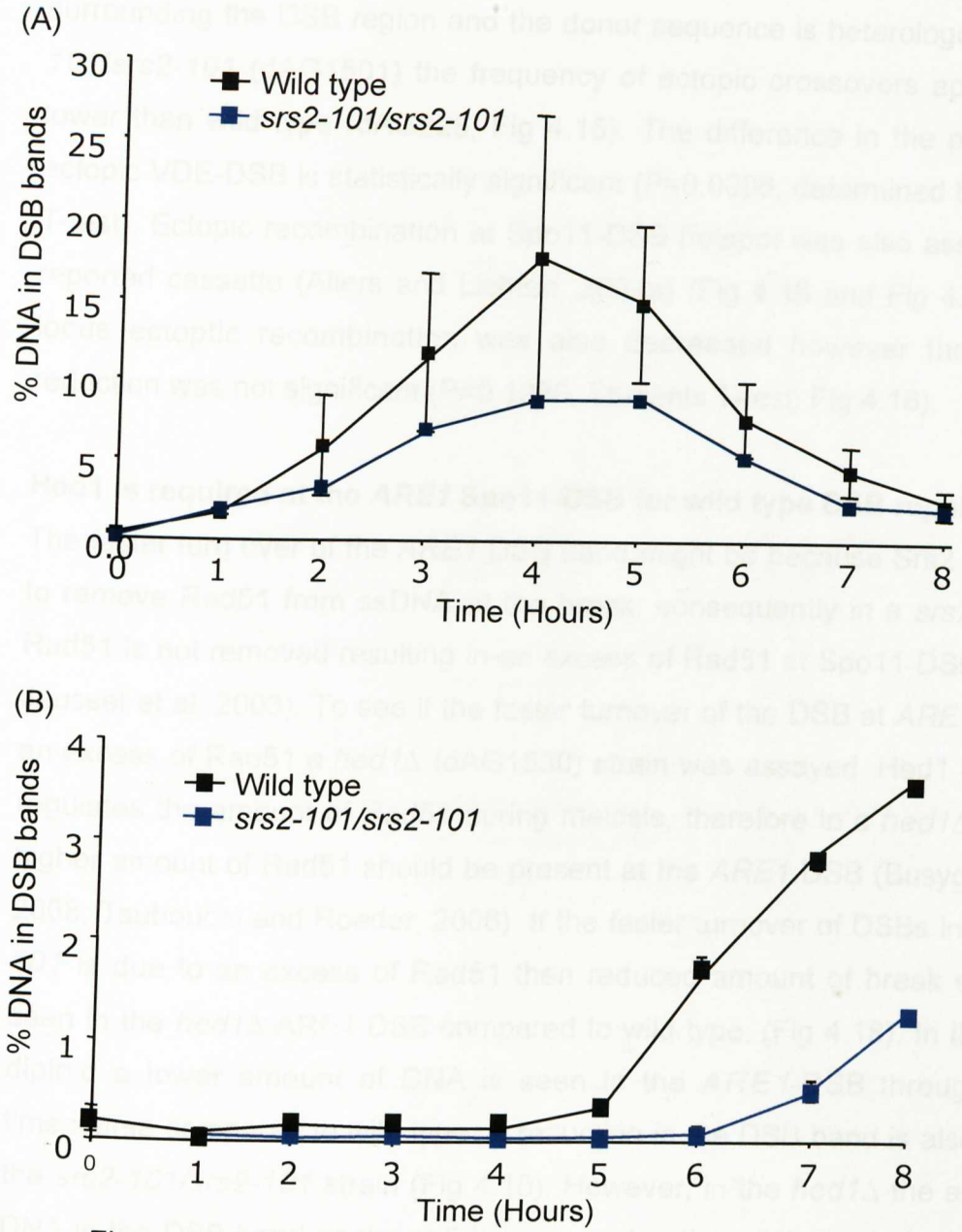


Figure 4.15 Ectopic recombination is reduced in *srs2-101/srs2-101* cells compared to wild type.

(A) DNA was extracted from synchronous meiotic cultures of *srs2-101* homozygous and wild type cells that contain the ectopic cassette and Southern blotted. The amount of DNA in the VDE-DSB in wild type and *srs2-101* cells. There is slightly more DNA in the VDE-DSB band in wild type cells, however there is no statistical difference between *srs2-101* and wild type VDE-DSB formation. (B) shows the amount of ectopic repair in *srs2-101* homozygous cell is reduced 1.3 % when compared to wild type 3.6 %.

chromosome III. Ectopic crossovers can be detected because the sequence surrounding the DSB region and the donor sequence is heterologous. In *srs2-101/srs2-101* (dAG1501) the frequency of ectopic crossovers appears to be lower than wild type (dAG228; Fig 4.15). The difference in the proportion of ectopic VDE-DSB is statistically significant (P=0.0006, determined by Student's T-test). Ectopic recombination at Spo11-DSB hotspot was also assayed using reported cassette (Allers and Lichten, 2001a) (Fig 4.16 and Fig 4.17). At this locus ectoptic recombination was also decreased however the observed reduction was not significant (P=0.1280, Students T-test; Fig 4.16).

Hed1 is required at the *ARE1* Spo11-DSB for wild type DSB repair

The faster turn over of the *ARE1* DSB band might be because Srs2 is required to remove Rad51 from ssDNA at the break; consequently in a *srs2-101* cells Rad51 is not removed resulting in an excess of Rad51 at Spo11-DSB (Veaute, Jeusset et al. 2003). To see if the faster turnover of the DSB at *ARE1* is due to an excess of Rad51 a *hed1*Δ (dAG1530) strain was assayed. Hed1 negatively regulates the amount of Rad51 during meiosis, therefore in a *hed1*Δ diploid a higher amount of Rad51 should be present at the *ARE1* DSB (Busygina et al., 2008; Tsubouchi and Roeder, 2006). If the faster turnover of DSBs in the *srs2-101* is due to an excess of Rad51 then reduced amount of break should be seen in the *hed1*Δ ARE1 DSB compared to wild type. (Fig 4.18). In the *hed1*Δ diploid a lower amount of DNA is seen in the *ARE1*-DSB throughout the timecourse compared to wild type. A reduction in the DSB band is also seen in the *srs2-101/srs2-101* strain (Fig 4.10). However, in the *hed1*Δ the amount of DNA in the DSB band peaks at 5 h compared to the wildtype strain that peaks at 4 h.

(A)

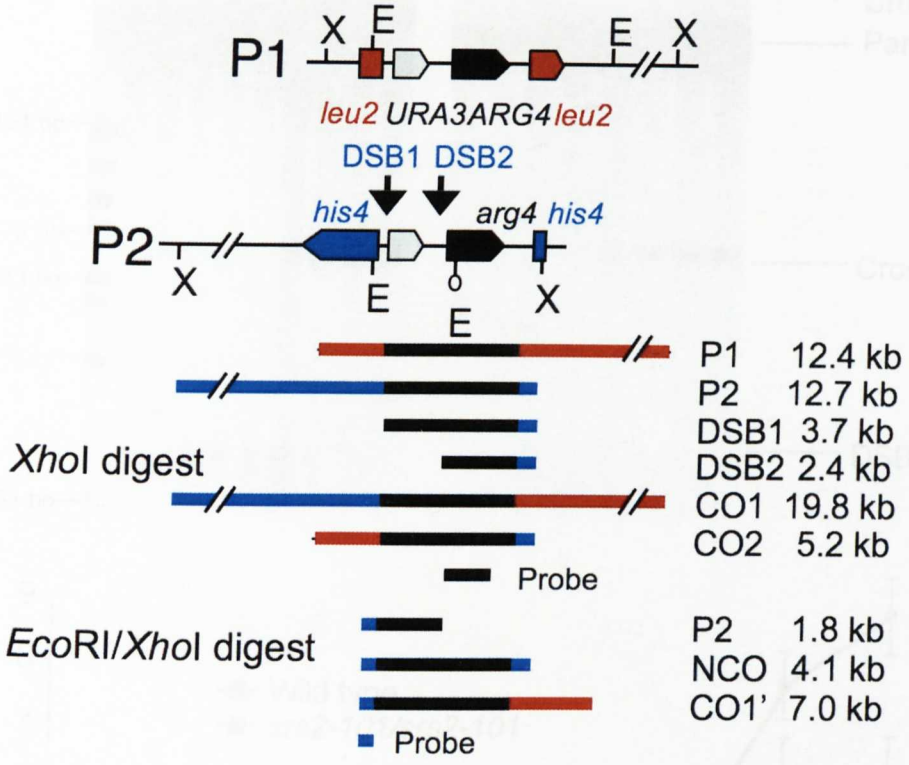


Figure 4.16 Reporter cassette to detect ectopic crossovers at the *URA3 ARG4* interval.

The Spo11-DSBs flank *URA3,* a donor sequence is inserted at *his4.* DNA can be digested with Xho1 and probed with the *ARG4* sequence to detected DSBs and COs. The crossover product can be seen at 5 h, 6 h, 7 h and 8 h as a 19.8 Kb band above the parental band and 5.2 kb below the parental band. NCO and CO products can be detected by digesting with *EcoR*1 and *Xho*1 and probing with *HIS4* sequence. NCO can be seen as a 4.1kb band COs can be seen as 7.0 kb band.

Figure taken from (Jessop, Rockmill et al. 2006).

