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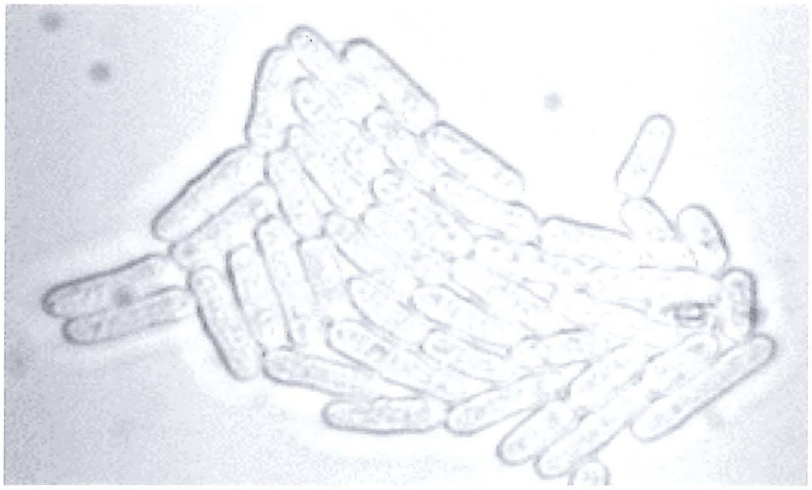
The Roles of Rad21p and Rec8p Cohesins During the Mitotic and Meiotic Cell Cycles in Fission Yeast



A thesis submitted for the degree of Doctor of Philosophy

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Declaration

The research reported in this thesis is my own work except where otherwise stated, and has not been submitted for any other degree.

Tracy Riddell

September, 2003

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Abstract

DNA is replicated during S phase of the *S. pombe* cell cycle so that each chromosome is composed of two identical sister chromatids. During M phase condensed chromosomes align at the cells equator and sister chromatids are attached to microtubules emanating from opposite spindle poles. At anaphase, sister chromatids are triggered to separate rapidly and completely and move to opposite ends of the dividing cell in the equational pattern of nuclear division forming two identical daughters. During sexual development pre-meiotic replication is followed by homologous recombination and two successive rounds of nuclear division, MI and MII. During MI sister chromatids remain joined and homologues migrate to opposite spindle poles (reductional division). During MII, sister chromatids separate as in mitosis, forming four haploid spores enclosed in an ascus.

Cohesin is a multi-subunit complex that controls chromosome segregation by providing a physical linkage between sister chromatids. Cohesin is loaded onto newly replicated chromosomes during G1/S phase and associates with chromatin both at the centromeres and chromosome arms. When cohesion is dissolved at the onset of anaphase (meiotic anaphase II), sister chromatid separation is triggered.

In *S. pombe* Rad21p is an essential cohesion protein first identified for its role DNA repair, while Rec8p cohesin is essential for meiosis, having additional roles in homologous recombination and spore formation. Both Rad21p and Rec8p occupy critical positions in the mitotic and meiotic cohesin complexes, respectively. The aim of this study was to investigate the mitotic and meiotic functions of Rad21p and Rec8p. To this end their specific patterns of expression were exchanged so that *rad21*$^+$ was expressed during meiotic development and *rec8*$^+$ during the mitotic cell cycle. This was achieved by swapping their promoter regions so that each would be ectopically expressed both at physiological levels and at the appropriate time in the cell cycle.

Replacing Rad21p with Rec8p during the mitotic cell cycle was found to rescue the lethal effect of a *rad21*$^+$ deletion with cells having wild type morphology. No S phase delay was detected in these cells though a ~5% increased division time was seen. This rescue was also accompanied with a ~30% loss of viability, and a proportion of cells exhibited the lethal *rad21* null phenotype.

In the absence of Rec8p cells underwent aberrant meioses with abnormal chromosome segregation, reduced spore viability and impaired ascospore development. When Rec8p was replaced with Rad21p, although aberrant meiotic divisions occurred, 66% of asci contained four DNA nuclei in comparison to 51% for *rec8* null meioses. Furthermore, ascospores developed normally and spore viability was improved by ~30% when compared to *rec8* null. No delay in S phase progression was detected in cells lacking Rec8p, while expressing Rad21p in place of Rec8p also did not alter S phase timing.

These data demonstrate that the essential mitotic function of Rad21p can be partially complemented by the meiotic cohesin Rec8p. Conversely, the role of Rec8p in ascospore development appeared to be fully complemented by the mitotic cohesin Rad21p. Taken together, the findings of this study indicate that, while *S. pombe* Rad21p and Rec8p cohesins have specialised mitotic and meiotic roles, the functional overlap between them is greater than previously described. They also suggest a novel role for Rad21p in septation and/ or ascospore development.

Abbreviations

µg	microgram
µl	microlitre
µM	micromolar
A	adenine
A. nidulans	*Aspergillus nidulans*
ACS	AS Consensus Sequence
APC	Anaphase Promoting Complex
Arabidopsis	*Arabidopsis thaliana*
ARS	Autonomously Replicating Sequence
ATP	adenosine triphosphate
bp	base pairs
C	cytosine
C. elegans	*Caenorhabditis elegans*
cAMP	cyclicAMP
cdc	cell division cycle
CDK	cyclin-dependant kinase
ChIP	Chromatin Immunoprecipitation
C-terminus	carboxy terminus
cut	cell untimely torn
DNA	deoxyribonucleic acid
Drosophila	*Drosophila melanogaster*
DSB	double strand break
DSC1	DNA Synthesis Control transcription factor
E. coli	*Escherischia coli*
FACS	Fluorescence-Activated Cell Sorting
G	guanine
g	gram
G1 phase	Gap 1 phase
G2 phase	Gap 2 phase
HU	hydroxyurea
Kb	kilobase

KDa	kilodalton
KMT	kinetochore microtubule
L	litre
M	molar
M phase	mitosis phase
MAP kinase	mitogen-activated protein kinase
Mb	megabases
MCB	*Mlu* I Cell Cycle Box
MCM	mini chromosome maintenance protein
MCS	multiple cloning site
MeiRNA	meiotic RNA species
mg	milligrams
MI	meiosis I
MII	meiosis II
ml	millilitre
mM	millimolar
MT	microtubule
NDJ	non-disjunction
N-terminus	amino terminus
ORC	Origin Recognition Complex
PCNA	Proliferating Cell NuclearAntigen
Polη	DNA polymerase η
pre-RC	pre-Replicatinve Complex
PSS	premature separation of sister chromatids
RFC	Replication factor C
RNA	ribonucleic acid
S phase	DNA synthesis phase
S. cerevisiae	*Saccharomyces cerevisiae*
S. macrospora	*Sordaria macrospora*
S. pombe	*Schizosaccharomyces pombe*
SC	Synaptonemal Complex
SCC	Sister Chromatid Cohesion protein
SIN	Septation Initiation Network
SMC	Structural Maintenance of Chromosomes protein
SPB	Spindle Pole Body
T	thymine

T167	threonine 167
Tm	annealing temperature
UV	ultraviolet
Xenopus	*Xenopus laevis*
Y15	tyrosine 15

List of Figures

Chapter 5

Chapter 6

List of Tables

Table of Contents

Chapter 1:

General Introduction

1.1 PREFACE

The faithful transmission of genetic material to newly formed daughter cells is the ultimate objective of the eukaryotic cell cycle. The highly complex system of pathways and events preceeding this end-point are in place to ensure that this process occurs precisely, even when malfunctions occur. Defects in this system can lead to the generation of progeny with abnormal DNA contents, termed aneuploidy. Many abnormal human conditions are caused by aneuploidy, such as spontaneous abortion, Downs' syndrome and infertility. It is therefore essential that the complex mechanisms controlling chromosome segregation are understood.

Cohesins are a group of proteins that serve a crucial function in controlling the segregation of chromosomes during nuclear division. The subjects of this study are Rad21p and Rec8p, two fission yeast cohesins that have roles in the mitotic and meiotic cell cycles.

The following introduction details the events of the cell cycle, how they are co-ordinated, and the regulatory mechanisms that are in place to ensure each daughter cell receives the full genetic complement. It then goes on to describe cohesins, their role(s) in the cell cycle, and their genetic and biochemical interactions. Finally, it introduces the cohesins Rad21p and Rec8p, which are the subjects of this study.

1.2 THE EUKARYOTIC CELL CYCLE

The cell cycle can be described as an ordered sequence of events whereby a single parent cell duplicates its contents and then divides to generate two identical daughter cells. In eukaryotic organisms the cell cycle can be divided into Interphase and M phase (mitosis). Interphase is further subdivided into gap phase 1 (G1), S phase (for DNA synthesis), and gap phase 2 (G2; Figure 1.1).

In higher eukaryotic organisms, including humans, newly formed daughter cells enter the next cell cycle at G1 phase, a period of rapid growth and high metabolic activity. If division is triggered, the cell will traverse the so called 'restriction point' in late G1 and proceed into S phase (for review, see Pardee, 1989). During S phase chromosomal DNA is duplicated so that each chromosome is now composed of two sister chromatids joined at the centromere. This is followed by a short G2, when the cell continues to grow and prepares for division. At the subsequent M phase, nuclear and cellular division takes place, leading to the formation of two identical daughter cells, each containing the full complement of chromosomes.

A number of regulatory mechanisms are in place to ensure the orderly progression of these cell cycle events. These 'checkpoints' ultimately serve to ensure that daughter cells each receive a complete set of intact genetic material.

1.3 FISSION YEAST AS A MODEL ORGANISM

The fission yeast, *Schizosaccharomyces pombe*, is an excellent model organism in which to study the basic eukaryotic cell cycle. Several key cell cycle regulators functioning in higher eukaryotes were first characterised in *S. pombe*, and many regulatory networks elucidated in *S. pombe* are conserved in higher eukaryotes (for review, see Nurse, 1990).

S. pombe is a unicellular, rod-shaped organism with a uniform diameter of about 3.5 μm, ranging from 7-16 μm in length. It grows by apical extension and divides by medial fission, a feature that permits the prediction of an individual cell's position in the cell cycle by measuring its length. Mutants deficient in cell cycle progression were first identified as recessive temperature-sensitive mutants that were unable to complete the cell division cycle. These '*cdc*' (cell division cycle) mutants were characterised by continued growth without division, and as a result had an elongated cell length phenotype, and defined unlinked genes involved in DNA synthesis, nuclear division and cell plate formation (Nurse *et al.*, 1976).

S. pombe has a genome size of 14 Mb, a similar size to that of the more traditional unicellular eukaryotic model organism, the budding yeast *Saccharomyces cerevisiae*. However, in *S. pombe* the DNA is distributed amongst three relatively long chromosomes, compared to sixteen chromosomes in *S. cerevisiae*.

Another advantage of *S. pombe* is that its life cycle is easily manipulated in the laboratory by altering the nutritional status. Up until the restriction point in G1, called Start in both yeast species (Hartwell *et al.*, 1974), several alternative developmental fates are possible. If adequate nutrients are present cells can pass Start, and enter a round of vegetative growth, the 'mitotic cell cycle'. If deprived of nutrients, however, cells accumulate in G1. When only one mating type is present, cells exit the cell cycle and enter stationary phase until nutrients are re-supplied. These cells become small and rounded in appearance. If, instead, both mating types are present the sexual pathway of development, the 'meiotic cell cycle', is triggered, and two cells of opposite mating types conjugate to form a diploid zygote (Figure 1.2). *S. pombe* cells naturally exist in the haploid state and are unstable as diploids. After crossing Start the diploid cell enters pre-meiotic S phase, followed by two successive rounds of nuclear division, thereby returning to the haploid state. Four haploid spores are produced enclosed in an ascus, collectively termed an

ascospore, or tetrad. A particular experimental advantage of this latter feature is that it permits the analysis of the products of a single parent cell meiosis using tetrad dissection. In addition, *S. pombe* mutants can be studied by analysing the meiotic products of diploid strains heterozygous for the gene of interest (Leupold, 1970, Gutz *et al.*, 1974, for review, see Hayles and Nurse, 1992).

1.4 FISSION YEAST CELL CYCLE

As mentioned earlier several checkpoints are in place to ensure orderly progression of cell cycle events. In *S. pombe* the two major control points are at G1/S and G2/M (Hartwell *et al.*, 1974; Nurse and Bissett, 1981). At both of these points Cdc2p, a cyclin-dependant kinase (CDK), appears to be the key regulator, with its activation being the rate-limiting step controlling the timing of entry into S phase and mitosis (Nurse *et al.*, 1976; Nurse and Bissett, 1981). Cyclin-dependant kinases are proteins whose concentration/activity fluctuate during specific points during the cell cycle and are regulated by binding to kinases. Association of Cdc2p with B-type cyclins regulates its cell stage-specificity of action (Figure 1.3). Cdc2p has homologues in all eukaryotes studied to date, so is likely to be a crucial component of the cell cycle machinery in all eukaryotes (for review, see Norbury and Nurse, 1990).

1.4.1 G1/S Control

Two separate mechanisms are in place that control entry into S phase; one involving regulation of Cdc2p kinase activity, and the other controlling the transcription of genes required for DNA replication (for review, see Okayama *et al.*, 1996).

1.4.1.1 REGULATION OF Cdc2p KINASE ACTIVITY

The catalytic activity of Cdc2p is determined by two main factors: the availability of cyclin partners and Cdc2p phosphorylation. (for review, see Moser and Russell, 2000). Cdc2p forms heterodimers with B-type cyclins, these associations being essential for Cdc2p catalytic activity (Moreno et el, 1989; Connolly and Beach, 1994; Martín-Castellanos *et al.*, 1996). The key cyclin partners of Cdc2p are Cig2p, required for S phase (Bueno and Russell, 1993; Martín-Castellanos *et al.*, 1996); and Cdc13p and Cig1p, both being required for entry into mitosis (Booher *et al.*,, 1989; Moreno *et al.*, 1989).

Phosphorylation at the Y15 residue by Wee1p and Mik1p, which inhibits Cdc2p activity, is cell cycle regulated, (Gould and Nurse, 1989). Cdc2p is also phosphorylated at

T167 by the cyclin-kinase complex Mcs6p/Crk1p/Mop1p-Mcs2p, which is closely related to the mammalian CDK7 protein and cyclin H. Phosphorylation of Cdc2p at this residue is essential for Cdc2p activity (Gould *et al.*, 1991). The overall activity of Cdc2p is regulated by changes in balance between phosphorylation at these two residues.

At Start Cdc2p associates with the cyclin Cig2p to form an active protein kinase complex required for the passage of Start and for progression through S phase (Martín-Castellanos *et al.*, 1996). The exact role of Cdc2p during S phase and its targets are still not clearly defined but potential candidates are Cdc18p and Orp1p, (Nishitani and Nurse, 1995; Leatherwood *et al.*, 1996), both of which contain several target Cdc2p-phosphorylation sites and are required for the early stages of DNA synthesis (Nishitani and Nurse, 1985; Grallert and Nurse, 1996), and Rum1 (Labib *et al.*, 1995). During G1, Cdc2p kinase activity is inhibited by Rum1p, which binds both Cdc2p-Cig2p and Cdc2p-Cdc13p, to regulate the timing of S phase and prevent premature mitosis (Booher and Beach, 1987; Bueno and Russell, 1993; Figure 1.3).

1.4.1.2 TRANSCRIPTIONAL REGULATION

The transcription factor complex DSC1 controls the periodic expression of several genes required for DNA replication (McIntosh *et al.*, 1991; Lowndes *et al.*, 1991). DSC1 binds to a consensus DNA sequence, ACGCGT, called an MCB (for *Mlu*I cell cycle box) in the promoters of a small group of target genes such as *cdc22*$^+$, *cdc18*$^+$ and *cdt1*$^+$ (Gordon and Fantes, 1986; Cailiguiri and Beach, 1993; Kell *et al.*, 1993; Zhu *et al.*, 1994). However, the majority of genes required for DNA replication in *S. pombe* are not cell cycle regulated (White *et al.*, 1986).

DSC1 is made up of several subunits that share sequence similarity (Lowndes *et al.*, 1992; Baum *et al.*, 1997; Whitehall *et al.*, 1999; White *et al.*, 2001) and have homologues in other eukaryotes (Lew *et al.*, 1997). *cdc10*$^+$ encodes the core transcriptional activator of DSC1 (Lowndes *et al.*, 1992; Reymond and Simanis, 1993): its expression in G1 is essential for passage through Start and for S phase progression (Nurse *et al.*, 1976; Nurse and Bissett, 1981). In contrast to the situation in *S. cerevisiae*, activity of DSC1 is not dependant on Cdc2p kinase (Breeden and Nasmyth, 1987; Baum *et al.*, 1997). However, *cdc10*$^+$ is required both for the expression of *cig2*$^+$ (Baum *et al.*, 1997) and for activity of the Cdc2p-Cig2p complex (Obara-Ishihara and Okayama, 1994; Martín-Castellanos *et al.*, 1996).

res1$^+$ and *res2*$^+$ encode structurally homologous transcriptional activators of DSC1 (Tanaka *et al.*, 1992; Caligiuri and Beach, 1993; Miyamoto *et al.*, 1994; Zhu *et al.*, 1994). Both interact with DNA directly via their amino-termini, associate with Cdc10p via their

carboxy termini, and have central ankyrin repeats (Caligiuri and Beach, 1993; Miyamoto *et al.*, 1994; Zhu *et al.*, 1994). Until recently DSC1 was thought to exist in two alternative forms: Res1p associated with Cdc10p for transcriptional activation in the mitotic cell cycle and that Res2p-Cdc10p regulating meiotic genes (Tanaka *et al.*, 1992; Miyamoto *et al.*, 1994). However, it has since been shown that Cdc10p and both Res subunits bind MCBs throughout the cell cycle, and that their life cycle-specific activities are regulated by co-activators (Whitehall *et al.*, 1999; Figure 1.4).

Res1p is required for the passage of Start and inhibits sexual development (Tanaka *et al.*, 1992). Res1p associates with Cdc10p to activate S phase transcription in the mitotic cell cycle (Caligiuri and Beach, 1993). Res2p is required for transcriptional activation during mitotic S phase, the activity of this subunit being dependant on the co-activator Rep2p (Nakashima *et al.*, 1995; Sturm and Okayama, 1996; Figure 1.4A). In the absence of Rep2p during G2 phase, Res2p has an inhibitory effect and MCB gene transcription is repressed (Figure 1.4B). During pre-meiotic S phase, Res2p associates with the co-activator Rep1p to activate transcription of S phase genes and meiotic *rec* genes (Figure 1.4C). Both Rep1p and Rep2p are under strict control by nutritional and pheromone signalling (Sugiyama *et al.*, 1994; Nakashima *et al.*, 1995).

In addition to *cig2*$^+$, known gene targets of DSC1 are *cdc18*$^+$, required for initiation of DNA replication, *cdc22*$^+$ that encodes the catalytic subunit of ribonucleotide reductase, and *cdt1*$^+$. Binding of DSC1 to the promoters of these genes at G1/S activates their transcription. Expression levels remain high throughout S phase and fall during G2 (Gordon and Fantes, 1986; Kelly *et al.*, 1993). However DSC1 remains bound to MCBs until M phase (Whitehall *et al.*, 1999). It is not clear how this periodic expression is regulated in *S. pombe*, though it may reflect changes in activity of the individual DSC1 components.

1.4.2 DNA Replication

During S phase chromosomal DNA is replicated so that each chromosome is composed of two identical sister chromatids. In *S. cerevisiae*, replication is initiated at ARS (Autonomously Replicating Sequence) elements. These are 100-200 bp long A/T-rich regions, distributed among the genome at 20-50 kb intervals. ARS elements can function as origins of replication both in plasmids and the chromosome (Lew *et al.*, 1997). ARS elements are also found in *S. pombe* and are essential for initiation of DNA replication. ARS Consensus Sequences (ACSs) have been identified in both *S. cerevisiae* and *S. pombe* ARS elements (Caddle and Calos, 1994; Wohlgemuth *et al.*, 1994). However, in *S. pombe*

the ACS is not absolutely required for ARS function and its role remains unclear (Maundrell *et al.*, 1988; Zhu *et al.*, 1994; Clyne and Kelly 1995).

ARS elements are bound throughout the cell cycle by a multi-subunit complex called the ORC (Origin of Recognition Complex), which recruits DNA replication proteins to the chromosome (Grallert and Nurse, 1996). In *S. pombe* five genes have been identified that encode ORC subunits (*orp1*$^+$, *orp2*$^+$, *orp3*$^+$, *orp4*$^+$, *orp5*$^+$) all of which are essential for DNA replication (Grallert and Nurse, 1996; Moon *et al.*, 1999; Lygerou *et al.*, 1999).

Inactivation of Cdc2p kinase at anaphase leads to stabilisation of the DNA replication initiation protein, Cdc18p (Jallapelli *et al.*, 1997; for review, see Toone *et al.*, 1997). During G1, Cdc18p binds ORC and then recruits other replication initiation factors, including the MCM proteins (encoded by *mcm2*$^+$-*7*$^+$; Nishitami and Nurse, 1997). MCM proteins are a highly conserved family of proteins, each being essential for DNA replication (for reviews, see Kearsey *et al.*, 1996; Kearsey and Labib, 1998). At this cell cycle stage the inactive ORC becomes an active pre-replication complex (pre-RC) that is capable of binding DNA replication factors to complete DNA replication. These factors include leading and lagging strand DNA polymerases, DNA ligase (encoded by *cdc17*$^+$), ribonucleotide reductase (*cdc22*$^+$ and *suc22*$^+$) and the polymerase processivity factor PCNA (*pcn1*$^+$; Barker *et al.*, 1987; Fernandez-Sarabia *et al.*, 1993; Arroyo and Wang, 1998, 1999). The active replication complex unwinds the DNA double helix at the replication origin, and DNA replication proceeds in both directions forming a structure called the replication fork.

As replication proceeds replication proteins are displaced and ORC re-formation is blocked by stable association of the Cdc2p-Cdc13p complex to replication origins, until late mitosis (Waurin *et al.*, 2002; Wolf *et al.*, 1996).

1.4.3 G2/M Control

The G2/M control point regulates timing of entry into mitosis. To progress into mitosis two criteria must be met: the cell must reach a critical mass (Nurse *et al.*, 1976; Nurse and Thuriaux, 1977), and the chromosomes must be intact. Progression into mitosis is delayed in the presence of DNA damage resulting from incomplete DNA replication, or from DNA breaks caused by environmental agents (Rhind and Russell, 1998a,b; 2001). Failure to meet these criteria leads to cell cycle arrest until the critical mass is reached, or until DNA repair is completed.

Cdc2p activity at G2/M is controlled through the balance of phosphorylation and de-phosphorylation. *cdc25*$^+$ encodes a tyrosine phosphatase that de-phosphorylates Cdc2p

at Y15 to trigger mitotic entry (Millar *et al.*, 1991; for review, see Millar and Russell, 1992). Conversely, the tyrosine kinases Wee1p and Mik1p inactivate Cdc2p by phosphorylating the same residue. Cdc25p, Wee1p and Mik1p are in turn subject to regulation by phosphorylation (Peng *et al.*, 1997; Boddy *et al.*, 1998; Furnari *et al.*, 1999).

Activation of Cdc2p kinase is the rate-limiting step required for mitotic entry (Booher and Beach, 1987). Although Cdc2p associates with the B-type cyclin Cdc13p after S phase, this complex is inactive until Cdc2p is de-phosphorylated by Cdc25p at G2/M (Russell and Nurse, 1986; Gould and Nurse, 1989). The Cdc2p-Cdc13p complex then remains active until proteolytic degradation of Cdc13p at anaphase (Su and Yanagida, 1997; Figure 1.3). Cdc2p forms an alternative complex at G2/M by associating with the cyclin Cig1p (Moreno *et al.*, 1989; Bueno *et al.*, 1991). The activities of both Cdc2p-Cdc13p and Cdc2p-Cig1p are regulated by the cyclin Suc1p.

When DNA damage or DNA replication defects are sensed, overlapping signal transduction pathways are triggered that ultimately leads to inactivation of Cdc2p causing cell cycle arrest. This is achieved through up-regulation of the tyrosine kinases Chk1p and Cds1p (Walworth *et al.*, 1993; O' Connell *et al.*, 1997; Lindsay *et al.*, 1998). Targets of these tyrosine kinases are thought to be both the Mik1p/Wee1p kinases and Cdc25p (O' Connell *et al.*, 1997; Boddy *et al.*, 1998; Furnari *et al.*, 1999; Rhind *et al.*, 2001). Chk1p and Cds1p are therefore able to regulate Cdc2p activity by altering the activities of both Cdc25p and Wee1p/Mik1p. DNA damage checkpoint mutants fail to elicit cell cycle arrest in response to HU (hydroxyurea; DNA synthesis inhibitor) and radiation-induced DNA damage (for review, see Humphrey *et al.*, 2000).

1.4.4 M Phase

During M phase sister chromatids separate and move to opposite poles of the cell (mitosis). This is followed by septation and cytokinesis, generating two identical daughter cells each containing the full genetic complement. A number of regulatory mechanisms are in place to ensure these events occur in the correct temporal order. Mitosis is historically divided into prophase, metaphase, anaphase and telophase.

1.4.4.1 CHROMOSOME SEGREGATION

Histones are a family of proteins that are highly conserved in evolution. Nucleosomes are histone octamers composed of two of each of the histones H2A, H2B, H3 and H4, which are regularly spaced along eukaryotic DNA, at intervals of 180-200bp (Blank & Becker, 1995). Histone proteins are rich in positively charged basic amino acids that have an

intrinsic affinity for the negatively charged phosphate groups in DNA. The presence of additional anionic factors that shield the charge of the histones from DNA, allow chromatin assembly to occur in a regulated and ordered manner. Histone chaperones are ATP-dependant chromatin remodelling machines that cooperate to assemble DNA and histone proteins into chromatin.

In higher eukaryotes, metaphase chromosomes are up to 10,000 times more compacted than in interphase (Trask *et al.*, 1994). At interphase, chromatin is packaged at two levels (Figure 1.5). The first level of chromatin packaging is the direct wrapping of DNA around the nucleosomes, forming chromatin fibre with the appearance of beads on a string. This structure is stabilised on the outer surface by the association of the histone H1 (Figure 1.5A). DNA in direct contact with the nucleosome is protected from hydrolysis. At this level of packing chromatin has the appearance of beads on a string (Figure 1.5B). Chromatin fibres are then further packed, 3-5 nucleosomes thick (Figure 1.5C), though the exact arrangement of nucleosomes appears to vary between cell types (for review, see Sharp and Kaufman, 2003).

At the onset of mitosis, packaging increases further, in a process termed 'chromosome condensation', so that chromatin is now arranged into a tightly coiled, highly organised structure (Paulson and Laemmli, 1977). The chromatin fibre forms loops that protrude from either side of a central protein scaffold (Figure 1.5D). At metaphase chromosomes are in their most condensed state, and these loops are further coiled and folded. Individual chromosomes are now visible as two distinct sister chromatids joined at the centromere.

Condensin is a highly conserved, multi-subunit complex that is essential for chromosome condensation during metaphase (Saka *et al.*, 1994; Strunnikov *et al.*, 1995). In *S. pombe* the 13S condensin complex is composed of two SMC (Structural Maintenance of Chromosomes) and three non-SMC subunits, each of which are essential for viability (Saka *et al.*, 1994; Sutani and Yanagida, 1997). Condensin contains members of the SMC2 and SMC4 subfamilies (*S. pombe* Cut14p and Cut3p; *S. cerevisiae* Smc2p and Smc4p; *Xenopus* XCAP-C and XCAP-E; Saka *et al.*, 1994; Strunikov *et al.*, 1995; Hirano *et al.*, 1997; Sutani and Yanagida, 1997). In *S. pombe* the three non-SMC subunits are Cnd1p, Cnd2p and Cnd3p (Sutani *et al.*, 1999).

SMC proteins have a central hinge domain, flanked by two coiled-coil domains, with putative ATP-binding and DNA-binding domains at their amino- and carboxy termini, respectively (for reviews, see Hirano *et al.*, 1998, 1999; Jessberger *et al.*, 1998; Figure 1.6A). SMC molecules are thought to function as heterodimers, with their coiled-coil regions arranged in anti-parallel, so that the amino- and carboxy-termini together form

complete DNA and ATP-binding domains (Hirano *et al.*, 1997; Sutani *et al.*, 1999; Figure 1.6B). It is also suggested that, rather than the two SMC subunits associating along their lengths, the subunits fold back on themselves and associate with each other via the hinge domains (for review, see Nasmyth *et al.*, 2000).