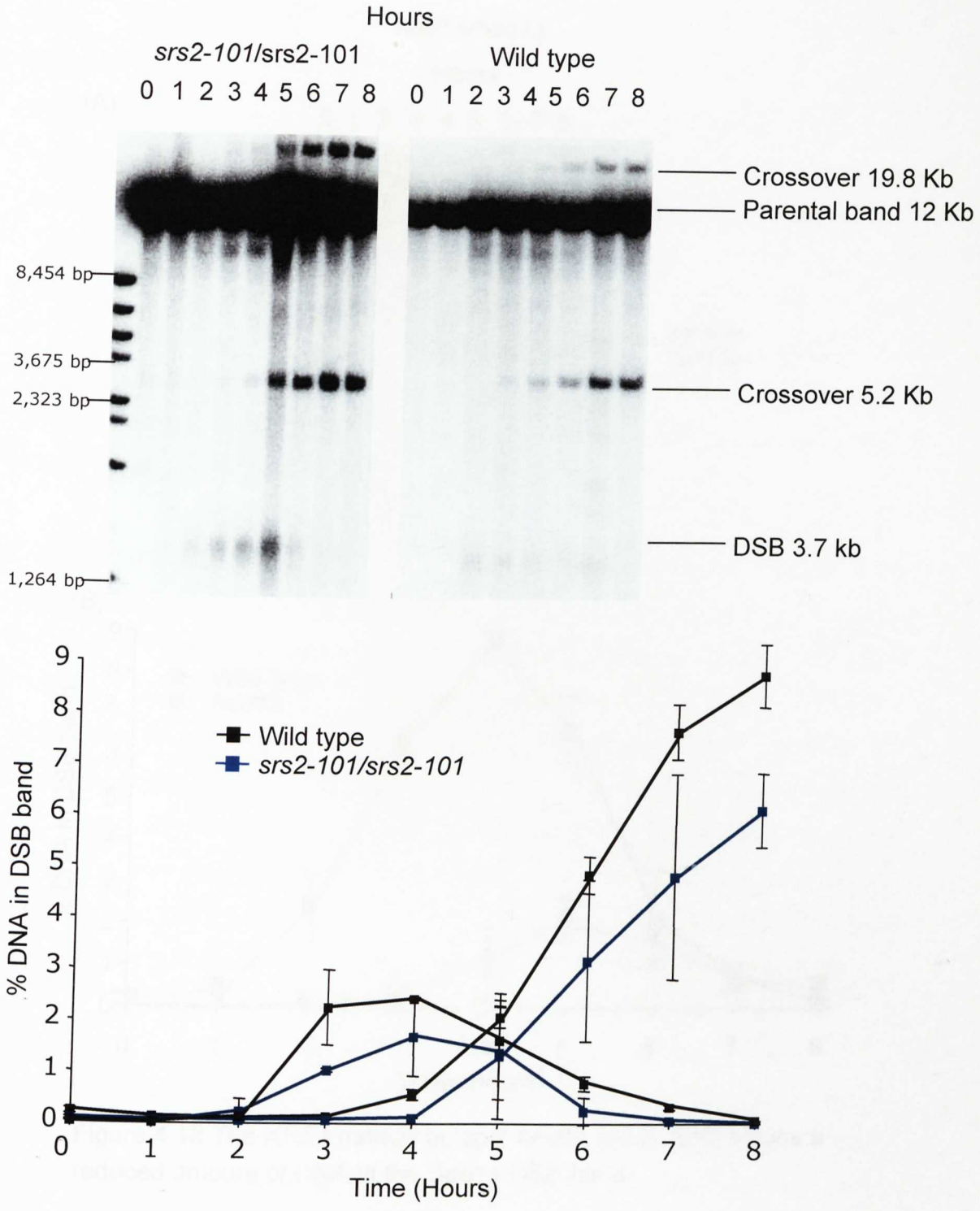


Figure 4.17 Ectopic recombination reduced in at Spo11-DSB.

(A) DNA was extracted from synchronous meiotic cultures of *srs2-101* homozygous and wild type cells. (A) Quantification of blot in (A). There is slightly more DNA in the DSB band in wild type cells, however there is no statistical difference between *srs2-101* and wild type. The amount of ectopic repair in *srs2-101* homozygous cell is reduced when compared to wild type.

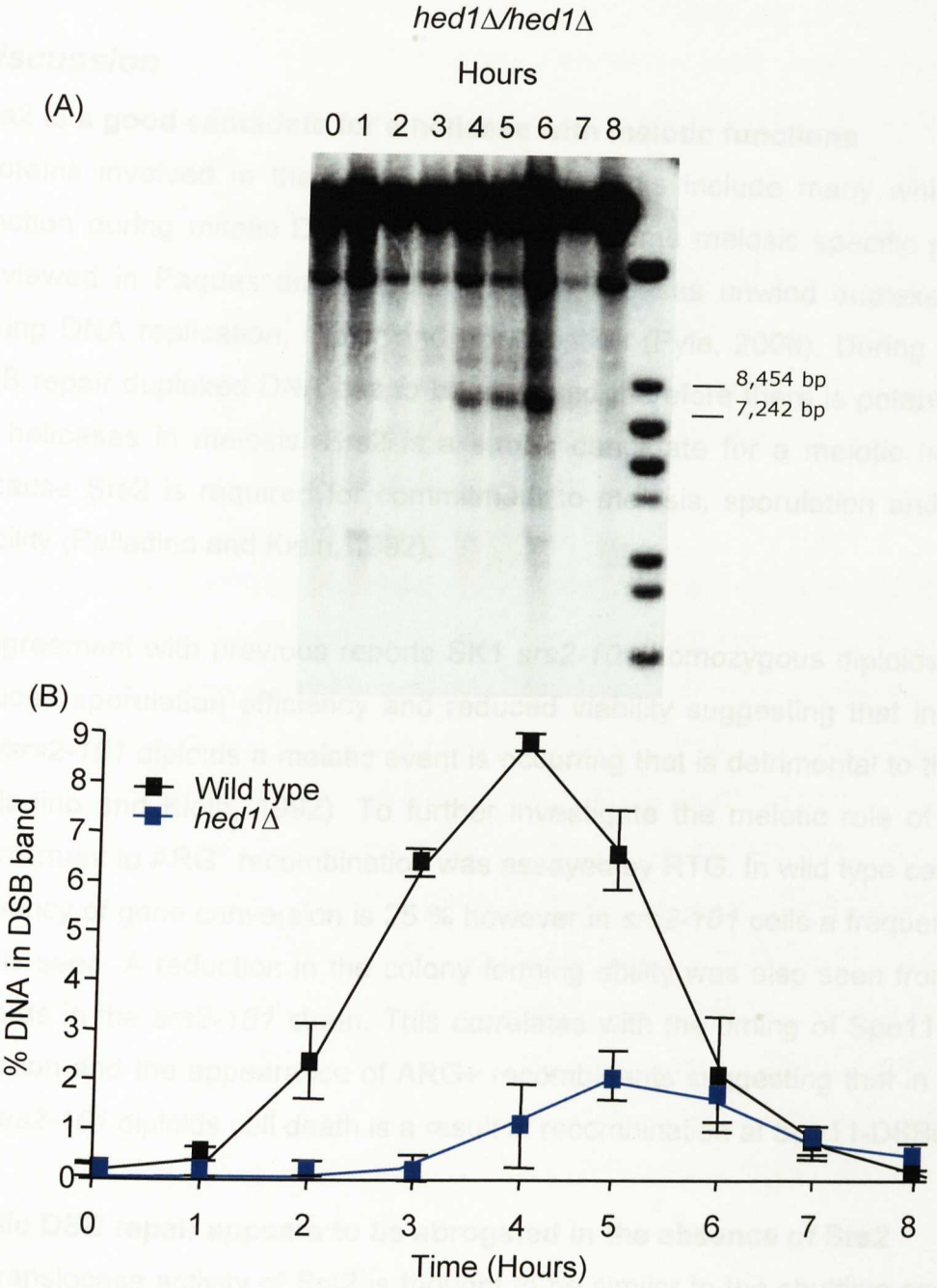


Figure 4.18 The *ARE1* natural hotspot *hed1Δ* (dAG1530) shows a reduced amount of DNA In the Spo11-DSB band.

(A) DNA was extracted from wild type and mutant *hed1Δ* synchronous meiotic and southern blotted. (B) Quantification of blot in (A). When compared to wild type the amount of DNA in the Spo11-DSB is reduced in *hed1Δ*. The maximal amount of DNA in the Spo11-DSB band is 2 % compared to 9 % in wild type cells. Also a delay is seen in *hed1Δ* cells maximal amount of DNA in the Spo11-DSB band is reached at 5 h, however in wild type cells peak is reached earlier at 4 h.

Discussion

Srs2 is a good candidate for a helicase with meiotic functions

Proteins involved in the repair of Spo11 breaks include many which also function during mitotic DNA repair, and also some meiosis specific proteins (reviewed in Paques and Haber, 1999). Helicases unwind duplexed DNA during DNA replication, repair and transcription (Pyle, 2008). During meiotic DSB repair duplexed DNA has to be unwound therefore there is potential role for helicases in meiosis. Srs2 is a strong candidate for a meiotic helicase because Srs2 is required for commitment to meiosis, sporulation and spore viability (Palladino and Klein, 1992).

In agreement with previous reports SK1 *srs2-101* homozygous diploids show reduced sporulation efficiency and reduced viability suggesting that in *srs2-101/srs2-101* diploids a meiotic event is occurring that is detrimental to the cell (Palladino and Klein, 1992). To further investigate the meiotic role of Srs2, commitment to ARG^+ recombination was assayed by RTG. In wild type cells the frequency of gene conversion is 25 % however in *srs2-101* cells a frequency of 5 % is seen. A reduction in the colony forming ability was also seen from 3 h onwards in the *srs2-101* strain. This correlates with the timing of Spo11-DSB formation and the appearance of ARG+ recombinants suggesting that in *srs2-101/srs2-101* diploids cell death is a result of recombination at Spo11-DSBs.