Precisely how condensin brings about chromosome condensation remains to be revealed, though it has been postulated that condensin acts as an intra-molecular DNA cross-linker, that promotes the compaction of a single DNA molecule by utilizing the energy of ATP hydrolysis (Kimura and Hirano, 1997; see Figure 1.12). In *S. pombe* the topoisomerase II enzyme, which is able to relax DNA supercoils in an ATP-dependant manner, is also thought to be involved in condensation (DiNardo *et al.*, 1984; Saka *et al.*, 1994). Histone modification is also thought to contribute to chromosome condensation by altering the affinity of histones for DNA, therefore regulating access of condensation factors (for review, see Roth and Allis, 1992; Sauve *et al.*, 1999). Condensin has recently been found to have additional roles during interphase, functioning in post-replicative DNA repair and DNA replication checkpoint activation (for review, see Jessberger *et al.*, 1999; Aono *et al.*, 2002).

1.4.4.2 SPINDLE DYNAMICS

During prophase, sister chromatids each become associated with a structure called the kinetochore. The kinetochore is a complex of proteins that assemble on the centromere, which has a high affinity for the microtubule protein, tubulin (Chan *et al.*, 1999: Nakaseko *et al.*, 2001). In the first stage of mitotic spindle assembly, the spindle pole body (SPB), which is located at the outer nuclear membrane, is duplicated. Microtubule (MT) fibres emanate from the SPBs as they split and begin to move to opposite poles of the cell, forming the mitotic spindle (Hiraoka *et al.*, 1984; Masuda *et al.*, 1992).

MTs are highly unstable structures, with polymerisation and de-polymerisation occurring continuously and simultaneously. Stabilisation of MTs is achieved by 'capping', a process whereby de-polymerisation is prevented by interaction with certain proteins (Rodionov *et al.*, 1999). During spindle formation, MTs are stabilised by association with the SPB. The growing ends are stabilised by attaching to kinetochores (kinetochore microtubules; KMTs), and by cross-linking at the cells equator with MTs from the opposite spindle pole (polar MTs; for review, see Hogan and Cande, 1990).

In most eukaryotes the nucleus disassembles during prophase, and reforms once mitosis has ended. In contrast, both yeast species undergo a 'closed mitosis' in which the nucleus remains intact.

At metaphase the SPBs occupy opposite poles of the cell and the condensed chromosomes align on spindle, attached via their associated kinetochores. At this time chromosomes are seen to move back and forth on the spindle, a process thought to assist their accurate spindle attachment (Nabeshima *et al.*, 1998). Kinetochores of sister chromatids attach to MT fibres from opposite poles. As a result, once correctly aligned at the cells equator, tension is generated between sister chromatids, triggering their separation (Goshima and Yanagida, 2000; Tanaka *et al.*, 2000; He *et al.*, 2000).

The transition from metaphase to anaphase is a critical event that sees the rapid and complete separation of sisters, to opposite poles of the cell. This is achieved by dissolution of the connections between sisters, allowing them to migrate to opposite poles, pulled by KMTs. This event involves both de-polymerisation of KMTs at their kinetochore end (anaphase A), and polar MT polymerisation that leads to elongation of the parent cell (anaphase B; Yamamoto *et al.*, 2001).

At telophase the spindle is dissolved followed by the rapid regeneration of cytoplasmic MT arrays. This is coupled to reorganisation of cellular structures at each end of the dividing cell, gene transcription is resumed and the chromosomes decondense. At the same time, a contractile actomyosin ring appears, pinching the dividing cell into two equal halves and a septum is formed at the cells equator. When the parent cell is eventually cleaved into two daughter cells by cytokinesis, cell division is complete.

1.4.4.3 ANAPHASE PROMOTING COMPLEX

The major control at the metaphase/anaphase transition involves regulation of the Anaphase Promoting Complex (APC). This is a highly conserved structure consisting largely of the ubiquitin ligase enzyme, that polyubiquitinates specific proteins, targeting them for proteolytic degradation by the 20S proteasome (for review, see Hochstrasser, 1996). Association with the highly conserved WD-repeat proteins, Slp1p and Ste9p, regulates both the activity and substrate specificity of APC (Kominami *et al.*, 1998; Yamada *et al.*, 2000).

APC is activated from anaphase through to Start and has two key roles during this time. First, APC promotes chromosome segregation by triggering the destruction of specific proteins that inhibit sister chromatid separation, *S. pombe* Cut2p, *S. cerevisiae* Pds1p, commonly termed securin (for reviews, see Peters, 2002; Yanagida, 2000). This will be discussed in more detail later. Secondly, APC triggers degradation of B-type cyclins to inhibit Cdc2p kinase activity. This has two major effects: destruction of Cdc13p inhibits MT polymerisation causing spindle breakdown and the destabilising effect of

Cdc2p on the DNA replication initiation protein, Cdc18p, is released to allow re-setting of DNA replication origins (Blanco *et al.*, 2000; Pomerening *et al.*, 2003).

To ensure accurate transmission of genetic material at mitosis, it is essential that chromosome segregation occurs only after the spindle is fully formed and chromosomes are correctly aligned on the spindle. The spindle checkpoint is a system that monitors these events and delays APC activity until completed. This inhibits destruction of cyclin B (*S. pombe* Cdc13p) and securin (*S. pombe* Cut2p, Section 1.7.5) to prevent chromosome segregation (for reviews, see Elledge, 1996; Rudner and Murray, 1996; Hardwick, 1998). In *S. cerevisiae*, activation of the spindle checkpoint leads to the formation of a complex composed of Mad1p, Mad2p, Mad3p and Bub3p, that binds and inhibits the APC activator, Cdc20p (for review, see Amon, 1999; Kotani *et al.*, 1999). Mad2p and Bub1p are components of the spindle checkpoint in *S. pombe* which operates in both mitosis and meiosis (Millband and Hardwick, 2002; Ikui *et al.*, 2002). Components of the spindle checkpoint are recruited to unattached kinetochores and inhibit anaphase until proper attachment of chromosomes to the spindle (Nabetani *et al.*, 2001; Ikui *et al.*, 2001; Millband and Hardwick, 2002). Defects in chromosome attachment, kinetochore-microtubule interactions or centromeric cohesion lead to activation of the spindle checkpoint and mitotic arrest (Murone and Simanis, 1996; He *et al.*, 1997). Spindle checkpoint mutants fail to elicit this arrest, and proceed through mitosis and cell division in the absence of nuclear division, generating mutants with the '*cut*' phenotype (cell untimely torn; Craig and Norbury, 1998; for review see Yanagida, 1998; Vardy and Toda, 2000). The cytological phenotypes of *cut* mutants can be classified into three groups. One group consists of mutants in which a portion of the nuclear chromatin is stretched by the elongated spindle, but the entire nucleus is not separated. Another group exhibits non-disjunctioned and condensed chromosomes in the presence of the spindle. The remaining group has a mixed phenotype of the above two groups; namely, stretched chromatin and condensed chromosomes.

1.4.4.4 Plo1p KINASE

Another system regulating M phase centres around the Plo1p serine/threonine kinase. Plo1p is activated after mitotic onset and is required for septation (Ohkura *et al.*, 1995; Tanaka *et al.*, 2001). This kinase is thought to act upstream of Spg1p, a G-protein that regulates the onset of septation (Mulvihill *et al.*, 1999; Cullen *et al.*, 2000). Plo1p localises to the SPB, in association with the Septation Inducing Network (SIN), a signal transduction network essential for correct regulation of cytokinesis, and whose activation is dependent on Plo1p (Mulvihill and Hyams, 2002). Plo1p has also recently been shown to

activate transcription of genes required for septation and cytokinesis e.g. *cdc15*$^+$, *sid2*$^+$ and *ppb1*$^+$ (Anderson *et al.*, 2002). Plo1p may also have an earlier role in activating the APC, as does the *S. cerevisiae* homologue Cdc5p (Charles *et al.*, 1998; Shirayama *et al.*, 1998).

1.5 SEXUAL DEVELOPMENT

Under conditions of nitrogen limitation, transcription of genes essential for sexual development is initiated by activating the key meiotic-specific transcription factor, Ste11p (Sugimoto *et al.*, 1991; Figure 1.7). This should be distinguished from the situation with glucose limitation, which, although causing fission yeast cells to accumulate in G1, does not cause sexual development. Stress also triggers a MAP kinase pathway, activating the heteromeric transcription factor complex Atf1p/Pcr1p, which leads to Ste11p expression (Warbrick and Fantes, 1991; Shiozaki and Russel, 1995, 1996; Wilkinson *et al.*, 1996; Kato *et al.*, 1996).

Nitrogen limitation causes a drop in intracellular cAMP levels. This in turn leads to inhibition of cAMP-dependant protein kinases and activation Ste11p (Sugimoto *et al.*, 1991; Hoffman and Winston, 1991). This transcription factor has two meiosis-promoting effects. First, it stimulates the rate-limiting step in meiotic entry, expression of Mei2p (Sugimoto *et al.*, 1991). Secondly, Ste11p triggers expression of the mating type genes (Mat1-P and Mat1-M), stimulating the production of pheromones, P-factor (h^+) and M-factor (h^-) and their receptors (Sugimoto *et al.*, 1991; Aono *et al.*, 1994). Cells of opposite mating type respond to pheromones by conjugating and fusing to form a diploid zygote. Co-expression of the mating type genes in the diploid cell leads to expression of *mei3*$^+$, which binds and inhibits the key meiotic suppressor, Pat1p kinase (McLeod *et al.*, 1987; McLeod and Beach, 1988; Willer *et al.*, 1995; Li and McLeod, 1996).

During vegetative growth, sexual development is inhibited by the serine/threonine kinase Pat1p, which inhibits both Ste11p and Mei2p. Binding and inactivation of Pat1p by Mei3p, relieves this inhibition permitting Mei2p activity and entry into meiosis (Nurse *et al.*, 1985; Willer *et al.*, 1995; Watanabe *et al.*, 1997; Wang *et al.*, 1998).

Mei2p is the key determinant for commitment to meiosis. Mei2p is an RNA-binding protein whose function is required for both pre-meiotic DNA synthesis and for the first meiotic division (MI). Mei2p complexes with meiRNA, a small meiotic RNA encoded by the *sme2*$^+$ gene, to promote MI (Watanabe and Yamamoto, 1994). Mei2p targets during premeiotic S phase, however, remain to be identified.

1.5.1 Meiotic Recombination

1.5.1.1 LINEAR ELEMENTS

Once homologues have been brought into alignment, connections are stabilised by large protein structures that assemble on the chromosomes, an event termed 'synapsis'. These structures assist meiotic recombination and subsequent chromosome segregation. In *S. cerevisiae* and higher eukaryotes these are large tripartite structures called the Synaptonemal Complex (SC). Formation of the SC begins with the appearance of two axial elements, one running the length of each sister chromatid. When homologues are brought into alignment their axial elements fuse creating two lateral elements with a central protein-rich core, collectively forming the SC (for reviews, see Hawlwy and Arbel, 1993; Roeder, 1995; Kleckner, 1996).

S. pombe does not form SCs. Instead, structures similar to the axial elements of *S. cerevisiae* appear, called 'linear elements' (Bahler *et al.*, 1993). In contrast to axial elements, these are discontinuous structures that do not run the length of the chromosome. Linear elements are required for homologous recombination and chromosome segregation in *S. pombe*, indicating a role similar to that of the SC. Linear elements may have additional roles in chromatin condensation and pairing of homologous chromosomes (Bahler *et al.*, 1993; Scherthan *et al.*, 1994; Kohli and Bahler, 1994). Linear elements are also implicated in sister chromatid cohesion, as meiotic cohesin *rec8*$^{-}$ mutants are defective in linear element formation and meiotic chromosome segregation (Molnar *et al.*, 1995).

1.5.1.2 NUCLEAR MORPHOGENESIS

During prophase of the first meiotic division (prophase I) homologous chromosomes align together and swap genetic information by homologous recombination.

Accurate alignment of homologous chromosomes is driven by dramatic changes in nuclear morphology (Chikisage *et al.*, 1994, 1997; Figure 1.8). During mitosis, chromosomes cluster at the SPB, associating with it via their centromeres (Funabiki *et al.*, 1993). In contrast, during meiotic prophase I, chromosomes associate with SPBs via their telomeres (Chikisage *et al.*, 1994; Svoboda *et al.*, 1995). These are non-transcribed regions at the ends of chromosomes, essential to prevent degradation of linear DNAs. The telomere-associated proteins, Taz1p and Lot2p, have been shown to be required for wild type levels of meiotic recombination, suggesting that this arrangement has a role in meiotic recombination (Nimmo *et al.*, 1998). This association is coupled with dramatic back-and-forth movements of the nucleus, driven by cytoskeletal rearrangements, termed 'horse-tail' movements due to the characteristic elongated shape of the nucleus at this time (Robinow,

1977; Hiraoka *et al.*, 2000). Tethering of chromosomes to the SPB by their telomeres, together with these horsetail movements, is thought to aid alignment of homologous DNA sequences.

1.5.1.3 CROSSOVER FREQUENCY

The mechanics of homologous recombination in *S. pombe* are poorly understood and most of what is known has been derived from studies in *S. cerevisiae*. In *S. cerevisiae*, crossovers are known to occur at regular intervals of ~50 kb. This even spacing is due to 'crossover interference', a physical block, perhaps involving the SC, which prevents crossovers from occurring too close together (Molnar *et al.*, 1995).

In *S. pombe* crossovers also occur at ~50 kb intervals though are not evenly distributed throughout the genome (Snow, 1979; Egel *et al.*, 1980; Deveaux and Smith, 1994). The reason for this is unclear but it might be linked to differences in distribution of specific meiotic recombination proteins (Section 1.7.9). The lack of crossover interference in *S. pombe* might reflect the absence of a SC.

1.5.1.4 RECOMBINATION HOT-SPOTS

Certain chromosomal loci in *S. pombe* have been found to undergo homologous recombination at either significantly increased or significantly reduced frequencies. These loci have been termed recombination 'hot-spots' or 'cold-spots' respectively.

The most well documented recombination hot-spot in *S. pombe* is encoded by the *ade6-M26* mutation, caused by a G→T transition at the *ade6* locus. These mutants undergo meiotic recombination at 15-fold higher frequency compared to wild type (Gutz *et al.*, 1971). The *ade6-M26* mutation encodes the consensus sequence ATGACGT, that has the ability to confer hot-spot activity on heterologous DNAs (Klar and Miglio, 1986; Schuchert and Kohli, 1988). However, this consensus sequence is not always essential for hot-spot activity in *S. pombe*, and additional factors are now thought to be required (Zahn-Zabal *et al.*, 1995). The *ade6-M26* hot-spot has been found to bind the Atf1/Pcr1 protein complex *in vivo* (for review, see Davis and Smith, 2001). This complex acts as a transcription factor in the meiotic induction pathway (Section 1.5), and the significance of this connection is still unclear. It has also been shown that this hot-spot only functions in the context of the chromosome, suggesting that gross chromosomal features are important for its function (Ponticelli and Smith, 1992; Virgin *et al.*, 1995).

A meiotic recombination cold-spot is located between the $mat2^+$ and $mat3^+$ mating type loci. This region is designed as a recombination cold-spot to permit repeated transfer

of information from *mat2*$^+$ and *mat3*$^+$ to *mat1*$^+$ by unidirectional gene conversion, which results in mating type switching, and the ability of cells in a culture derived from a single cell to mate (Beach and Klar, 1984; Thon and Klar, 1993).

1.5.1.5 DOUBLE STRAND BREAKS

DNA double strand breaks (DSBs) are now known to occur and be required for meiotic recombination in both *S. pombe* and *S. cerevisiae* (Lichten and Goldman, 1995; Roeder 1997; Cervantes *et al.*, 2000). However, details of how this process is controlled in the two yeasts are slightly different.

In *S. cerevisiae*, pre-meiotic DNA replication is essential for DSB formation whereas in *S. pombe* this is not the case (Merino *et al.*, 2000; Murakami and Nurse, 2001). Instead, some other S phase factor is thought to be required. In *S. cerevisiae* DSB formation is a pre-requisite for reductional division at MI (Roeder, 1997). In contrast, in *S. pombe* meiotic divisions can proceed normally in the absence of DSB formation (Molnar *et al.*, 2001).

1.5.2 Meiotic Chromosome Segregation

In *S. pombe* meiotic development DNA replication is followed by two successive rounds of chromosome segregation. In the first meiotic division (MI) sister chromatids remain joined and homologous chromosomes segregate to opposite poles of the cell. This results in the transient formation of two 2C daughters from a 4C parent cell, in what is termed the 'reductional' pattern of segregation.

The second meiotic division (MII) proceeds as for mitotic division (equational segregation); sister chromatids separate as connections between them are lost, and migrate to opposite poles. The four haploid products of meiosis are enclosed in an ascus.

1.5.3 Sporulation

S. pombe ascospore formation is similar to that in *S. cerevisiae*. The SPBs differentiate into multi-layered plaques at MII, and a flattened sac called the forespore membrane develops at the cytoplasmic side of these plaques. The forespore membrane grows to eventually enclose each individual nucleus. The spore wall then begins to form between the inner and outer membranes of the forespore membrane (Yoo *et al.*, 1973, Tanaka & Hirata, 1982). The mature spore wall is rich in starch and stains brown with iodine vapour, which is a convenient marker for sporulation (Gutz *et al.*, 1974).

Several sporulation-deficient mutants have been identified that are defective not just in spore formation, but in various aspects of meiotic development. For example, *spo5* mutants are thought to be defective in the completion of MII, while SPB modification and initiation of forespore membrane formation are apparently normal (Kishida & Shimoda, 1986). In contrast, *spo4* mutants duplicate their SPBs and undergo normal nuclear divisions. However, SPBs do not differentiate and forespore membranes fail to develop in these mutants (Hirata & Shimoda, 1992, 1994). Furthermore, both *cdc25*$^{-}$ and *cdc2*$^{-}$, mutations in which MII is blocked, generate two diploid meiotic products each with intact spore walls (Grallert & Sipiczci, 1991). Together these data indicate that the meiotic processes of spore formation and nuclear division are genetically separable.

Following completion of meiotic division and sporulation, the ascus breaks down and the haploid spores are released. If adequate nutrients are available, the protective spore walls autolyse, and spores mature to enter a new round of vegetative growth. If conditions are hostile, spore walls remain intact until conditions improve.

1.6 THE CENTROMERE-KINETOCHORE COMPLEX

The centromere is the constricted region of a metaphase chromosome that has both a structural and a biological role. First, this is the site where the two sister chromatids of a duplicated chromosome are tethered together, due to a high density of cohesin in this region (Tanaka *et al.*, 1999; Laloraya, 2000; Warren *et al.*, 2000). Cohesin is a multi-subunit complex that binds sister chromatids together to regulate chromosome segregation (see below). Centromeric cohesin is essential to prevent premature separation of sister chromatids (Tatebayashi *et al.*, 1999; Watanabe & Nurse, 1999). The second function of the centromere is to provide the point of assembly of the kinetochore, a multi-protein complex that binds MTs, attaching the chromosomes to the mitotic spindle (Doe *et al.*, 1998; Holy & Liebler, 1996). Together, the centromere and its associated proteins form the 'centromere-kinetochore complex'.

Each sister chromatid becomes associated with a kinetochore during metaphase, when chromosomes are most highly condensed. Alternative kinetochore orientations mediate the different patterns of chromosome segregation in mitosis and meiosis (Tanaka, 2000). In mitosis, sister kinetochores are bi-orientated so that they attach to MTs from opposite spindle poles. As a result, sisters are pulled to opposite poles during chromosome segregation. In contrast, during the first meiotic division (MI), sister kinetochores are co-oriented so that they capture MTs from the same spindle pole. However, the kinetochores of homologues attach to opposite spindle MTs, so that sister chromatids remain joined and homologues are pulled to opposite poles. The second meiotic division (MII) proceeds as in

mitosis: sister kinetochores face opposite poles and sister chromatids are pulled away from each other. Defects in centromere or kinetochore function lead to inaccurate chromosome segregation and elevated rates of chromosome gain and loss (Nabeshima *et al.*, 1998; Nakaseko *et al.*, 2001).

The centromere appears to be organised in different, separable domains in order to carry out its role. However, despite centromere functions being evolutionarily conserved, molecular and genetic analysis of centromeres across species have revealed little conservation at the level of centromere DNA sequences. In addition, few centromere proteins are conserved, and many are unique to the different organisms. However, striking similarities have been found at the overall structural level between centromeres of *S. pombe*, *Drosophila* and humans (Blower *et al.*, 2001; Howe *et al.*, 2001; Kniola *et al.*, 2001; Pearson *et al.*, 2001).

The structural organisation of the centromere is generally multi-layered with a heterochromatin domain at the outer repeats, and a central core that harbours the outer plate structures of the kinetochore, flanked by repetitive DNA sequences. *S. cerevisiae* has a small 'point' centromere (< 2.0 kb) composed of a short AT-rich sequence flanked by two very short conserved regions (for reviews, see Pluta *et al.*, 1995; Pidoux & Allshire, 2000). Higher eukaryotes have centromeres composed of long sequences flanked by large quantities of repeated DNA, known as satellite DNA (for review, see Bjerling & Ekwall, 2002). *S. pombe* centromeres are 40-100 kb in length and have a symmetric organisation. These heterochromatic centromeres are composed of distinct domains, which have different qualities of transcriptional silencing and are associated with specific proteins (Figure 1.9). The central core sequence (*cnt*), of roughly 5 kb, is flanked by arrays of repeated inner (*imr*) and outer (*otr*) sequences that consist of a limited set of DNA elements, *dg* and *dh* (for review, see Clarke & Carbon, 1985; Fishel *et al*, 1988).

The key factors for centromere assembly and function are specialised and modified histones. The centromeres in *S. pombe*, like heterochromatic regions in vertebrates, are hypo-acetylated on histones (Ekwall *et al.*, 1997). In addition, chromatin of the central core (*cnt*) contains a unique histone H3 variant, Cnp1p. This H3 variant was first identified in humans as CENP-A (Takahashi *et al.*, 2000; for review, see Choo, 2001), an essential centromere component required for the recruitment of other centromere proteins, such as CENP-C (*S. pombe* Cnp3p), and INCENP (Howman *et al*, 2000). Other proteins that associate with the centromere are the central domain proteins Mis6p and Mis12p (Saitoh *et al*, 1997; Goshima *et al.*, 1999). Mis6p alleviates transcriptional silencing at this region but not at the outer repeats (Partridge *et al*, 2000). The central core and inner repeat regions

have a large number of transfer RNA (tRNA) genes and experience weak silencing (Takahashi *et al.*, 1991; Kuhn *et al.*, 1991).

Swi6p and Chp1p are chromodomain proteins found at the outer repeats (Ekwall *et al.*, 1995; Doe *et al.*, 1998; Partridge *et al.*, 2000), and this localisation requires Rik1p and Clr4p (Ekwall *et al.*, 1996; Thon & Verhein-Hansen, 2000). This association mediates the silencing of marker genes placed within these regions (Thon & Verhein-Hansen, 2000). Clr4p also methylates histone H3 at the outer repeats (Nakayama *et al.*, 2001). Histone hypo-acetylation is important for Swi6p binding at the outer repeats both in fission yeast and mammalian cells (Ekwall *et al.*, 1997; Taddei *et al.*, 2001), and binding of cohesin proteins, Rad21p and Psc3p, is dependant on Swi6p (Bernard *et al.*, 2001; Nonaka *et al.*, 2002). The spindle checkpoint protein, Bub1p, is also recruited to the kinetochore in mitotic prophase (Toyuda *et al.*, 2002).

In *S. pombe*, electron microscopy and immunofluorescence studies of interphase cells show that the central core and flanking regions occupy cytologically distinct positions within a heterochromatin domain, and that an 'anchor' structure containing Ndc80p is present between the heterochromatin domain and the spindle pole body (Kniola *et al.*, 2001).

1.7 COHESIN

After completion of DNA replication, sister chromatids are physically associated by two different mechanisms: sister DNA strands are intertwined, thus providing a topological linkage, and sisters are bound together along their length by a protein complex called cohesin. Cohesin is a multi-subunit complex whose primary function is to regulate chromosome segregation by physically connecting sister chromatids (for review, see Nasmyth, 2001). In *S. pombe and S. cerevisiae* cohesin is loaded onto newly replicated chromosomes and/or activated during S phase (Uhlmann & Nasmyth, 1998; Watanabe & Nurse, 1999). In metazoans cohesin binds to chromatin at the earlier cell cycle stage of telophase (for reviews, see Hirano, 2000; Nasmyth, 2001). Cohesin binding provides the molecular 'glue' that holds sisters together until M phase, when nuclear and cellular division takes place.

1.7.1 The Cohesin Complex

Cohesin is composed of at least 4 subunits that were first characterised by genetic studies in *S. cerevisiae* (Guacci *et al.*, 1997; Michaelis *et al.*, 1997; Toth *et al.*, 1999), and that are now known to be evolutionarily conserved (Losada *et al.*, 1998; Tomonaga *et al.*, 2000;

Sonoda *et al.*, 2001; Sumara *et al.*, 2000; Table 1.1). In *S. pombe*, cohesin consists of two members of the SMC family of proteins (Section 1.4.4.1), Psm1p and Psm3p, and two SCC (Sister Chromatid Cohesion) proteins, Rad21p and Psc3p (Tomonaga *et al.*, 2000). The *S. cerevisiae* homologues are Smc1p, Smc3p, Mcd1p/Scc1p and Scc3p, respectively (Guacci *et al.*, 1997; Toth *et al.*, 1999; Michaelis *et al.*, 1997). In fission yeast all four proteins are essential for viability, and their mutation leads to defective sister chromatid cohesion and premature separation of sister chromatids (Tomonaga *et al.*, 2000). The *S. pombe rad21*$^{+}$ gene will be discussed in more detail later.

Protein complexes containing vertebrate homologues of these subunits has been isolated in *Xenopus* and subunit homologues have been described in several species (Table 1.1), in which several alternative complexes have been described that contain different SMC and SCC variants. In *Xenopus*, the cohesin complexes exist as two major forms with sedimentation coefficients of 9S and 14S. 9S cohesin is a heterodimer of XSMC1 and XSMC3, whereas 14S cohesin contains three additional subunits, one of which is XRAD21 (Losada *et al.*, 1998, 2000). In humans, three SCC3 variants have been identified: STAG1 and STAG2 occupy alternative mitotic cohesin complexes while STAG3 is meiosis-specific (Carramolino *et al.*, 1997; Sumara *et al.*, 2000; Prieto *et al.*, 2001). *Caenorhabditis elegans* (*C. elegans*) has four SCC1 homologues (Parisi *et al.*, 1999; Pasierebek *et al.*, 2001), while *Arabidopsis thalianis* has three (Dong *et al.*, 2001; Cai *et al.*, 2003). In both species, one of the SCC1 homologues is meiosis-specific. It is not known what the individual functions of mitotic SCC1 variants are, and they may be partially redundant.

The recently identified *S. pombe* Pds5p protein associates with cohesin, and is required for maintenance of sister chromatid cohesion (Tanaka *et al.*, 2001; see below). Pds5p co-localises with Rad21p throughout the cell cycle and is thought to be an integral component of cohesin in *S. pombe*. Homologues in *S. cerevisiae* (Pds5p), *S. Macrospora* (Spo76) and *A. nidulans* (BimD) are involved in several aspects of chromosome morphology including sister chromatid cohesion and condensation (Denison *et al.*, 1993; van Heemst *et al.*, 1999; Hartman *et al.*, 2000; Panizza *et al.*, 2000). The co-localisation of *S. cerevisiae* Pds5p and Mcd1p to chromatin are inter-dependant, but they form only a weak association indicating that Pds5p is not an integral component of cohesin in this yeast (Tanaka *et al.*, 2001; Wang *et al.*, 2002). Pds5p homologues are also associated with the 14S cohesin complex in *Xenopus* and humans (Sumara *et al.*, 2000).