Meiotic DSB repair appears to be abrogated in the absence of Srs2

The translocase activity of Srs2 is thought to be similar to the shuttling seen in Rep, a bacterial Srs2 homologue involved in replication restart (Myong et al., 2005). A possible function of the repetitive shuttling exhibited by Rep is to remove unwanted proteins from ssDNA at a stalled replication forks (Myong et al., 2005). In an early step of recombination, proteins such as Rad51 bind to ssDNA forming a structure known as Rad51 nucleofilaments (reviewed in Paques and Haber, 1999). During meiosis these structures mark the sites of recombination (Rockmill et al., 1995). Srs2 has been suggested to prevent crossovers by dismantling Rad51 presynaptic filaments by its translocase

activity in mitosis (Veaute et al., 2003). If Srs2 is required to remove Rad51 from ssDNA during meiotic DSB repair then an increase in recombination is could occur in *srs2-101* mutant strains. Hyper-recombination mutants exhibit altered SC formation this is also seen in our *srs2-101* strain (Rockmill, Fung et al. 2003). An increased frequency of recombination could also result in chromosomal re-arrangements or a change in crossover frequency (discussed in Foiani, 2003). In meiosis a change in the frequency of crossovers between homologous chromosomes could prevent the homologues from segregating correctly, potentially explaining the reduced viability in *srs2-101* diploids after 3 h of meiosis.

A hyper-recombination phenotype could increase the rate of meiotic DSB repair, because recombination events that are normally prevented in wild type are to mature into crossovers and noncrossover products. At the VDE-DSB the rate of DSB turn over was wild type in the heterozygote as was the proportion of repair by SSA. In the homozygote strain a higher percentage of DNA is in the VDE-DSB between 2 h and 6 h compared to wild type. This suggests that the rate of VDE-DSB is altered and there is a delay in repair. Repair at the VDE-DSB site is normally indicative of the kinetics of repair at the natural Spo11-DSBs. A natural Spo11-DSB hotspot was also assayed by Southern blot. Unlike the VDE-DSB the heterozygote strain showed a change in repair kinetics. A reduction in the proportion of DNA in the DSB band is seen in both the homozygote and the heterozygote. This was shown to be a result of faster DSB repair.

In meiosis recombination can occur between the DSB and one of three templates the: homologous chromosome, the sister chromatid or an ectopic locus. However for a successful meiotic division repair using the homologue is imperative. An increase in recombination could result in reduced regulation of partner choice. To see if there is an increased frequency of ectopic recombination an ectopic reporter cassette was used to assay the VDE-DSB and a Spo11-DSB. In both experiments the frequency of ectopic recombination was reduced in the *srs2-101* strain. A reduction in gene conversion events and

a decrease in ectopic recombination suggests that less recombination is happening in the cell. An alternative explanation is that DSB repair is being directed towards the sister chromatid which can be detected by 2D gels.

Srs2 might prevent the inter sister repair.

Rad51 mediated repair is biased towards the sister chromatid during mitosis. Dmc1 is only expressed in meiosis and is thought to direct repair towards the homologous chromosome (reviewed in Paques and Haber, 1999). If Srs2 is required to remove Rad51 from meiotic DSB sites in the absence of Srs2 an excess of Rad51 might push repair towards the sister chromatid rather then the homologous chromosome. The faster turn over of the *ARE1* DSB seen in the *srs2-101/srs2-101* strain might be a result of an excess of Rad51, because repair of the *ARE1* DSB in *srs2-101/srs2-101* diploids is similar to that seen in the *hed1*Δ mutant, which negatively regulates transcription of Rad51 during meiosis (Busygina et al., 2008; Tsubouchi and Roeder, 2006). The increased rate of DSB repair at the Spo11 hotspot is consistent with repair being directed towards the sister. In meiosis the sister chromatids are held together via the cohesion complex and consequently the search for homology could be quicker if the strand invasion event occurs between the broken duplex and the sister. This can also explain the different repair profiles of the VDE-DSB and the natural Spo11-DSB, because at the VDE-DSB site both the sisters are broken. Consequently Rad51 maybe unable to direct repair towards the sister and the template has to originate from an alternative locus, such as the homologous chromosome. To achieve this the DSB ends may have to undergo more resection therefore repair is slowed.

Additionally Srs2 might also dismantle strand invasion events that occur between the sister chromatids. Sgs1 is suggested to dismantle recombination intermediates that are not protected by ZMM proteins (Jessop et al., 2006). Also *sgs1*Δ*srs2*Δ mutants are lethal due to excessive recombination, indicating the lethality in *sgs1*Δ*srs2*Δ cells is due to a accumulation toxic recombination intermediates normally dismantled by Srs2 or Sgs1 (Gangloff et al., 2000; discussed in Foiani, 2003). The substrate might be Rad1 mediated inter sister

recombination intermediates. Therefore in an *srs2-101* diploid, not only is there an excess of Rad51 at the DSBs directing repair towards the sister, but also Srs2 is not dismantling recombination intermediates formed between the sisters. Therefore in the absence of Srs2 meiotic DSB repair is more rapid but toxic recombination intermediates are formed. However when *srs2-101* viable tetrads are dissected the result is not indicative of increased intersister repair, in *srs2-101* cells Dmc1 is still present pushing repair towards the homologous chromosome also the barrier to intersister repair is present. Consequently in an *srs2-101* diploid repair using the sister is possibly increased but repair using the homologous chromosome is not absent.

If repair in *srs2-101/srs2-101* is directed towards the sister chromatid Srs2 has an extremely important role. Srs2 is potentially required to prevent Rad51 mediated repair during meiosis ensuring that crossovers occur between the homologue to help guarantee correct alignment during meteaphase. If in the absence of Srs2, repair is directed towards the sister this strengthens the theory that Dmc1 directs repair towards the homologous chromosome. In this model the reduced viability in *srs2-101* cells would be due to incorrect homologue alignment and toxic recombination intermediates and chromosomal rearrangements causing cell death.

Chapter Five – *RAD6* influences meiotic DSB repair

Brief introduction

Histones are components of nucleosomes in which DNA is packaged, allowing the genome to be organised but accessible for replication, transcription and DNA repair. Histones form an octomer comprised of one H3-H4 tetramer between two H2A-H2B dimers (Luger, Mader et al. 1997). DNA is wound around the octomer and each nucleosome is joined to its neighbour by a stretch of linker DNA; histone H1 binds the linker DNA and the nucleosome (Kaczanowski and Jerzmanowski 2001). In the presence of H1 the "chain" structure can form a helical structure 30 nm in diameter termed the 30 nm fibre (Felsenfeld and Groudine 2003). Histones have tail extensions which project from the nucleosome and are therefore easily accessible and have been shown to be modified.

Post-transcriptional modification of histones can occur in a variety of ways including ubiquitination, acetylation, methylation and phosphorylation and are thought to be important for the interaction between the dimers and the tetromer therefore possibly influencing nucleosome structure (Felsenfeld and Groudine 2003). The modifications normally occur in the N-terminal tails however modifications in C-tails have also been reported. Histone modifications are implicated in regulating DSB repair events in mitosis (Tsukuda et al., 2005).

The ubiquitin system is an enzymatic cascade which allows long chains of ubiquitin to be formed by attaching a UBIQ to a Lysine of another UBIQ (Li and Ye 2008). Ubiquitin chains are normally associated with protein degradation, however mono-ubiquitination might be involved in protein signalling (Raiborg, Slagsvold et al. 2006). Deubiquitination or ubiquitylation depends upon an activating enzyme (E1), a conjugating enzyme (E2) and an ubiquitin ligase (E3). For ubiquitination to occur an E1 activates ubiquitin in the presence of ATP then transfers it to the active cysteine residue of an E2. In the final step UBIQ is transfered from the E2 to a lysine on the substrate protein in the presence of an

E3 (Li and Ye 2008). Malfunctions in the ubiquitin system have been associated with cancer, HIV and neurodegenerative diseases (Petroski 2008).

The ubiquitination of H2B is involved in both gene silencing and transcription. Ubiquitination is dependent upon the E3 Bre1 (Kao et al., 2004). H2B ubiquitination has been linked with regulating transcription of *GAL4* and *ACT1* and is absent from silent chromatin. Ubiquitination is normally associated with protein transportation and degradation (Ulrich, 2002). However while long chains of ubiquitin appear to signal protein turnover, monoubiquitination does not. Consequently monoubiquitination is thought to be associated with signalling and structural modifications (Ulrich, 2002).

Histone modifications are also suggested to affect the distribution of Spo11-DSB, which is known to relate to chromatin structure. Sir2 is a deacetylase, one of whose substrates is H4K16. In *sir2*Δ the distribution of Spo11-DSB is altered, this has been attributed to the H4 modification possibly allowing Spo11 / recombination machinery access to the DNA (Kao et al., 2004). Also Set1 a known H3 methyltransferase is required for wild type DSB frequency at the *CYS3* hotspot (Sollier et al., 2004).

Rad6 is an E2 ubiquitin-conjugating enzyme that has been shown to monoubiquitinate the histone H2B at lysine 124 in *S. cerevisiae* (Robzyk et al., 2000) Rad6 is implicated in diverse cellular functions including post replication repair, transcription and gene silencing. In mitosis *rad6*Δ cells show growth retardation on rich medium and are sensitive to IR, UV and MMS (Prakash et al., 1993). Regulation of meiotic events by Rad6 is also reported including chromosome compaction and expansion that possibly aids pairing (Kleckner, Zickler et al. 2004). During the meiotic division *rad6*Δ cells exhibit a delayed entry in to pre-meiotic S-phase and late prophase I arrest. The formation of Spo11-induced DSBs is also delayed with a reduction in the formation of DSBs at stronger hotspots (Yamashita et al., 2004)..

Histone modifications possibly allow or regulate Spo11 or recombination machinery access to the DNA or stabilise DNA protein interactions. Therefore Rad6 dependent ubiquitination of H2B might influence the processing of meiotic DSBs. This is supported because Rad6 dependent ubiquitination of histone H2B has been suggested as the direct cause for *rad6*Δ mutants failing to sporulate, and their reduced amount of DSBs. To establish if Rad6 ubiquitination of H2B important for wild type timing and processing of meiotic DSBs the VDE-DSB was assayed in a non-ubiquitinatable H2B mutant and in *rad6*Δ cells.

Results

Meiotic progression and viability of the *rad6Δ* strain

In previous reports the majority of synchronously sporulated *rad6Δ* cells arrest during prophase and are unable to form spores (Robzyk et al., 2000; Yamashita et al., 2004). To analyse meiotic progression of *rad6Δ* in our hands diploid cells (dAG1313) were synchronously sporulated and treated with the DNA stain DAPI. Consistent with previous reports, *rad6Δ* cells are delayed entering the first meiotic division (Fig 5.1). In wild type cells the majority of the cells have two DAPI stained nuclei at 6 h, at 7 h 50 % have more than 2 nuclei. In the *rad6Δ* strain only cells with one DAPI stained body are visible at 6 h and at 7 h only 40 % have more then one nucleus. Surprisingly in the *rad6Δ* strain 90 % of cells have four DAPI stained bodies at 8 h comparable to wild type, however no mature tetrads are formed (Fig 5.1).

Spo11-DSB are reduced in *rad6Δ* cells

Previously a reduction in the frequency of Spo11-DSB has been reported at Spo11 hotspots in *rad6Δ* cells (Yamashita et al., 2004). To check that our *rad6Δ* diploid behaved consistently with previous reports, the frequency of DSB formation at the *ARE1* hotspot was assayed (Fig 5.2). In wild type cells the amount of DNA in the Spo11-DSB band reaches a peak of 9 % at 4 h. In *rad6Δ* cells the amount of DNA in the Spo11-DSB band reaches a peak of 6 % at 5 h. The result indicates that Spo11-DSBs at this locus are reduced and are formed later compared to wild type consistent with the reported *rad6Δ* mutant phenotype. The delay in Spo11-DSB formation is also consistant with the reported delay in replication seen in *rad6Δ* cells (Yamashita, Shinohara et al. 2004).

Timely repair of the VDE-DSB is dependent on Rad6

The VDE reporter cassette was assayed to see if repair of the VDE-DSB is changed when fewer Spo11-DSB were present in the cells (Fig 5.3). In a wild type cell the VDE homing endonuclease forms DSBs at the same time as Spo11. In *rad6Δ* cells Spo11-DSBs appear one hour later than in a wild type cell

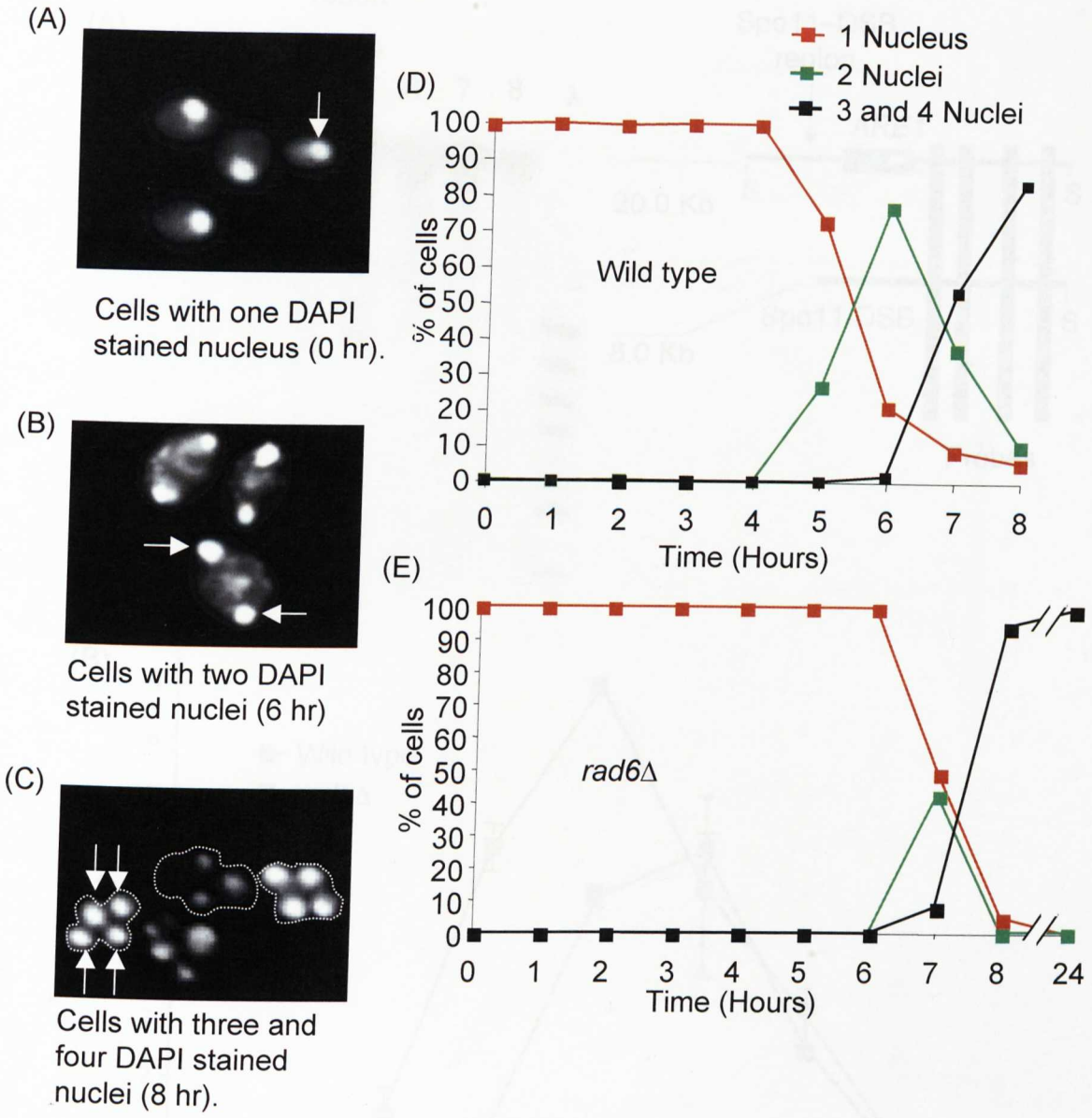


Figure 5.1 Meiotic checkpoint progression in *rad6*Δ cells (dAG1313).

Meiotic progression was determined by scoring MI and MII nuclea divisions. Cells were fixed in ethanol and stained with DAPI. Samples were visualised using fluorescence microscopy and scored for the number of DAPI–staining bodies. completion of meiosis I is indicated by cells containing 2 nuclei. Cells with 3 or 4 nuclei represent the cumulative total of cells that have passed meiosis I. (A)-(C) Examples of wild type cells at t=0. t=6 and t=8. (D) wild type meiotic progression. Only cells with 1 nucleus are visible between 1 h and 4 h, cells with 2 nuclei are prevalent at 6 h. At 8 h the majority have 4 nuclei. (E) Meiotic progression of *rad6*Δ cells both divisions are delayed compared to wild type cells. At 7 h in the *rad6*Δ mutant there is an equal amount of cell with 1,2,3 and 4 DAPI stained bodies. In wild type at 7 h only a small number of cells with 1 DAPI stained body is visible. In both strains both meiotic divisions are completed by 8 h.

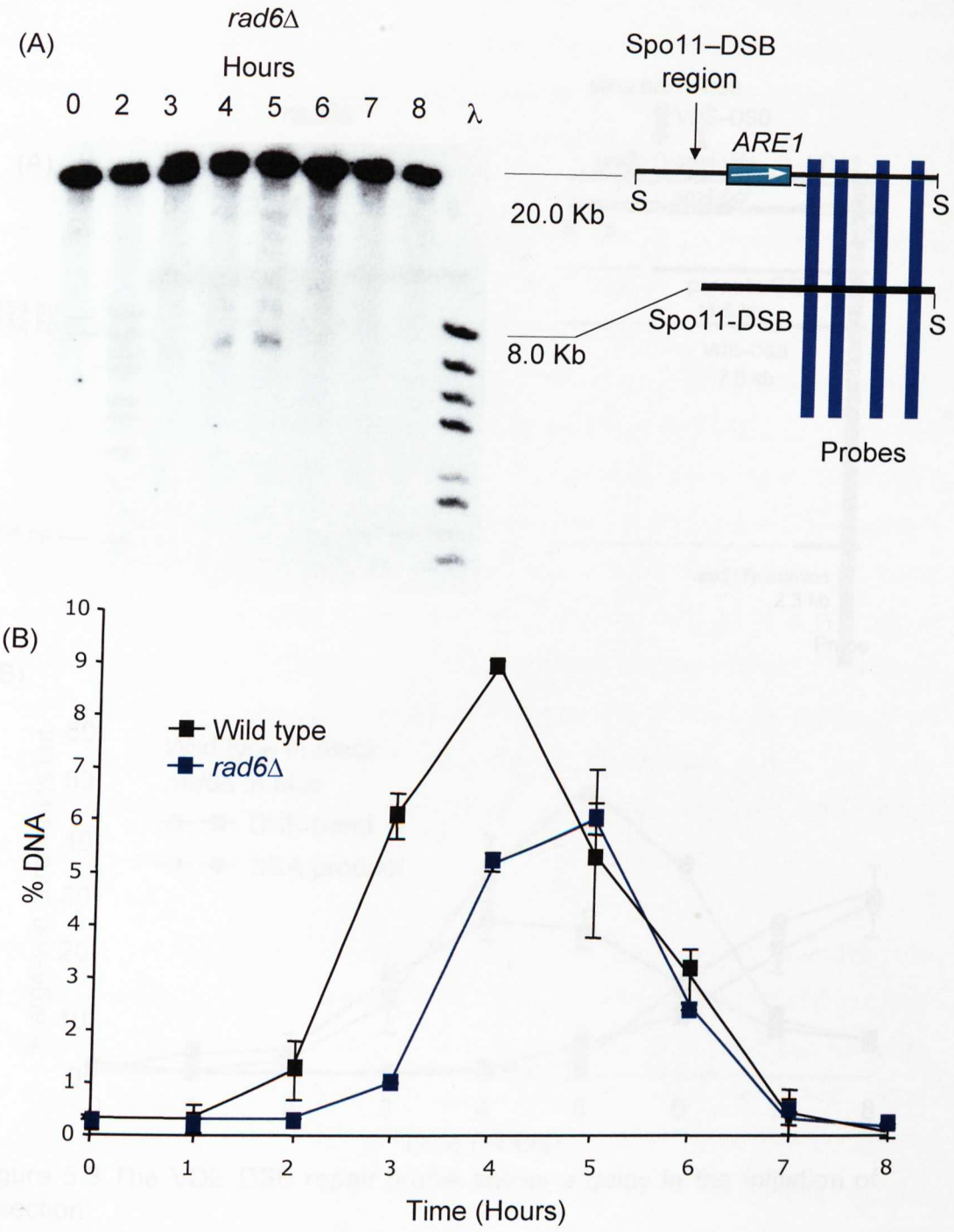


Figure 5.2 A reduced amount of DSB is seen at the natural *ARE1* Spo11 DSB hotspot.