1.7.2 Meiotic Cohesin

The meiotic-specific cohesin protein, Rec8p, was first identified in *S. pombe* (Molnar *et al.*, 1995; Watanabe & Nurse, 1999) and homologues have since been identified in several species including *S. cerevisiae* and humans (Klein *et al.*, 1999; Parisi *et al.*, 1999). *S. pombe rec8* mutants are defective in meiotic chromosome segregation, homologous recombination, and linear element formation indicating a crucial role for this gene in several aspects of meiosis (Molnar *et al.*, 1995; Parisi *et al.*, 1999; Watanabe & Nurse, 1999). The *S. pombe* $rec8^+$ gene will be discussed in more detail later (Section 1.7.9). Meiotic cohesin is thought to be required not only for cohesion between sister chromatids, but also for the association of homologous chromosomes during meiotic recombination (Section 1.5.1.3).

Although a meiotic cohesin complex remains to be defined, several candidates have been identified that may be involved in meiotic cohesin function. The $rec10^+$ and $rec11^+$ genes of *S. pombe*, are variously required for aspects of chromosome dynamics ranging from the initial pairing of homologous chromosomes to chromosome segregation in MII, and interact with $rec8^+$ to bring about normal levels of meiotic recombination (DeVeaux & Smith, 1994; Krawchuk *et al*, 1999; Molnar *et al.*, 2003). Rec11p is homologous to *S. cerevisiae* Scc3p (Table 1.1) and is required for meiotic sister chromatid cohesion (Krawchuk et al., 1999). The cohesin Psc3p is also present on chromatin during meiosis, though it doesn't co-localise with Rec11p, and its role in meiosis is unclear (Kitajima *et al.*, 2003). A direct role for Rec10p in sister chromatid cohesion has not been demonstrated. Mammalian Rec8 has been shown to co-localise with the mitotic cohesin component, Smc3p, throughout the first and second meiotic divisions (Lee *et al.*, 2003). In *Drosophila* MEI-S332 is a centromere-specific protein that is present during both mitosis and meiosis. During meiosis MEI-S332 is essential for the maintenance of cohesion, while it is disposable for cohesion maintenance in mitosis (Bickel *et al.*, 1998; Tang *et al.*, 1998). Another meiotic *Drosophila* cohesin is ORD, which acts earlier in the meiotic cell cycle than MEI-S332, and may be involved in establishment of cohesion (Bickel *et al.*, 1996, 2002).

1.7.3 Establishment of Cohesion

In *S. pombe*, cohesin genes are expressed at G1/S phase, and delaying this expression until G2 leads to defects in sister chromatid cohesion and chromosome segregation (Watanabe *et al*, 2001). This indicates that cohesin must be loaded onto the chromosomes and/or activated at G1/S for normal cohesin function. One explanation for this is that S phase is a

time in the cell cycle when newly replicated sister chromatids are in close association, so is also a time when cohesion can be established most efficiently. In *S. pombe*, cohesin is able to bind chromatin when expressed in G2, but does not cross-link sister chromatids (Watanbe *et al.*, 2001). The association of cohesin with chromatin and the establishment of cohesion are therefore separable functions. When DNA replication is blocked no effect is seen on the ability of cohesin to bind chromatin. Instead, DNA replication is thought to provide a mechanism whereby cohesin present on newly replicated chromosomes is activated to establish cohesion between sister chromatids (Section 1.2.3).

S. pombe Eso1p is composed of two functionally distinct domains: the N-terminal two thirds homologous to *S. cerevisiae* DNA polymerase η (Pol η) and the C-terminal one third homologous to *S. cerevisiae* Eco1p/Ctf7p (Tanaka *et al*, 2000). The Eco1p/Ctf7p-homologous domain of Eso1p is essential for establishing sister chromatid cohesion whereas the Pol η domain presumably catalyses translesion DNA synthesis during S phase. Eso1p physically interacts with the recently identified cohesin subunit Pds5p that has homologues in *S. cerevisiae*, *Xenopus* and humans (Hartman *et al.*, 2000; Panizza *et al.*, 2000; Sumara *et al*, 2000). In *S. pombe* Pds5p constitutively bound to cohesin hinders the formation of cohesion until counteracted by Eso1p via a physical interaction through its Eco1p/Ctf7p domain, but functions to stabilise cohesion once it is formed (Tanaka *et al.*, 2001). One benefit of this mechanism is that it ensures cohesion is established only between emerging sister chromatids during replication, but not before or afterwards between unrelated sequences.

In *S. cerevisiae* two members of the SCC family, Scc2p and Scc4p, form a complex that is required for the loading of cohesin onto chromatin (Ciosk *et al.*, 2000). However, Scc2p and Scc4p do not co-localise with cohesin, indicating that their role is not in recruitment (Toth *et al.*, 1999; Ciosk *et al.*, 2000). Mis4p is a *S. pombe* homologue of Scc2p that is essential for loading of Rad21p onto chromatin, and co-localises with Rad21p at the centromere (Furuya *et al.*, 1998). Nipped B is a *Drosophila* Scc2p homologue involved in the long-range activation of gene expression by enhancers. This has led to the postulation of a role for the Scc2p/Scc4p in global modulation of chromatin for cohesion establishment (Rollins *et al.*, 1999).

1.7.4 Chromosomal Distribution

The chromosomal distribution of cohesin has been analysed by chromatin immunoprecipitation (ChIP), and it has been shown, in both *S. pombe* and *S. cerevisiae*, to localise preferentially to heterochromatic regions (for review, see Bernard & Allshire,

2002; Bernard *et al.*, 2002; Nonaka *et al.*, 2002). In *S. cerevisiae* cohesin preferentially binds intergenic regions along the length of chromosome arms (Blat & Kleckner, 1999), in areas of high local A/T base composition, and is highly enriched at the centromere, telomeres and mating type locus (Nonaka *et al.*, 2002). In *S. pombe* localisation of cohesin is mediated by the heterochromatin component Swi6p which recruits Rad21p and Psc3p to heterochromatic regions. Consequently, a role has been implicated for cohesin in gene silencing (Section 1.7.7.5). It is not yet known how cohesin binding sites are distributed in higher eukaryotes.

1.7.5 Cleavage of Cohesin

In both yeasts anaphase is triggered by the cleavage of cohesin subunit Rad21p/Mcd1p, to release cohesion between sister chromatids. At metaphase, sister centromeres are under tension from opposing kinetochore microtubule forces. The loss of cohesion, therefore, sees the separation of sister chromatids and their rapid migration to opposite spindle poles.

The timing of cohesin cleavage differs between species. In *S. pombe* Rad21p is cleaved at anaphase (Tomonaga *et al.*, 2000) while in *S. cerevisiae* Mcd1p is cleaved at the metaphase/anaphase transition (Uhlmann *et al.*, 2000). In both yeast species cleavage of cohesin occurs simultaneously with loss of sister chromatid cohesion. In eukaryotes, sister chromatid cohesion is dissolved in two stages. Cohesion along chromosome arms is dissolved at prophase/prometaphase, when chromosome condensation takes place (Losada *et al.*, 1998; Waizenegger *et al.*, 2000; Sumara *et al.*, 2000). Condensation is thought to contribute to loss of cohesion by decatenating DNA strands, leading to displacement of cohesin. However, in the absence of cohesin, arm cohesion is somehow maintained, and this may be mediated, directly or indirectly, by condensin. Centromeric cohesin persists until metaphase/anaphase when dissolution of cohesin allows sister chromatids to separate (Uhlmann *et al.*, 2000; Hauf *et al.*, 2001). The significance of these species variations is unclear.

This stepwise loss of cohesion is also seen in eukaryotic meioses (for review, see Rieder & Cole, 1999). Cohesin along chromosome arms is dissolved at anaphase I allowing resolution of chiasmata and separation of homologous chromosomes. Centromeric cohesin persists, resisting the pulling forces of kinetochore microtubules and sisters remain joined. At anaphase II, loss of centromeric cohesion allows sister chromatids to separate. It is not known how centromeric cohesin is protected from proteolysis, though phosphorylation of cohesin subunits is known to be important for regulation of its cleavage and may be involved (Birkenbihl & Subramani, 1995; Uhlmann *et al.*, 2000; Hoque &

Ishikawa, 2001; Alexandru *et al.*, 2001). It is possible that cohesin subsets i.e. arm versus centromeric are differentially phosphorylated and it has been suggested that in metazoan mitosis, regulating factors that localise to the centromere might be involved in this process (Cooke *et al.*, 1987; Rattner *et al.*, 1988;Kamieniecki *et al.*, 2000). In support of this, rat Cor1/SCP3 is thought to protect centromeric cohesion by relocating from dissolving linear elements to the centromere at anaphase II (Tarsounas *et al.*, 1999). A similar role has been suggested for Drosophila MEI-S332 (Kerrebrock *et al.*, 1995).

Loss of cohesion is controlled by the APC (Section 1.4.4.3). However, the APC is inactive until metaphase/anaphase in vertebrate mitosis, so it is not known how dissolution of arm cohesion is controlled in these species (Fang *et al.*, 1998; Kallio *et al.*, 1998).

The APC has two major roles in mitosis: degradation of cyclin B (*S. pombe* Cdc13p) to inactivate Cdc2p, and degradation of securin, an inhibitor of sister chromatid separation (for reviews, see Cohen-Fix & Koshland, 1997; Townsley & Ruderman, 1998; Figure 1.10). Securin is largely non-conserved between species apart from an N-terminal domain that targets the protein for ubiquitination by the APC (Yamada *et al.*, 1997; Yamashita *et al.*, 1999). Securin is encoded by Esp1p in *S. cerevisiae* (McGrew *et al.*, 1992; Ciosk *et al.*, 1998) and *cut2*$^{+}$ in *S. pombe* (Funabiki *et al.*, 1996a, 1996b), and is required to prevent premature sister chromatid separation (Ciosk *et al.*, 1998; Uhlmann *et al.*, 1999). The primary target of securin is thought to be separase, which has sequence homologues in several species (*S. cerevisiae* Pds1p; *S. pombe* Cut1p), with a conserved C-terminus and unique N-terminus (Ross & Cohen-Fix, 2002). In *S. cerevisiae* the N-terminus of Pds1p is thought to contain protease function, and binding of Esp1p to this region of Pds1p regulates its activity (Ciosk *et al.*, 1998; Uhlmann *et al.*, 1999).

In *S. pombe* Rad21p has two highly conserved separase cleavage sites in its carboxy-terminus, also found in the meiotic cohesin Rec8p (Uhlmann *et al*, 1999; Buonomo *et al.*, 2000). In *S. cerevisiae*, ectopically expressed Rec8p is cleaved at consensus separase cleavage sites during the mitotic metapahase/anaphase transition, indicating that the same mechanism operates in meiosis (Buonomo *et al.*, 2000; Uhlmann *et al.*, 2000).

Cut2p regulates anaphase onset in two ways: during metaphase it binds Cut1p, and traffics it to the mitotic spindle. However, at this time separase activity is inhibited by securin binding (Cohen-Fix *et al.*, 1996; Funabiki *et al.*, 1996b); at anaphase, the APC triggers degradation of securin, releasing its inhibitory effect on separase activity (Uhlmann *et al.*, 1999, 2000).

The precise role of separase in different organisms is unclear, but its activity is thought to ultimately lead to the release of cohesion. In *S. cerevisiae*, recombinant Esp1p

has been shown to cleave Mcd1p directly. However, so far it hasn't been possible to demonstrate protease activity for Cut1p and no genetic interaction has been found between Cut1p and Rad21p/ Psc3p (for review, see Yanagida, 2000). How or even whether Cut1p leads to cleavage of cohesin remains to be revealed.

Securin is a large polypeptide that might have many functional domains, and it is suggested that it may have important roles in several varied cell cycle functions in addition to regulation of sister chromatid cohesion (Funabiki *et al.*, 1996b; Kumada *et al.*, 1998; Zou *et al.*, 1999). It is also possible that the unique N-terminus of separase has alternative functions in different species. Another explanation for species differences in this system is that several different subsets of securin and separase are present in the cell that have related but specific cell cycle functions (for review, see Yanagida, 2000).

1.7.6 Cohesin Model

In *S. pombe* only 5% of Rad21p is cleaved at anaphase and the majority remains associated with the chromosome throughout the cell cycle (Tomonaga *et al.*, 2000). The reason for this is unclear, but a model has been proposed to explain this phenomenon. It is suggested that, rather than the two SMC subunits associating along their lengths, as described in Section 1.4.4.3 (Figure 1.6B), the subunits fold back on themselves and associate with each other via the hinge domains (for review, see Nasmyth, 2002). In this model, two SMC subunits form a V-shaped heterodimer, and interaction of amino-termini (ATP-binding) and carboxy-termini (DNA-binding) from each SMC subunit would form domains with both DNA- and ATP-binding properties at each end of the molecule. These domains would directly bind DNA, cross-linking sister chromatids, and ATP hydrolysis may provide a mechanism to alter the configuration of the complex (for reviews, see Peterson, 1994; Gasser, 1995). However, in contrast to condensin, no role has been found for ATP hydrolysis in cohesin function (for review, see Losada & Hirano, 2001). Both SCC proteins would have a regulatory role in this model. Binding of Scc1p to the amino-terminus (ATP-binding) of Smc1p, and the carboxy-terminus (DNA-binding) of Smc3p, would form a ring structure that surrounds both DNA strands (Figure 1.11). Scc3p would associate directly with Scc1p, making no contact with the SMC subunits and might have a regulatory, rather than a structural role (for review, see Uhlmann, 2001; Anderson *et al.*, 2002). This proposed model helps to explain why cleavage of only a tiny proportion of Rad21p is required to release chromosome cohesion in *S. pombe*. In this model it is also propose that the SMC subunits of condensin form a heterodimer in the same way, and that the ring structure formed may stabilise chromatin loops during metaphase in vertebrate

cells, when arm cohesin has been dissolved (Hirano *et al.*, 1997; for review, see Nasmyth *et al.*, 2002; Figure 1.12).

1.7.7 Additional roles for Cohesin

Because chromosome cohesion is established at G1/S in *S. pombe* and *S. cerevisiae*, and at telophase in metazoans (for reviews, see Hirano *et al.*, 2000; Uhlmann *et al.*, 2001), it must be flexible to the dramatic changes in chromosomal structure that take place during the subsequent processes of DNA replication, DNA repair, meiotic recombination and chromosome condensation. Loss of this co-ordination leads to defects in gross chromosomal architecture and chromosome loss. Mechanisms are therefore in place to ensure these events are co-ordinated spatially and temporally. In addition, it is now becoming increasingly clear that cohesin has critical roles in some of these processes. The following sections describe how cohesin function may be important for various chromosomal functions.