(A) DNA was extracted from *rad6Δ* synchronous meiotic time course and southern blotted. The filter was hybridized with a probes specific to a regions downstream of *ARE1* on Chr. *III*. (B) Quantification of the southern blot in (A) compared to the wild type strain. The maximal amount of DNA in the *rad6Δ* *ARE1*-DSB band peaks at a lower percentage then in wild type. Normally the maximal amount of DNA in DSB (9%) is reached at 4 h, in the *rad6Δ* strain 6 % reached by 5 h.

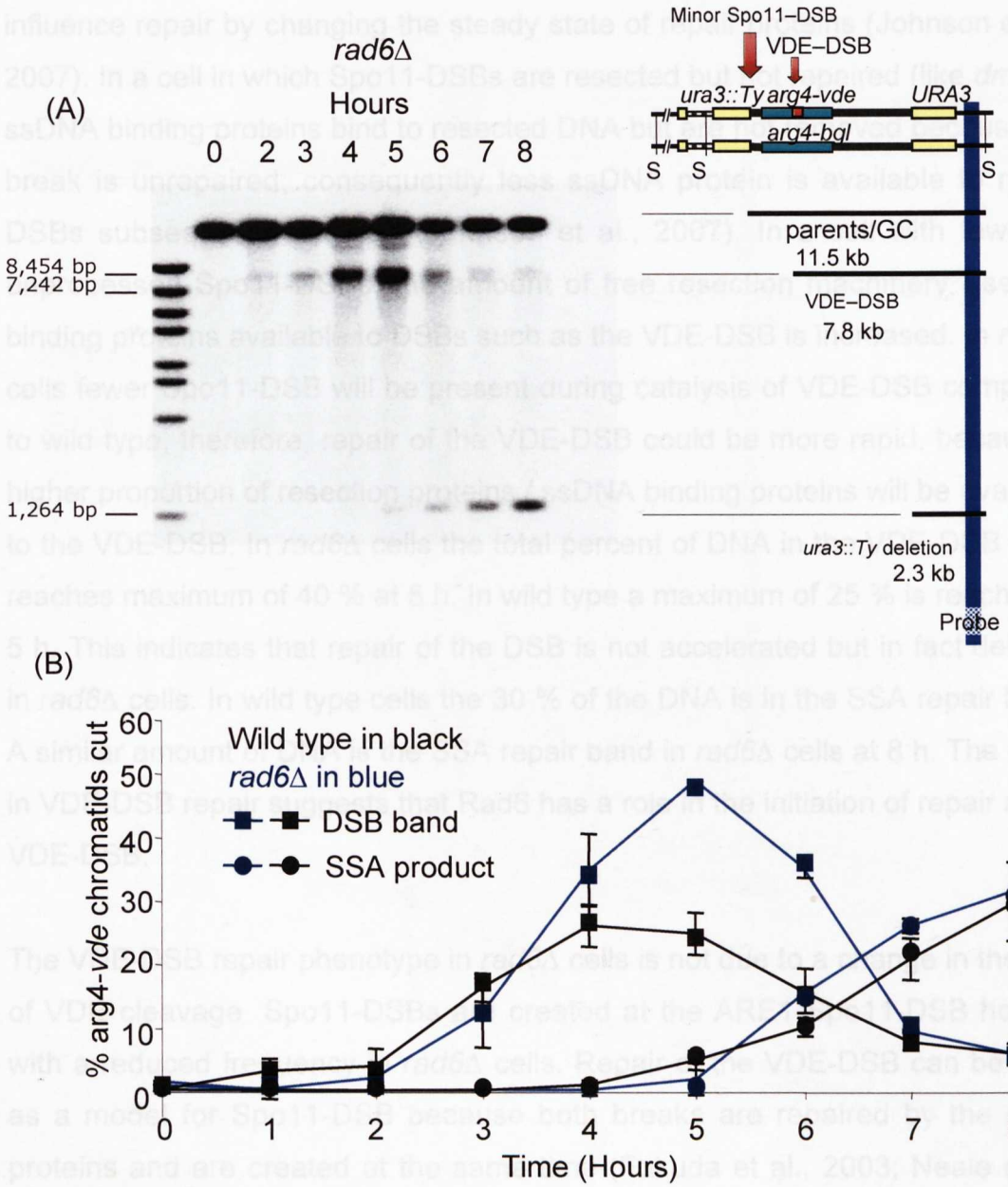


Figure 5.3 The VDE DSB repair profile shows a delay in the initiation of resection.

(A) *rad6Δ* DNA was extracted from synchronous meiotic cultures, digested with *SpeI* and fractionated on 0.5% agarsoe gel, Blotted on to a nylon membrane and hybridised with a 1 kb probe specific to the region of chromosome V. The VDE-DSB is not a discrete band as seen in wild type, a smear is seen under the DSB band, the smear is characteristic of a delay in resection containing accumulated unresected structures that are normally processed. (B) Quantification using scanning densitometry of the blot in (A). A higher percentage of DNA is seen in the maximal value of VDE-DSB band in the *rad6Δ* mutant 50 % compared to 20 % in wild type, this is indicative of a delay in repair at the VDE-DSB. In *rad6Δ* and wild type cells the proportion of the DSB by SSA is essentially the same.

and fewer are catalysed (Fig 5.2). Proteins required for DSB repair are limited during meiosis, therefore the amount of DSB created during meiosis can influence repair by changing the steady state of repair proteins (Johnson et al., 2007). In a cell in which Spo11-DSBs are resected but not repaired (like *dmc1*Δ) ssDNA binding proteins bind to resected DNA but are not removed because the break is unrepaired; consequently less ssDNA protein is available to repair DSBs subsequently created (Johnson et al., 2007). In a cell with fewer or unprocessed Spo11-DSBs, the amount of free resection machinery / ssDNA binding proteins available to DSBs such as the VDE-DSB is increased. In *rad6*Δ cells fewer Spo11-DSB will be present during catalysis of VDE-DSB compared to wild type, therefore, repair of the VDE-DSB could be more rapid, because a higher proportion of resection proteins / ssDNA binding proteins will be available to the VDE-DSB. In *rad6*Δ cells the total percent of DNA in the VDE-DSB band reaches maximum of 40 % at 5 h. In wild type a maximum of 25 % is reached at 5 h. This indicates that repair of the DSB is not accelerated but in fact delayed in *rad6*Δ cells. In wild type cells the 30 % of the DNA is in the SSA repair band. A similar amount of DNA is the SSA repair band in *rad6*Δ cells at 8 h. The delay in VDE-DSB repair suggests that Rad6 has a role in the initiation of repair at the VDE-DSB.

The VDE-DSB repair phenotype in *rad6*Δ cells is not due to a change in the rate of VDE cleavage. Spo11-DSBs are created at the ARE1 Spo11-DSB hotspot with a reduced frequency in *rad6*Δ cells. Repair of the VDE-DSB can be used as a model for Spo11-DSB because both breaks are repaired by the same proteins and are created at the same time (Fukuda et al., 2003; Neale et al., 2002). Therefore the efficiency of VDE cleavage might also be affected by the absence of Rad6, consequently the kinetics of VDE-DSB repair seen in the *rad6*Δ cells might be due to a change in the rate of VDE-DSB creation. The rate of cleavage by VDE can be assayed by southern blots. DNA was extracted from *rad6*Δ cells and processed as described in chapter 3. The rate of VDE cleavage in *rad6*Δ cells and wild type cells is essentially the same (Fig 5.4).

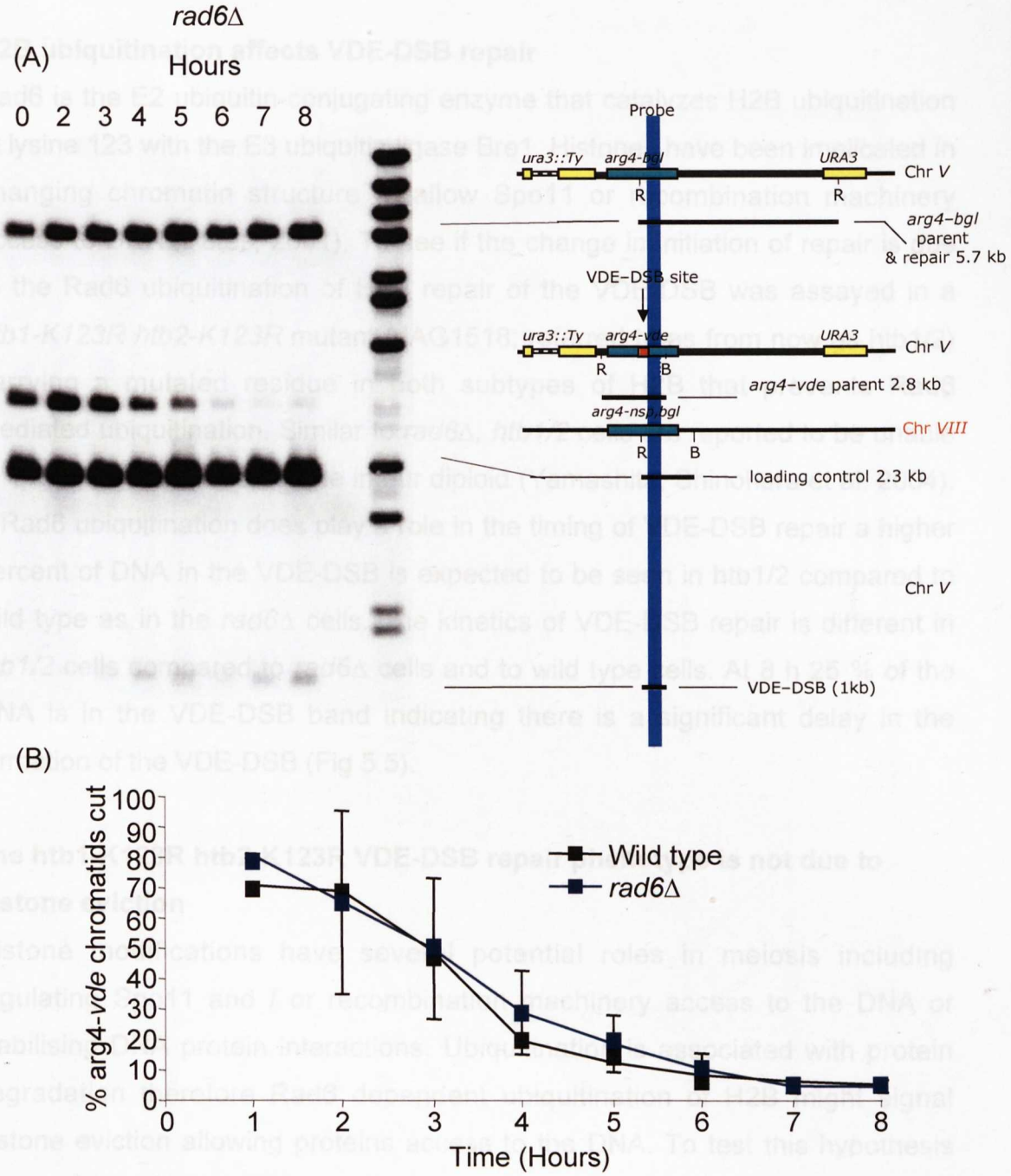


Figure 5.4 Proportion of uncut arg4-VDE chromatids.

(A) Cells from a *rad6*Δ sporulation were removed at hourly intervals and processed as described in chapter 3. (B) The rate of cleavage in *rad6*Δ is similar to wild type.

H2B ubiquitination affects VDE-DSB repair

Rad6 is the E2 ubiquitin-conjugating enzyme that catalyzes H2B ubiquitination at lysine 123 with the E3 ubiquitin ligase Bre1. Histones have been implicated in changing chromatin structure to allow Spo11 or recombination machinery access to DNA (Petes, 2001). To see if the change in initiation of repair is due to the Rad6 ubiquitination of H2B repair of the VDE-DSB was assayed in a *htb1-K123R htb2-K123R* mutant (dAG1518; referred to as from now as htb1/2) carrying a mutated residue in both subtypes of H2B that prevents Rad6 mediated ubiquitination. Similar to *rad6Δ, htb1/2* cells are reported to be unable to sporulate this was also true in our diploid (Yamashita, Shinohara et al. 2004). If Rad6 ubiquitination does play a role in the timing of VDE-DSB repair a higher percent of DNA in the VDE-DSB is expected to be seen in htb1/2 compared to wild type as in the *rad6Δ* cells. The kinetics of VDE-DSB repair is different in *htb1/2* cells compared to *rad6Δ* cells and to wild type cells. At 8 h 25 % of the DNA is in the VDE-DSB band indicating there is a significant delay in the formation of the VDE-DSB (Fig 5.5).

The htb1-K123R htb2-K123R VDE-DSB repair phenotype is not due to histone eviction

Histone modifications have several potential roles in meiosis including regulating Spo11 and / or recombination machinery access to the DNA or stabilising DNA protein interactions. Ubiquitination is associated with protein degradation therefore Rad6 dependent ubiquitination of H2B might signal histone eviction allowing proteins access to the DNA. To test this hypothesis chromatin analysis was performed in collaboration with Dr. Nick Kent (Cardiff University). In this assay chromatin is digested with Micrococcal nuclease (MNase), chromatin is only accessible to MNase in the absence of nucleosomes therefore if H2B is degraded after Rad6 mediated mono ubiquitination the MNase digestion pattern should change in *rad6Δ* cells. The MNase cleavage pattern in *rad6Δ* cells does change during the timecourse therefore there is chromatin remodelling at the VDE-DSB between 2 h and 6 h

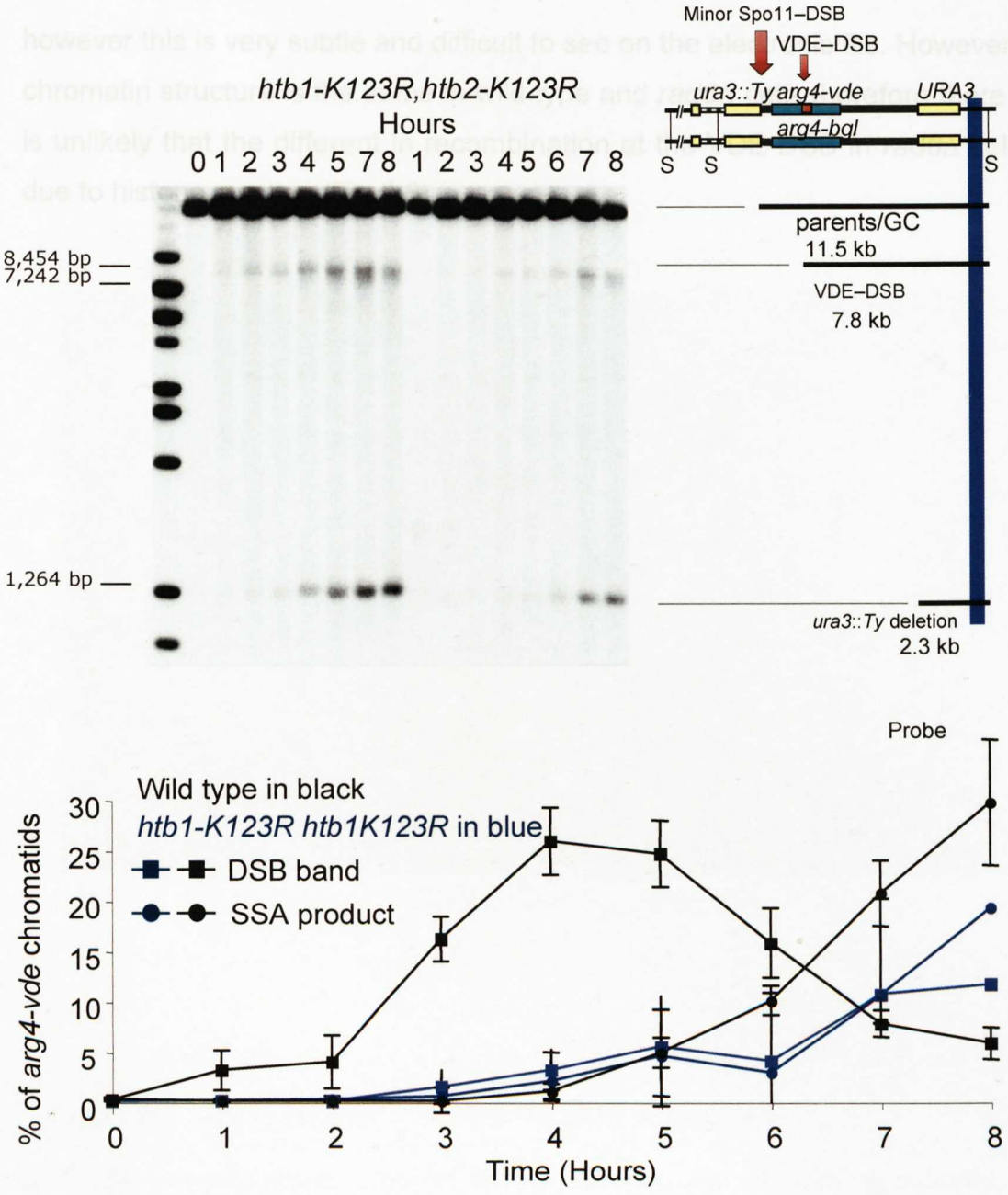


Figure 5.5 H2B ubquitination is required for wild type kinetics at the VDE-DSB.

(A) DNA was extracted from *htb1/2* cells and processed as for Fig 5.2 (B) Quantification of the blot in (A). In a wild type culture less then 10 % of the DNA is present at 8 h however, in the histone mutant 25 % of the DNA is present at 8 h. The amount of DNA in the SSA repair product band is also reduced at 8 h there is 15 % of the DNA in SSA repair band in the histone mutant compared to 30 % in wild type. This illustrates that H2B unbitination is required for normal DSB repair.

however this is very subtle and difficult to see on the electronic file. However the chromatin structure is the same in wild type and *rad6*Δ cells therefore there is it is unlikely that the different in recombination at the VDE-DSB in *rad6*Δ cells is due to histone eviction (Fig 5.6).