1.7.7.1 DNA REPLICATION

Several components of the replication machinery have been implicated in sister chromatid cohesion. *S. cerevisiae* DNA polymerases Trf4p, Trf5p and σ-DNA polymerases have all been shown to be required for the establishment of cohesion during S phase (Castano *et al.*, 1996; Wang *et al.*, 2000, 2002). *S. pombe* Hsk1p is a serine/threonine kinase required for G1/S that is essential for DNA replication initiation (Takeda *et al.*, 1999). *hsk1*$^+$ interacts genetically with *rad21*$^+$ and is required for cohesin function during S phase (Takeda *et al.*, 2001).

S. pombe eso1$^+$ encodes a product whose C-terminus is homologous to *S. cerevisiae* Eco1p, and is required for establishment of sister chromatid cohesion (Takeda *et al.*, 2000, 2001; Section 1.7.2). *eso1*$^+$ interacts genetically with *pcn1*$^+$ (encoding polymerase processivity factor PCNA) and *cdc20*$^+$ (a DNA polymerase ε gene; Tanaka *et al.*, 2000). Similarly, *S. cerevisiae* Eco1p interacts genetically with DNA replication genes Pol30p and Ctf18p (Skibbens *et al.*, 1999). These findings strongly support the idea that sister chromatid cohesion is established at the replication fork, and that the Eso1p family functions in close association with the replication machinery to establish linkages between sister chromatids.

In *S. cerevisiae* an alternative RFC (Replication Factor C) has been identified, containing homologues of RFC subunits, that is implicated in the loading of DNA polymerase κ (TRF4p; now known as DNA polymerase σ), and is required for sister

chromatid cohesion (Hanna *et al.*, 2001; Mayer *et al.*, 2001). It is postulated that modified RFCs might associate with chromatin during S phase to establish cohesion, and that this association is dependant on changes in gross chromatin structure elicited by the passage of the replication machinery (Wang *et al.*, 2000; Mayer *et al.*, 2001; Bellaoui *et al.*, 2003; Merkle *et al.*, 2003). Another proposal is that cohesin is loaded onto chromatin prior to replication and that the passing replication fork serves to activate cohesin function (Kenna & Skibbens, 2003). In either case, it is clear that DNA replication is important for establishment of sister chromatid cohesion. However, whether these functions are co-dependant i.e. if cohesin has a role in DNA replication, remains to be revealed.

1.7.7.2 DNA REPAIR

The DNA damage checkpoint of eukaryotic cells helps ensure that DNA damage is repaired before it causes permanent, genetic alterations. To accomplish this, the checkpoint monitors the genome for damaged DNA. Once damage is sensed, the checkpoint delays the cell cycle to allow DNA repair enzymes sufficient time to execute their activities. Several checkpoint control genes have been isolated from fission yeast genetic screens that are required for cell-cycle arrest in response to DNA damage (Weinert *et al.*, 1988; Al-Khodairy & Carr, 1992; Rowley *et al.*, 1992; Walworth *et al.*, 1993; Al-Khodairy *et al.*, 1994). The Rad26p/Rad3p complex in fission yeast detects genotoxic insults (Edwards *et al.*, 1999) and this leads to activation of, Chk1p (Lopez-Girona *et al.*, 2001), mitotic delay and DNA repair.

Several lines of evidence suggest that cohesin subunits play critical roles in DNA repair pathways. *S. pombe* Rad21p was first identified as a protein required for efficient DNA repair. Mutations in *rad21*$^+$ cause an increased sensitivity to UV and γ irradiation and other DNA damaging agents (Birkenbihl & Subramani, 1992). Rad50p is an SMC-like protein that is involved in mating type switching and DNA replication, and also promotes the use of sister chromatids as the template for DNA repair by homologous recombination (Hartsuiker *et al.*, 2001). *rad21*$^+$ interacts genetically with *rad50*$^+$, and they are thought to function in the same pathway during S phase to assist repair and possibly re-initiation of collapsed replication forks. In *Xenopus*, cohesins XSMC1 and XSMC3 form a complex *in vitro* that promotes the repair of DNA gaps and deletions by homologous recombination (Hirano *et al.*, 1997; Losada *et al.*, 1998; for review, see Hirano, 1998). In addition, *S. pombe* Eso1p is thought to have a role in both cohesion and repair of DNA during replication (Tanaka *et al.*, 2000, 2001; see above). Cohesin may therefore have a direct role in DNA repair, perhaps by recruiting repair proteins to damaged DNA strands.

However, homologous recombinational repair of DNA, damaged by environmental agents or incomplete DNA replication, requires an intact DNA strand to act as a template. The role of cohesin in DNA repair might therefore be indirect, with cohesin holding the intact and damaged sister chromatids in alignment for the DNA repair machinery. In this way, cohesin would promote the use of the sister chromatid as a template for repair rather than another chromosome, therefore preventing ectopic transfer of genetic information.

1.7.7.3 MEIOTIC RECOMBINATION

Sister chromatid cohesion is also required for homologous recombination in meiosis. Meiotic cohesin co-localises with the axial elements of the synaptonemal complex in *S. cerevisiae* (Klein *et al.*, 1999) and mammals (Eijpe *et al.*, 2000). However, it is not clear whether cohesin is an integral component of the axial elements or if cohesin runs alongside these structures. In *S. pombe* linear elements, which are analogous to the axial elements, are required for homologous recombination and reductional segregation of chromosomes at MI. The meiotic cohesin Rec8p, is required for homologue pairing, linear element formation and normal levels of meiotic recombination (Molnar *et al.*, 1998; Section 1.5.1.2). During meiosis, cohesin distal to crossovers stabilises connections between homologues, and this is required for normal MI segregation (Buonomo *et al.*, 2000).

As for DNA repair, homologous recombination requires that chromosomes be held in alignment, which might explain why cohesin is required for this process. Because meiotic cohesin stabilises interhomologue interactions, it would promote the use of homologues rather than sister chromatids for meiotic recombination. This would favour the exchange of non-identical genetic material (a crucial feature of meiosis), and promote the accurate segregation of chromosomes in subsequent meiotic divisions. Meiotic cohesin may also have a more direct role in recombination, such as recruitment of recombination proteins. In support of this, in mammalian meiotic cells, cohesin was found to recruit recombination proteins and promote synapsis between homologous chromosomes in the absence of an axial element (Pelttari *et al.*, 2001).

1.7.7.4 CHROMOSOME CONDENSATION

A connection between chromosome condensation and cohesion was first indicated in an *S. cerevisiae* Mcd1p mutant that was defective in both of these processes (Guacci *et al.*, 1997). The cohesin-associated protein Pds5p, which co-localises with Mcd1p, is thought to have a role in this association (Hartman *et al.*, 2000; Panizza *et al.*, 2000). Pds5p homologues in *S. macrospora* and *A. nidulans* are also components of the cohesion and

condensation mechanisms in both mitosis and meiosis (Denison *et al.*, 1993; van Heemst *et al.*, 1999).

It is not yet clear how cohesion and condensation might be linked mechanistically. One possibility is that the presence of cohesin promotes condensation, perhaps by causing gross changes in chromosome structure. The following model describes how this might occur (Nasmyth, 1999). Soon after replication, the sister chromatids are held together at sites of cohesin binding. In the first stage of condensation, sites of cohesion are brought together, and as a consequence, the chromosomal regions between these sites are looped out. Condensin may then bind and further condense DNA in the intervening loops (Figure 1.12). Proteins that mediate cohesion and condensation are distinct but might interact with common chromosomal core components such as Rad21p/Mcd1p and Pds5p. These processes would be regulated independently by controlling the association of factors involved exclusively in either cohesion or condensation. In eukaryotes, condensation occurs simultaneously with loss of arm cohesin at prophase/prometaphase. It has been suggested that binding of condensin may physically displace cohesin from chromatin, and then, in the absence of cohesin complex, facilitate sister chromatid arm cohesion until anaphase (Bhat *et al.*, 1996; Hagstrom *et al.*, 2002; Bhalla *et al.*, 2002).

The most striking relationship between cohesin and condensin concerns their similarity in subunit composition. In both yeast species, condensin and cohesin complexes are composed of two members of the SMC family (cohesin: SMC1 and SMC3; condensin: SMC2 and SMC4) that form a V-shaped hetero-dimer (Figure 1.6), and at least two non-SMC subunits (Hirano *et al.*, 1997; Haering *et al.*, 2002; Anderson *et al.*, 2002). This probably reflects parallels in their functions, with both complexes providing a structural role, by binding DNA strands to assist chromosome dynamics.

1.7.7.5 CENTROMERE-KINETOCHORE FUNCTION

The relationship between cohesin and centromere function is complex, with one function of the centromere being to localise cohesin, and cohesin possibly influencing centromere function in return. In both *S. pombe* and *S. cerevisiae* cohesin is recruited to heterochromatic regions of the chromosome, in particular the centromere, telomeres and mating type locus. In *S. pombe* recruitment of cohesin to these regions is dependant on centromere proteins Swi6p and Clr4p. Swi6p has been shown to bind *S. pombe* cohesin Psc3p, and both Swi6p and Clr4p are required for accurate chromosome segregation (for reviews, see Pidoux & Allshire, 2000; Bernard *et al.*, 2001).

Rad21p localises with Mis4p to the centromere and pericentric regions from S phase, and inhibits sister chromatid separation until it's cleavage in anaphase (Toyoda *et*

al., 2001). Bub1p is a protein kinase essential for the spindle checkpoint in both mitosis (Bernard *et al.*, 1998) and meiosis (Bernard *et al.*, 2001). Bub1p normally localises at the kinetochore briefly during prophase, before MT capture (Bernard *et al.*, 1998). However, in *mis4*$^{-}$ and *rad21*$^{-}$mutants, mitotic kinetochore-MT interactions are diminished and localisation of Bub1p to the inner centromeres is prolonged. This leads to activation the Mad2p/Bub1p-dependant spindle checkpoint, and mitotic arrest (Toyuda *et al.*, 2002). In *rad21*$^{-}$ and *mis4*$^{-}$ mutants that lack Mad2p, the spindle checkpoint is not activated, and full spindle extension occurs with unequal nuclear division. *S. pombe* Mis4p and Rad21p therefore act in establishing the normal spindle-kinetochore interaction in early mitosis.

In *S. pombe* the mitotic cohesin Rad21p is preferentially localised at the outer centromeric repeats (*dg* and *dh*) while the meiotic cohesin, Rec8p, is also present at the inner centromere (*cnt* and *imr*; Watanabe *et al.*, 2001). It has been suggested that this pattern of cohesin localisation might be important for kinetochore orientation, with the presence of Rec8p at the inner centromere promoting monopolar attachment of sister kinetochores during MI for reductional division. However, by swapping the mitotic and meiotic cohesins, it was recently shown that, in *S. cerevisiae*, that centromeric cohesion and kinetochore orientation are separate functions (Buonomo *et al.*, 2000). When centromeric Rec8p was replaced with Mcd1p in meiosis, kinetochore attachment in MI was monopolar, yet MI segregation was equational because Mcd1p was cleaved at anaphase I leading to a loss of centromeric cohesion. Therefore, centromeric localisation of Mcd1p at was not enough to protect it from proteolysis, suggesting other factors must be involved. This study also showed that monopolar attachment was due to co-orientation of kinetochores, rather than one being inactived during MI. The centromeric protein Mam1p/monopolin, was identified as being critical for monopolar kinetochore attachment (Toth *et al.*, 2000). *Drosophila* MEI-S332, which is important for maintenance of centromeric cohesion, may have a similar function (LeBlanc *et al.*, 1999).

Human hRAD21 mutants also do not establish normal kinetochore-microtubule associations (Hoque & Ishikawa, 2001). The *S. cerevisiae* centromere protein, Spo13p, provides another possible link between centromeric cohesion and kinetochore behaviour. Spo13p is important for centromeric Rec8p localisation, for sister chromatid cohesion and normal kinetochore behaviour during meiosis (Shonn *et al.*, 2002; Lee *et al.*, 2002). The function of Spo13p may be to delay MI until monopolar kinetochore orientation and meiotic cohesion are established (Birkenbihl & Subramani, 1992).

1.7.8 *S. pombe* Rad21p

S. pombe Rad21p is a 67 kDa nuclear protein that is essential for mitotic growth (Birkenbihl & Subramani, 1992). *rad21*$^+$ contains an MCB element in its promoter region and its expression is cell cycle-regulated, with a peak in transcript and protein levels occurring at G1/S. Following translation, Rad21p is hypo-phosphorylated, then becomes phosphorylated at multiple serine residues as the cell cycle progresses (Birkenbihl & Subramani, 1995). The most hyper-phosphorylated form of Rad21p appears at metaphase, and this may be essential for APC-mediated proteolysis of Rad21p during anaphase. In contrast to *S. cerevisiae* where the majority of MCD1p is cleaved at the metaphase/anaphase transition, only < 5% of Rad21p is cleaved at anaphase, though this is essential for sister chromatid separation (Tomonaga *et al.*, 2000).

The *rad21*$^+$ gene was first identified in *S. pombe* in a screen for DNA repair mutants, and was found to be involved in the DNA double strand break repair pathway, with mutants being sensitive to γ-irradiation (Birkenbihl & Subramani, 1992). The *rad21-45* mutant is defective in the DNA double strand break (DSB) repair pathway but is capable of cell cycle arrest following irradiation, indicating that the checkpoints recognising DNA damage and mediating cell cycle arrest are still intact (Birkenbihl & Subramani, 1992). Interactions of Rad21p with Rad50p and Eso1p (see above) also suggest a function in S phase DNA repair.

The *rad21-K1* mutant is sensitive to UV and γ-irradiation. It is also sensitive to the spindle inhibitor thiobendazole, and the DNA replication inhibitor hydroxyurea, indicating additional roles for Rad21p in microtubule function and S phase function (Tatebayashi *et al.*, 1998). *rad21*$^+$ also interacts with tubulin genes *nda2*$^+$ and *nda3*$^+$, further supporting a role in microtubule function. The *rad21-K1* mutant is also defective in chromosome segregation, with cells displaying a cut-like phenotype i.e. cell division takes place in the absence of nuclear division, leading to uneven separation of chromosomes or bisection of the nucleus with a septum.

rad21$^+$ also interacts genetically with *cut9*$^+$ which encodes a component of the APC, indicating it may be involved in the ubiquitin-mediated proteolysis pathway (Tatebayashi *et al.*, 1998). In addition to its roles in cyclin destruction and sister chromatid separation, the APC is important for various cellular functions such as regulation of DNA replication, microtubule function and DNA repair (Section 1.4.4.3). Rad21p may be involved in these biological events by directly or indirectly regulating APC function.

1.7.9 *S. pombe* Rec8p

S. pombe rec8$^+$ encodes a meiotic cohesin, that contains two MCB elements in its promoter region, and whose expression is induced from pre-meiotic S phase through to MI (Lin *et al.*, 1992). Rec8p is loaded onto chromatin and activated during premeiotic S phase, and this timing is essential for the meiotic pattern of chromosome segregation (Watanabe *et al.*, 2001). Pre-meiotic S phase is universally several times longer than mitotic S phase, and this is thought to relate to the laying down of specialised features required for meiosis, such as meiotic cohesin (Riley & Bennett, 1971; Callan, 1973). Homologues of Rec8p have been identified in several species including *S. cerevisiae*, *Xenopus* and humans (Klein *et al.*, 1999). Human hRec8 is expressed at high levels in germ line cells supporting a meiosis-specific role for this conserved protein (Parisi *et al.*, 1999).

Rec8p and Rad21p share 29% and 35% sequence homology in their amino- and carboxy-termini, respectively (Watanabe & Nurse, 1999). Similar to Rad21p, phosphorylation of Rec8p is cell cycle-regulated. Rec8p is phosphorylated from prophase I through to beyond MI (Parisi *et al.*, 1999), and this may be relevant in the regulation of APC-mediated proteolysis of meiotic cohesin (Rogers *et al.*, 2002).

Rec8p is concentrated at the centromeric regions, becoming less abundant along the chromosome arms (Watanabe *et al.*, 2001). Cohesion distal to the centromeres is thought to be mediated by cohesins other than Rec8p (Krawchuk *et al.*, 1999; Kitajima *et al.*, 2003). In *S. cerevisiae*, Rec8p on chromosome arms is cleaved during anaphase I, allowing resolution of crossovers between homologues. Centromeric Rec8p is somehow protected from proteolysis at MI, and sister chromatids remain joined. Cleavage of centromeric Rec8p at anaphase II leads to loss of sister chromatid cohesion, and separation of sister chromatids as in mitosis (Buonomo *et al.*, 2000). However it is not presently known how centromeric cohesion is maintained until MII in *S. pombe.*

Rec8p was first identified in a screen for mutants with reduced recombination frequency at the *ade6* locus. It was also found to be essential for the meiotic pattern of chromosome segregation with *rec8* mutants undergoing equational division at MI due to precocious separation of sister chromatids (Watanabe & Nurse, 1999; Krawchuk *et al.*, 1999). Rec8p has been found to be important for several other aspects of chromosome dynamics during meiosis including homologue pairing, linear element polymerisation and homologous recombination (Molnar *et al.*, 1995). In addition, spore formation and spore viability are reduced in *rec8* mutants.

Exactly how Rec8p functions in meiotic chromosome dynamics is unclear. Linear elements are required for stabilisation of homologous contacts, which is especially important at chromosome regions furthest from the telomeres i.e. the centromeres where

Rec8p is most abundant (Figure 1.8). In turn, stable homologous contacts are required for efficient meiotic recombination (for review, see Moore & Orr-Weaver, 1998). Rec8p may bind to sites of early homologous contact, and directly promote binding of proteins for linear element formation and homologous recombination. Rec8p might also act indirectly in these processes, holding homologous chromosomes in alignment to assist assembly of replication initiation complexes and axial elements.

1.8 THIS STUDY

In a recent study in *S. pombe*, the meiotic cohesin *rec8*$^+$ was ectopically over-expressed during the mitotic cell cycle in cells deleted for *rad21*$^+$ (*rad21*Δ). Rec8p was found to support vegetative growth in these cells, though a reduction in growth rate and viability was reported. Conversely, the mitotic cohesin *rad21*$^+$ was ectopically over-expressed in the meiotic cell cycle, in cells deleted for *rec8*$^+$ (*rec8*Δ). However, defects such as aberrant chromosome segregation and abnormal spore formation were seen. It was concluded that Rec8p has basal cohesin activity, but also has specialised activities required for meiosis (Watanabe & Nurse, 1999).

The aim of this study is to further investigate the specific roles of Rad21p and Rec8p cohesins in the mitotic and meiotic cell cycles in *S. pombe*. This was achieved by swapping the promoter regions of the two genes, so that *rad21*$^+$ was expressed in a *rec8*Δ meiosis, and *rec8*$^+$ in mitosis where Rad21p was depleted. Using this system, the cohesins were ectopically expressed but, in contrast to the previous study, they were expressed at the appropriate time in the cell cycle and at physiological levels.

Physiological expression of *rec8*$^+$ was found to support vegetative growth in the absence of Rad21p, without cell cycle delay or loss of viability. In contrast, Rad21p was unable to support meiotic functions in the absence of Rec8p with defects seen in chromosome segregation and spore viability. However, in comparison to *rec8*Δ meiosis, spore development was significantly improved in these mutants, indicating that Rad21p was able to support this meiotic cohesin function. These findings show that the essential function of Rad21p is conserved in Rec8p, and indicate that the functional overlap between mitotic and meiotic cohesins may be greater than originally considered.

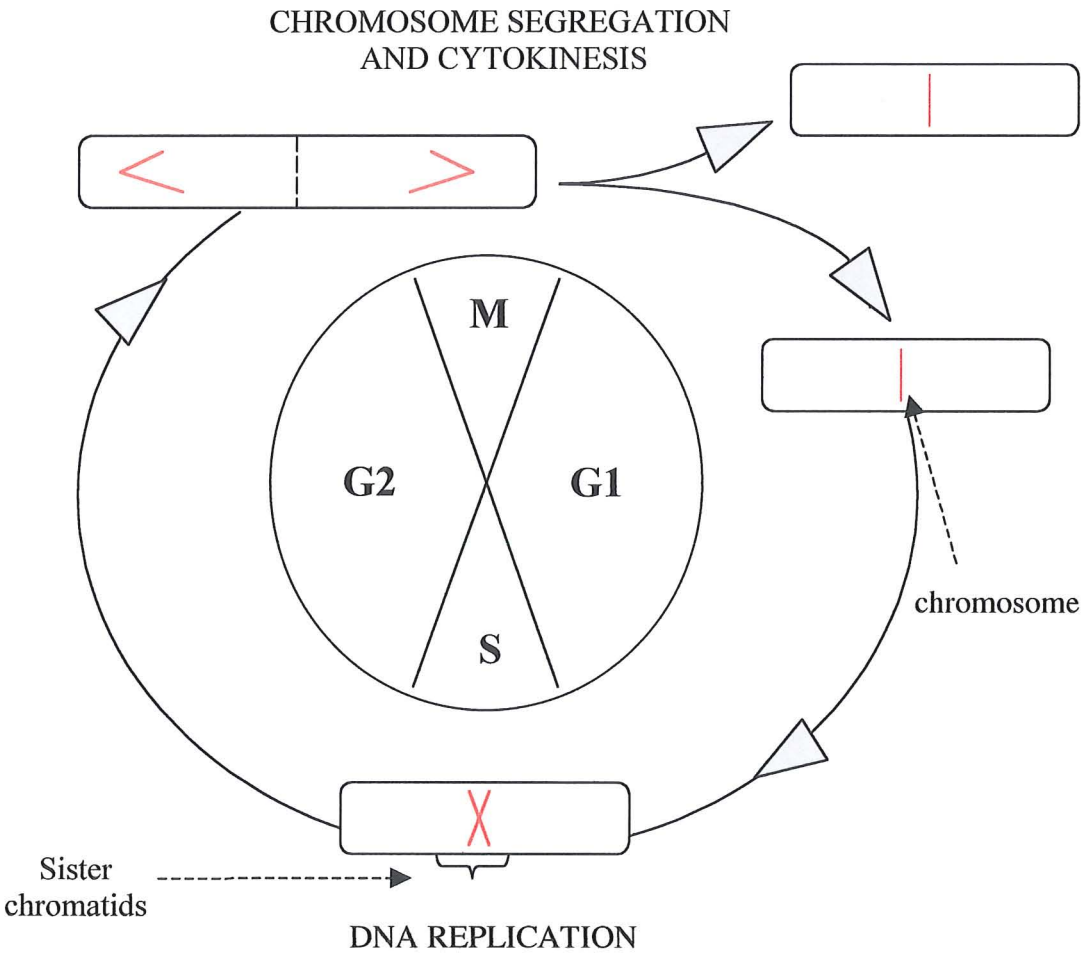


Figure 1.1 The Eukaryotic Cell Cycle The eukaryotic cell cycle can be divided into four phases. DNA is replicated at S phase so that each chromosome is now composed of two sister chromatids. At M phase sister chromatids separate and move to opposite poles of the parent cell. Nuclear and cellular division (cytokinesis) ensues with the generation of two identical daughter cells. For the purpose of clarity only one chromosome is shown.

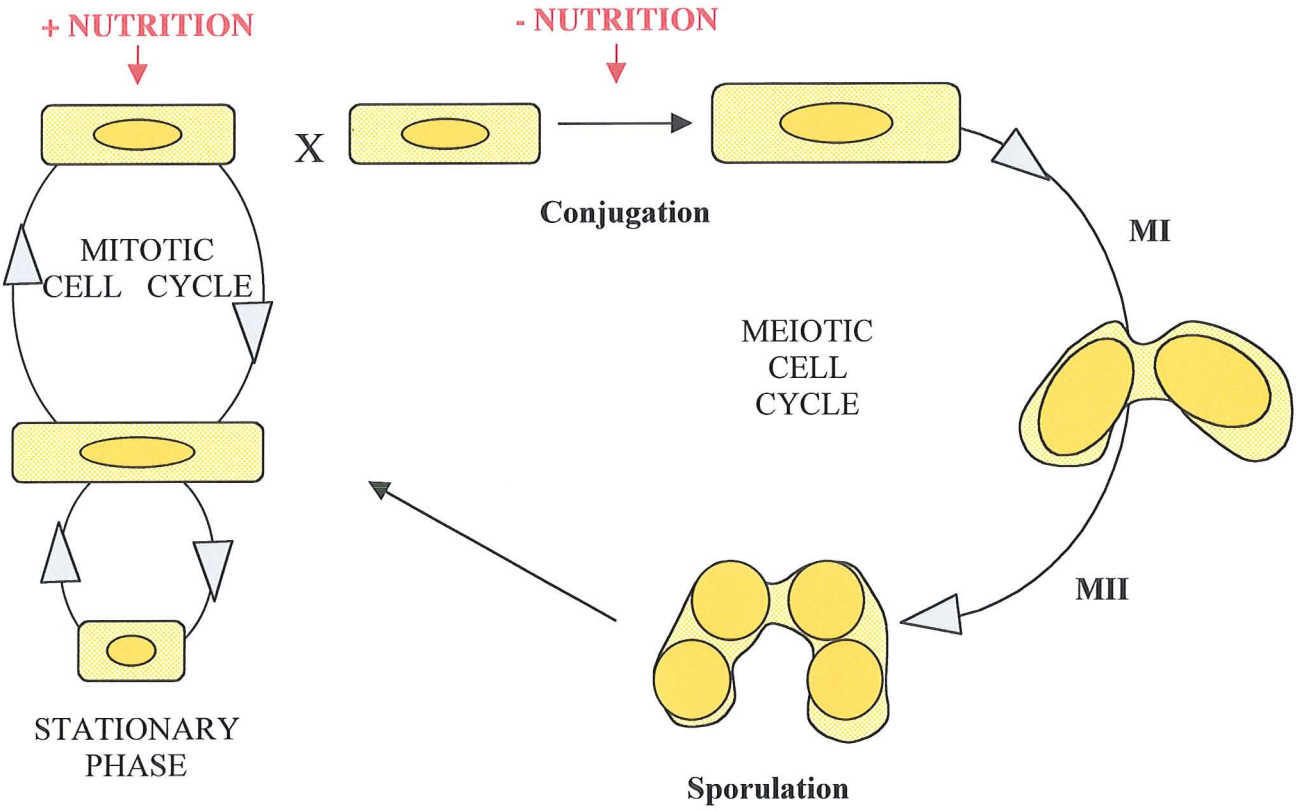


Figure 1.2 The *S. pombe* Life Cycle. In the presence of adequate nutrients, cells undergo repeated rounds of mitotic division as haploids. When nutrients are limiting, two cells of opposite mating type (h^+ and h^-) conjugate, forming a transient diploid that enters the meiotic cell cycle. Two rounds of meiotic division (MI and MII) lead to the formation of four haploid spores enclosed within an ascus.