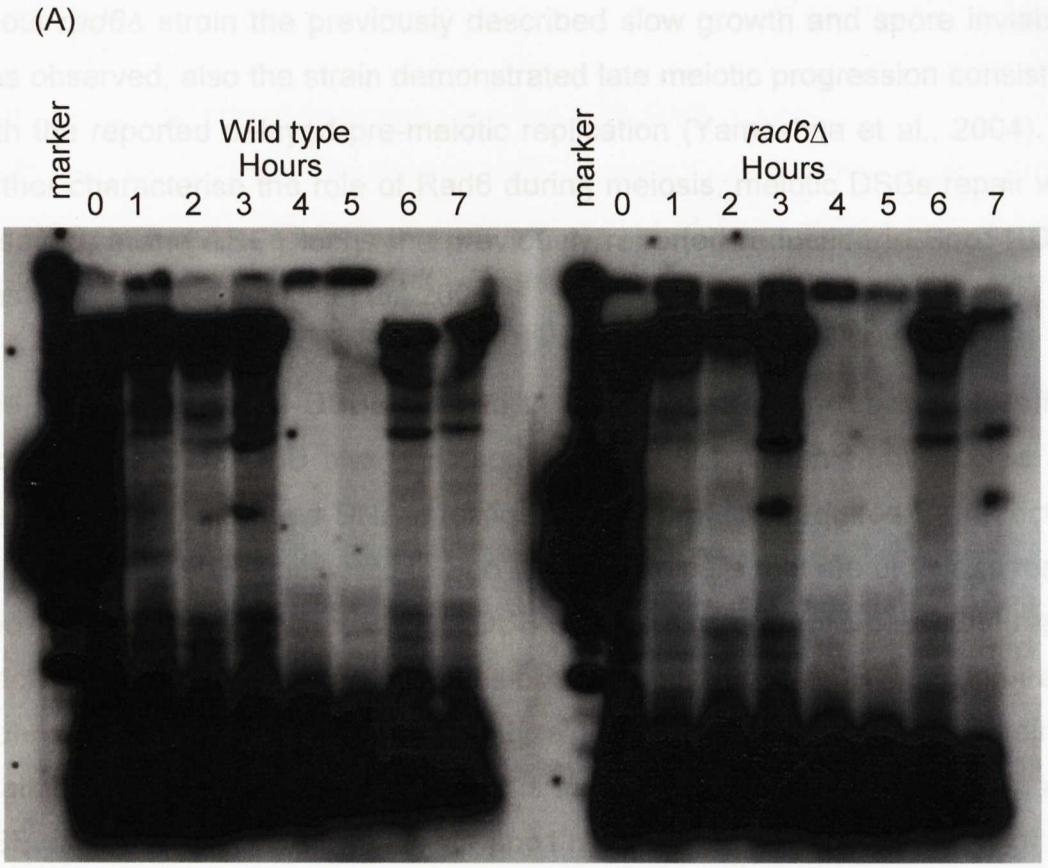


Figure 5.6 Mnase digestion of the VDE-DSB in *rad6* cells.

Hourly samples were removed from synchronously sporulating cultures of *rad6*Δ and wild type cells. The chromatin was digested with MNase and *Nru*I then fractionated on an agarose gel and southern blotted. The blots were probed with a PCR product specific to the *arg4* region (amplified by CA probe F and CA probe R). No change is visible in Mnase accessibility between *rad6*Δ cells and wild type cells.

Discussion

In our *rad6*Δ strain the previously described slow growth and spore inviabilty was observed, also the strain demonstrated late meiotic progression consistent with the reported delayed pre-meiotic replication (Yamashita et al., 2004). To further characterise the role of Rad6 during meiosis, meiotic DSBs repair was assayed, at the ARE1 locus the previously reported reduction in Spo11-DSB was detected (Yamashita et al., 2004).

The amount of Spo11-DSBs created in the cell has been reported to affect repair at the VDE-DSB site (Johnson et al., 2007). When Spo11-DSBs are resected single stranded DNA is exposed. The proteins required for resection and repair can be sequestered to Spo11-DSBs. In cells that are unable to repair Spo11-DSBs but are able to resect DSB ends (such as *dmc1*Δ cells), the repair machinery (for example RPA) remains bound to the Spo11-DSB resection tracts (Johnson et al., 2007). Consequently a lower proportion of free protein is available to the VDE-DSB and repair is inefficient. In cells with fewer Spo11-DSB or unresected DSB (such as spo11-Y135F) a higher proportion of the resection machinery is available to process the VDE-DSB and repair is efficient (Johnson et al., 2007). In *rad6*Δ cells few Spo11-DSBs are thought to be created at hot spots which was observed at the *ARE1* site, therefore repair of the VDE–DSB was expected to be efficient and more rapid however VDE-DSB repair in the *rad6*Δ strain is delayed.

In the absence of Rad6 VDE-DSB repair is delayed

In the *rad6*Δ strain VDE-DSB repair is rapid after 5 h into meiosis, the same Spo11-DSBs decline. This indicates both breaks start to repair at the same time therefore they might be repaired by a common mechanism that is dependent upon Spo11-DSBs. In the absence of Rad6 a delay has been reported in replication, a delay in replication causes a delay in Spo11-DSB break formation. Consequently the delay in VDE-DSB repair might be a result of late replication. However the delayed repair in the *rad6*Δ cells is not due a delay in replication because the VDE-DSB is formed on time and can be repaired in the absence of

Spo11-DSBs. The data suggests that Rad6 has a direct role in the initiation of DSB repair. One hypothesised is that this could be related to chromatin structure.

H2B monoubiquitination is required for VDE-DSB repair

Rad6 has been shown to modify H2B by monoubiquitination; the reduced amount of Spo11-DSBs seen in *rad6*Δ mutants has been attributed to the lack of Rad6 dependent monoubiquitination of H2B (Robzyk et al., 2000). To see if the delay in VDE-DSB repair seen in *rad6*Δ is a consequence of Rad6 mediated ubiquitination VDE-DSB repair was also assayed in a *htb1/2* diploid, in this strain the lysine residue cannot be ubiquitinated (Robzyk et al., 2000). In *htb1/2* cells the delay in VDE-DSB repair is more pronounced than the *rad6*Δ diploid. The formation and repair of the VDE-DSB appears to be severely delayed compared to wild type and even more delayed than a *rad6*Δ. During ubiquitination ubiquitin is conjugated to a lysine residue, ubiquitin also contains a lysine therefore can self-conjugate creating polyubiquitin chains. Normally multiubiquitination targets proteins for degradation by proteasome, although monoubiquitination is normally associated with signaling an association with protein degradation has also been reported (Sun and Chen, 2004). In mitosis it is suggested that Ino80-promoted nucleosome displacement is required for efficient Rad51 replacement of RPA during DSB repair at the HO break (Tsukuda et al., 2005). Therefore degradation of H2B at the VDE-DSB break site might be required to allow repair machinery access to the VDE-DSB. Consequently late repair of the VDE-DSB seen in *rad6*Δ and *htb1/2* might be caused by a lack of histone eviction. It is suggested that during transcription H2A and H2B ubquitination affects the higher structure of chromatin, or provides an interaction surface for transcription machinery (Berger, 2002; Berger, 2007). If this is true it could have an impact on meiotic DSB formation and repair; which often is sensitive to similar chromatin limitations as transcription. However there is no detectable change between wild type and the *rad6*Δ or in MNase sensitivity of the chromatin around the VDE-DSB site (In collaboration with Dr.Nick Kent, Cardiff University). The lack of chromatin modification suggests that the delay in VDE-DSB formation and repair is not

due to a lack of histone eviction. However the assay is only able to detect large chromatin modification therefore a small change would be undetectable by the assay.

Another possibility is that Rad6 is not the only E2 required for H2B monoubiquitination. In mice there are two Rad6 sequence homologues HR6A and HR6B. hr6b knockout mice are viable and male mice are sterile, however ubH2B is detected and no change in chromatin modification can be identifyed between wild type and hr6b mice (Baarends et al., 2003). This suggests that in mice HR6A and HR6B are redundant. Also another E2 UbcH6 has also been found that can interact with the human orthologues of Bre3 in vitro and is involved in transcription (Lee et al., 2008). If two E2 conjugating enzymes do function during yeast meiosis this would also explain the different severity of VDE-DSB repair in *rad6Δ* cells and htb1/2 cells. When the RAD6 sequence is entered into BLAST three sequences with a high degree of homology are returned including UBC11, 13 and CDC34. All are ubiquitin conjugating enzymes therefore are possible enzymes that might also be able to ubitquitinate H2B.

Ubiquitination cannot be thought of as a solitary event because it has been reported that Rad6, Bre1 mediated ubiquitination of H2B is a pre-requisite for H3 methylation (Bartova et al., 2008). This has been discovered to be a result of a second mutation in the *htb1/2* strain. This mutation may or may not be present in the assayed diploid because the haploid strains were crossed and then dissected to produce haploids that carried the reporter cassette and the *htb1/2* mutations. Therefore the late repair phenotype seen in the *htb1/2* cells might be more severe compared to *rad6Δ* diploid cells because H2B ubquitination absent but so is H3 methylation.

Chapter Six - General Discussion

Meiosis is a cell division in which one diploid parent cell divides producing four haploid daughter cells (Zickler and Kleckner, 1998; Zickler and Kleckner, 1999). Accurate alignment and segregation of homologous chromosomes during metaphase is critical for a successful meiotic division and viable gametes. Three concomitant events are required for correct homologue alignment and segregation: chromosome pairing, synapsis and recombination (Zickler and Kleckner, 1998; Zickler and Kleckner, 1999). Pairing is the close alignment of homologous chromosomes, synapsis is the formation of a tripartite proteinaceous structure between the homologues along their entire length. During meiosis recombination is initiated at meiotic DSBs. Interchromosomal repair of a meiotic DSB can form a crossover that creates genetic diversity by changing linkage; recombination is also required for the formation of chiasmata between the homologous chromosomes that resist the tension of the spindle. Control of recombination starts with regulation of Spo11-DSBs formation.

This thesis investigated the roles of the protein kinase Tel1, the Srs2 helicase and Rad6 mediated histone modification, in regulation of recombination during meiotic DSB repair. All of these protein are required for normal DSB repair. The diversity of the proteins investigated indicates how complex meiotic DSB repair regulation is and how many levels of control are required.

Control at the histone level

Histones have been implicated in two meiotic events, pairing and Spo11-DSB formation (Yamashita et al., 2004; Zickler and Kleckner, 1999). Spo11-DSB hotspots are associated with open chromatin. In *hb1/2*, which is unable to be ubiquitinated, the frequency of Spo11-DSB is reduced and at the VDE site DSBs are formed later. This suggests that histone modification regulates the formation of DSBs during meiosis. Ubiquitination is associated with protein degradation. Histone eviction due to ubiquitination might allow meiotic proteins access to chromatin. However the impaired VDE-DSB formation is not simply due to a lack of histone eviction because chromatin remodelling is not detected in *rad6*Δ cells. Histone modifications have been suggested to create new

binding sites during transcription; this might also be a possible mechanism during meiosis. The role of histones during meiosis is not just during DSB formation; during pairing homologous chromosomes have to recognise each other and become physically close. Chromosome expansion and compaction has been suggested to increase the chances of regions of homology finding each other. Chromosome compaction and expansion is mediated by histones consequently histone modifications is suggested to aid pairing (Zickler and Kleckner, 1999).

Control of meiotic DSB repair

All Spo11-DSBs are repaired by homologous recombination; some proteins that are involved in mitotic homologous recombination are also involved in meiotic DSB repair (Zickler, 2006; Zickler and Kleckner, 1998). Tel1 and Mec1 are protein kinases that function during mitosis and meiosis. In mitosis Tel1 and Mec1 activate a signalling cascade in response to DNA damage (Mantiero et al., 2007; Usui et al., 2001; Usui et al., 1998). In meiosis Tel1 is known to respond to meiotic DSBs therefore it potentially regulates the timing of meiotic DSB repair (Usui et al., 2001). Repair of the VDE-DSB initiates later in *tel1*Δ cells compared to wild type cells, however this late repair is not totally dependent upon the presence of Spo11-DSB. Tel1 has been shown to phosphorylate Sae2 in response to pre-meiotic replication, and the VDE-DSB repair phenotype of *tel1*Δ and *sae2*Δ cells show a similar delay in repair that is not observed in *tel1*Δ *sae2*Δ cells (Palladino and Klein, 1992). This suggests that Tel1 phosphorylation of Sae2 is required for timely repair of meiotic DSBs. In *tel1*Δ and *sae2*Δ strains the proportion of repair by SSA is different, suggesting that once the delay is overcome the method of repair is different in each strain. Tel1 appears to be required for a wild type proportion of recombination repair at the VDE-DSB because a high amount of SSA repair is detected in *tel1*Δ cells and *tel1*Δ *sae2*Δ cells. This indicates that Tel1 is required to ensure that the VDE-DSB is repaired by gene conversion, therefore Tel1 has a potential role ensuring meiotic Spo11-DSB are repaired by recombination. Consequently in the absence of both Tel1 and Sae2 the VDE-DSB is not regulated and a high proportion of DSB repair occurs by SSA.

How could Tel1 phosphorylation of Sae2 be required for prompt DSB repair? Sae2 has recently been proven to have endonuclease activity (Lengsfeld et al., 2007). In mitosis Sae2 is hypothesised to be involved in initiating resection at DSBs that have bulky adducts (discussed in Huertas et al., 2008). This is consistent with the meiotic requirement of Sae2 to remove Spo11 from Spo11-DSB. During meiosis Spo11-DSBs are unrepaired in cells in which Sae2 is present but is unable to be phosphorylated by Tel1 or Mec1, indicating that Sae2 has to be phosophorylated for repair. Sae2, can be phosphorylated by Mec1 therefore in a *tel1*Δ strain Sae2 is not completely unphosphorylated. However Tel1 is thought to respond to blocked DSBs and Mec1 is thought to respond to ssDNA consequently in the absence of Tel1 Sae2 will not be phosphorylated until Mec1 has sensed ssDNA. This might explain the late repair seen in a *tel1*Δ.

Control of the repair template

Helicases are proteins which unwind DNA and RNA duplexes during DNA replication, repair and transcription (Pyle, 2008). There is evidence to link certain helicases with meiotic break repair. For instance Bloom syndrome is caused by a mutation in a human helicase, and one symptom of this is male sterility (German, 1993; Karow et al., 1997). In yeast, Srs2 is a good candidate for a helicase of importance in meiotic recombination because *srs2*Δ cells exhibit delayed commitment to meiosis, a reduction in sporulation and decreased spore viability (Palladino and Klein, 1992). Srs2 belongs to the SF-1 superfamily which have an ATPase activity that possibly allow the proteins to translocate on ssDNA potentally allowing protein displacement from DNA (Myong et al., 2005).

When *srs2-101/srs2-101* cells are removed from meiosis after 3 h and forced to re-enter vegetative growth they show reduced cell viability compared to wild type. Meiotic DSB begin to form at 3 h into meiosis suggesting that in *srs2-101/srs2-101* diploids cell death is a result of meiotic recombination. To see if Srs2 is important for successful meiotic DSB repair, the VDE-DSB was

assayed. At the VDE-DSB *srs2-101/srs2-101* shows a delay in repair. Repair of the VDE-DSB can be used as a model for repair at Spo11-DSB. Therefore for comparison a natural Spo11-DSB hotspot was also assayed. Surprisingly, in *srs2-101/srs2-101* Spo11-DSB breaks were repaired with faster kinetics than wild type. One possible explantion for this result is that recombination is uncontrolled. A possible function of helicase translocation is protein displacement from DNA, perhaps to strip Rad51 from ssDNA preventing recombination. Mitotic Rad51 mediated repair is biased towards the sister chromatid, rather than the homologue. If Srs2 is required to remove Rad51 from meiotic DSB sites, then in the absence of Srs2 an excess of Rad51 at the break might bias repair towards the sister chromatid. The sister chromatids are held together via the cohesin complex and consequently the search for homology might be quicker if the strand invasion event occurs between the broken duplex and the sister than between the break and the homologue. The different repair profiles of the VDE-DSB and the Spo11-DSB can also be explained if template for repair is the sister; at the VDE-DSB site both the sisters are broken, consequently Rad51 is unable to direct repair towards the sister and consequently VDE break the template has to originate from an alternative locus and repair is slowed. Uncontrolled recombination can also occur between the DSB and an ectopic locus. When ectopic repair was assayed at both a Spo11-DSB and the VDE-DSB a decrease was observed consistent with repair using the sister chromatid as a template. Uncontrolled recombination can increase the frequency of COs. One example are *sgs1*Δ cells, these cells also exhibit a change in SC and SIC formation (Rockmill, Fung et al. 2003; Jessop, Rockmill et al. 2006). The SC is a proteinaceous structure, which forms between homologues and is initiated at sites of recombination that form COs. Therefore in cells with increased recombination SC formation might be changed. Results indicate that in *srs2-101/srs2-101* mature SCs persist for longer; the extended presence of SC suggests CO intermediates are being formed but are not resolved with normal kinetics.

If the sister is used as template for repair rather then the homologous chromosome this would explain the reduced viability of *srs2-101* mutants during

meiosis. When repair is directed towards the sister a lower proportion of chromosomes will receive a crossover preventing proper alignment during metaphase. This would increase non-disjunction during meiosis I however *srs2-101* homozygous tetrads do not (Mantiero et al., 2007; Usui et al., 2001; Usui et al., 1998) show an increase in two spore viable spores that normally indicate an increase in non-disjunction during meiosis I. However in an *srs2-101* Dmc1 is still present at the DSB site therefore is still directing repair towards the homologue and Hop1, Mek1 and Red1 are also present blocking sister chromatid repair. Srs2 might also dismantle Rad51 mediated recombination events between the broken duplex and the sister chromatid. Dismantling of recombination events is also suggested to be the role of Sgs1 during meiosis (Jessop et al., 2006). This would also explain the faster turnover of the Spo11-DSB and the difference in repair of the VDE and the Spo11-DSB.

Further work

The results so far suggest that Srs2 is involved in preventing intersister repair, and this hypothesis can be answered using 2-D gels as JM formed between sister chromatids can be detected.

SRS2 could also be placed under the *CLB2* promoter. Clb2 is expressed during the mitotic cell cycle but not during meiosis therefore we can assay the effect of deleting *SRS2* meiosis. Consequently this strain could also be used to see if the loss in viability seen in the RTG is due to DNA damage accumulated during mitosis or as a result of a meiotic defect. Because *srs2-101* cells are known to be hyper recombinant, therefore accumulate gross chromosomal rearrangements during the mitotic cycle. This strain could also be used to see if repair in *srs2-101* strain is dependent upon Rad54 which is required for intersister repair.

Srs2 has been shown to remove Rad51 from DNA *in vitro*. In mitosis the removal of Rad51 by Srs2 is thought to prevent Rad51 from binding to nicks at stalled replication forks, therefore allowing a translesion polymerase to bind and

continue replication. In meiosis Srs2 might prevent Rad51 and Dmc1 to bind to the ssDNA allowing RPA to bind and the resection tract to be extended until programmed strand invasion. Therefore in the absence of Srs2 Rad51 and Dmc1 might prematurely bind to the single stranded DNA. If the resection tract is short repair might be pushed towards the sister chromatid. Therefore during meiosis *srs2-101* cells should have a higher amount of Rad51 and possibly Dmc1 at early time points when compared to wild type cells.

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