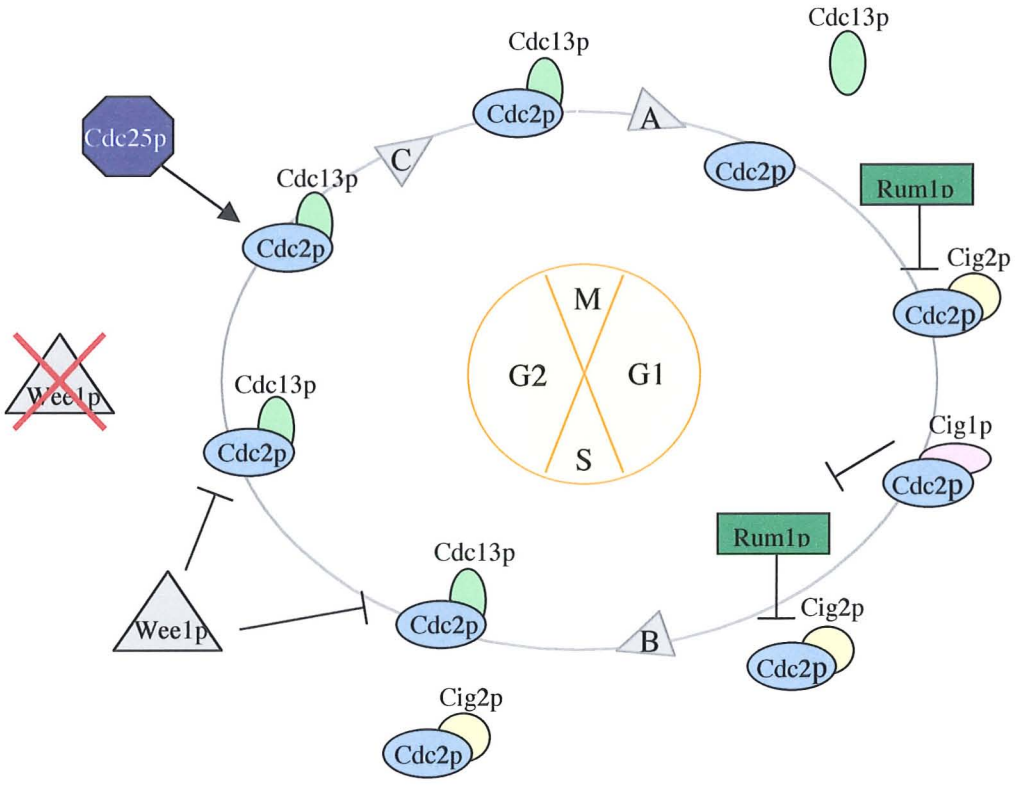


Figure 1.3 Regulation of the *S. pombe* Cell Cycle by Cdc2p. Cdc2p kinase activity is regulated by association with cyclins Cig1p, Cig2p and Cdc13p. **A,** Cdc2p activity is inhibited when cells exit mitosis by degradation of its associated cyclin Cdc13p. Accumulation of the Cdc2p inhibitor Rum1p ensures Cdc2p activity is kept low throughout late M phase and G1. **B,** In late G1 Cig1p-associated Cdc2p phosphorylates and inhibits Rum1p and Cig2p-associated Cdc2p activity rises, inducing entry into S phase. Cdc13p associates with Cdc2p from S phase, but its activity is inhibited by Wee1p phosphorylation. **C,** In late G2 Wee1p is inhibited, Cdc2p is de-phosphorylated by Cdc25p driving cells into mitosis.

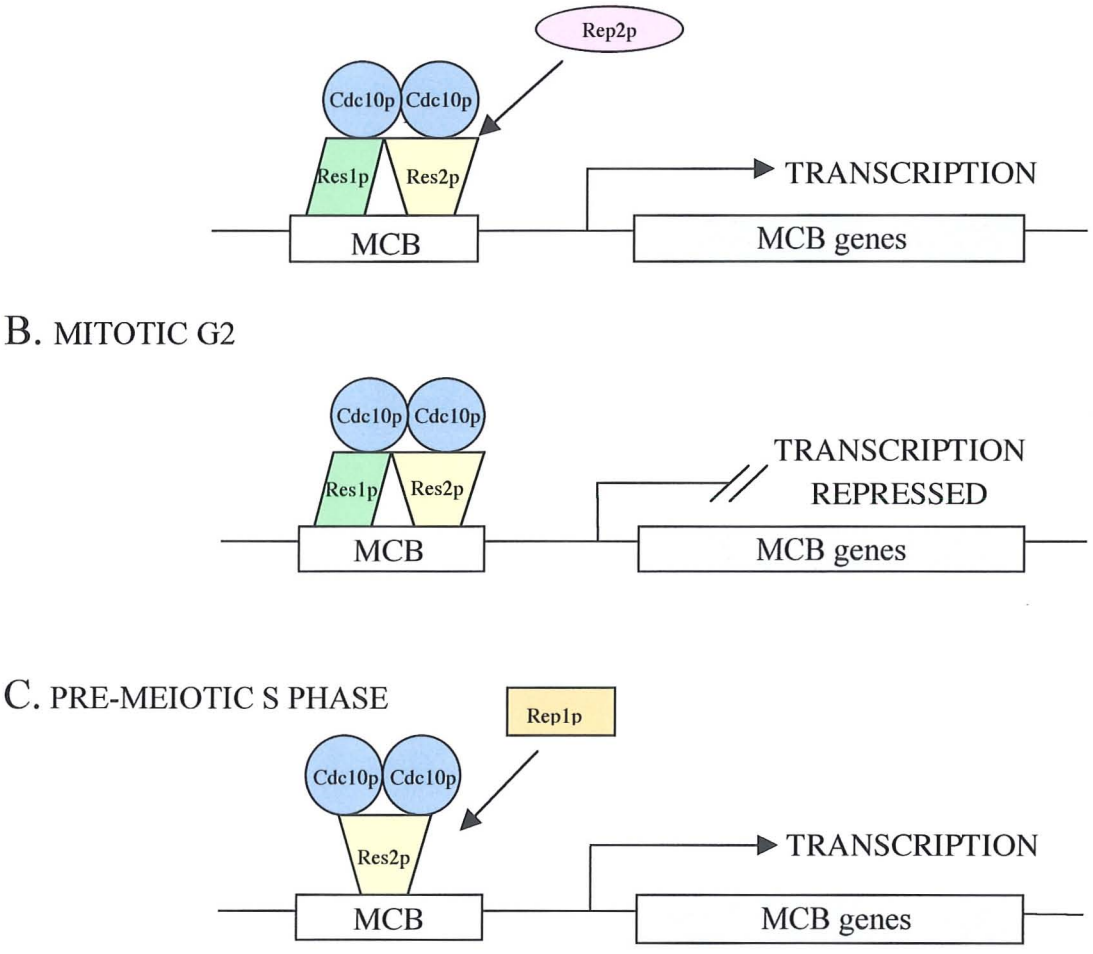


Figure 1.4 Transcriptional Regulation of the *S. pombe* Cell Cycle by DSC1. Res1p and Res2p bind Cdc10p at their C termini and MCBs via their N-termini. Both Res1p and Res2p are bound throughout the cell cycle. Transcriptional co-activators Rep1p and Rep2p regulate the periodic expression of MCB genes. **A,** At G1-S, Rep2p activates Res2p subunit, promoting transcription of MCB genes **B,** At G2, Res2p has inhibitory effect on DSC1 and MCB gene transcription is repressed **C,** Rep1p binds Res2p during pre-meiotic S phase to stimulate transcription of meiotic MCB genes.

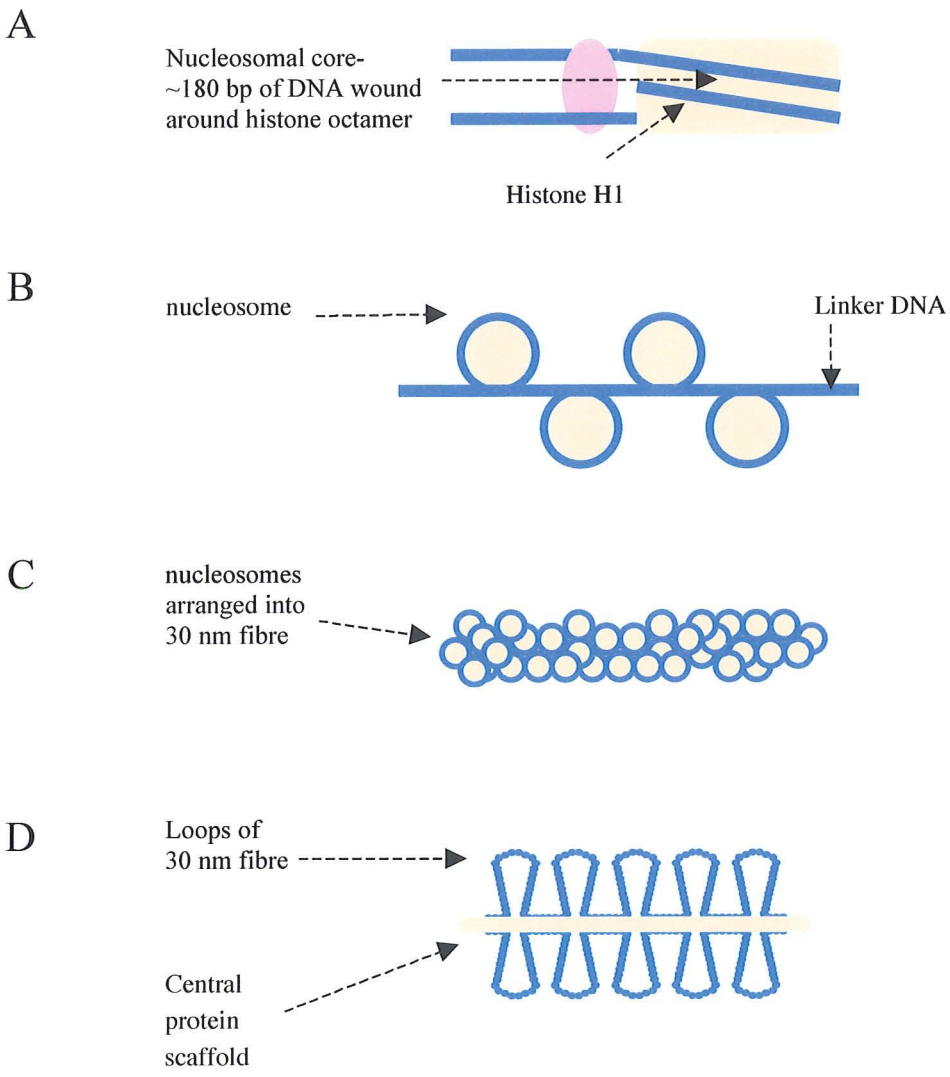


Figure 1.5 Eukaryotic Chromatin Packing. A, Diagram of a nucleosome. Each DNA strand wraps twice around a histone octamers, composed of two of each of the histones H2A, H2B, H3 and H4. The nucleosome is stabilised by a molecule of histone H1. **B,** The first order of packing gives a 10 nm fibre. **C,** The second order of packing forms a fibre 3 nucleosomes thick. **D,** Chromatin loops are attached at their base to a protein scaffold. In the metaphase chromosome these loops are further supercoiled to give the highest degree of chromatin condensation (not shown). Blue lines represent DNA.

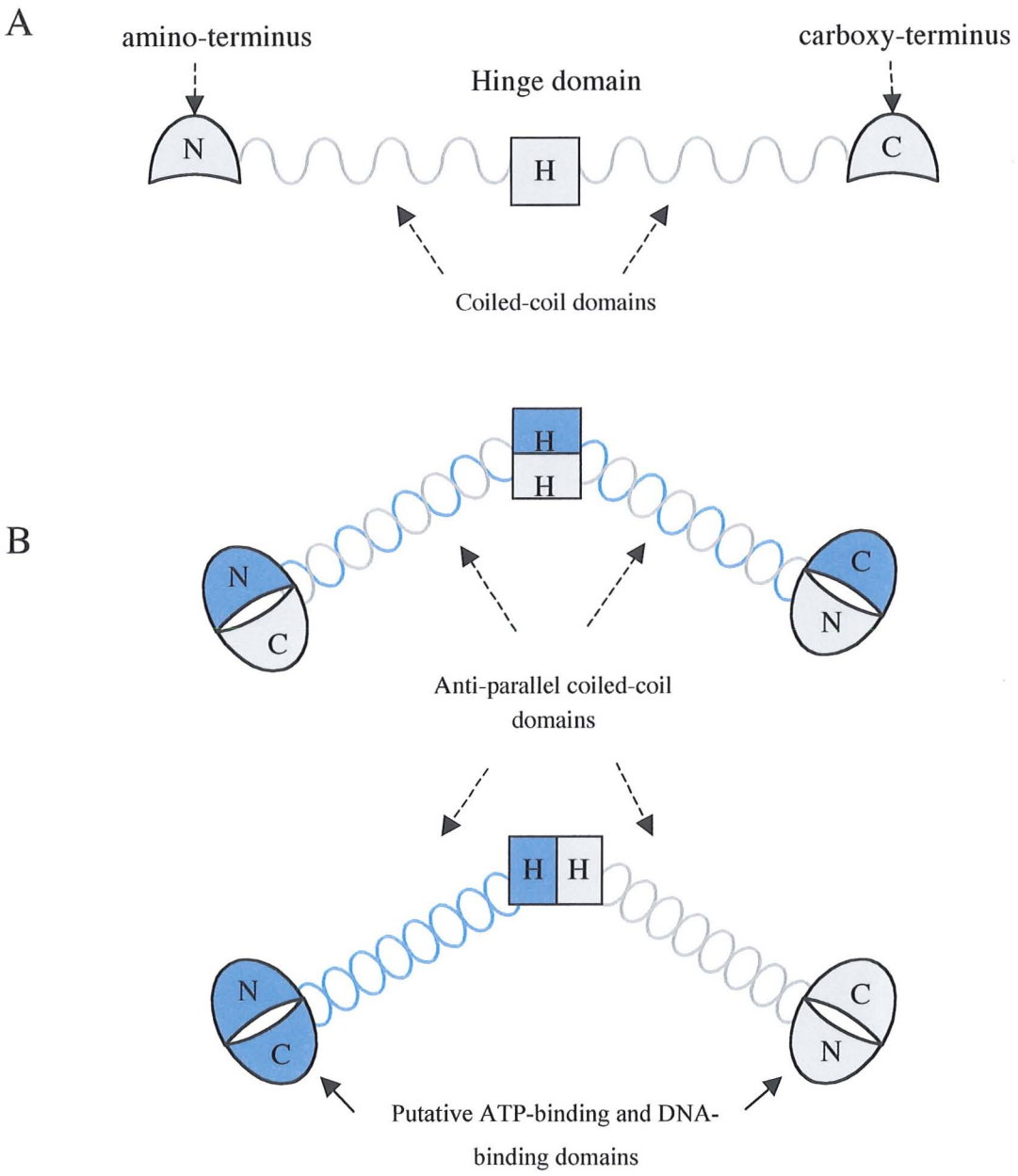


Figure 1.6 The Structure of SMC Dimers. A, Domain organisation of SMC proteins. **B**, Alternative proposed dimeric interactions of SMC molecules. **Top**: Smc1p (blue) and Smc3p (grey) associate with eachother along their lengths. **Bottom**: SMCs double back on themselves at the flexible hinge domain (H). Smc1p and Smc3p associate at their hinge regions. Association of amino- and carboxy-termini (N and C respectively) form putative ATP- and DNA-binding domains.

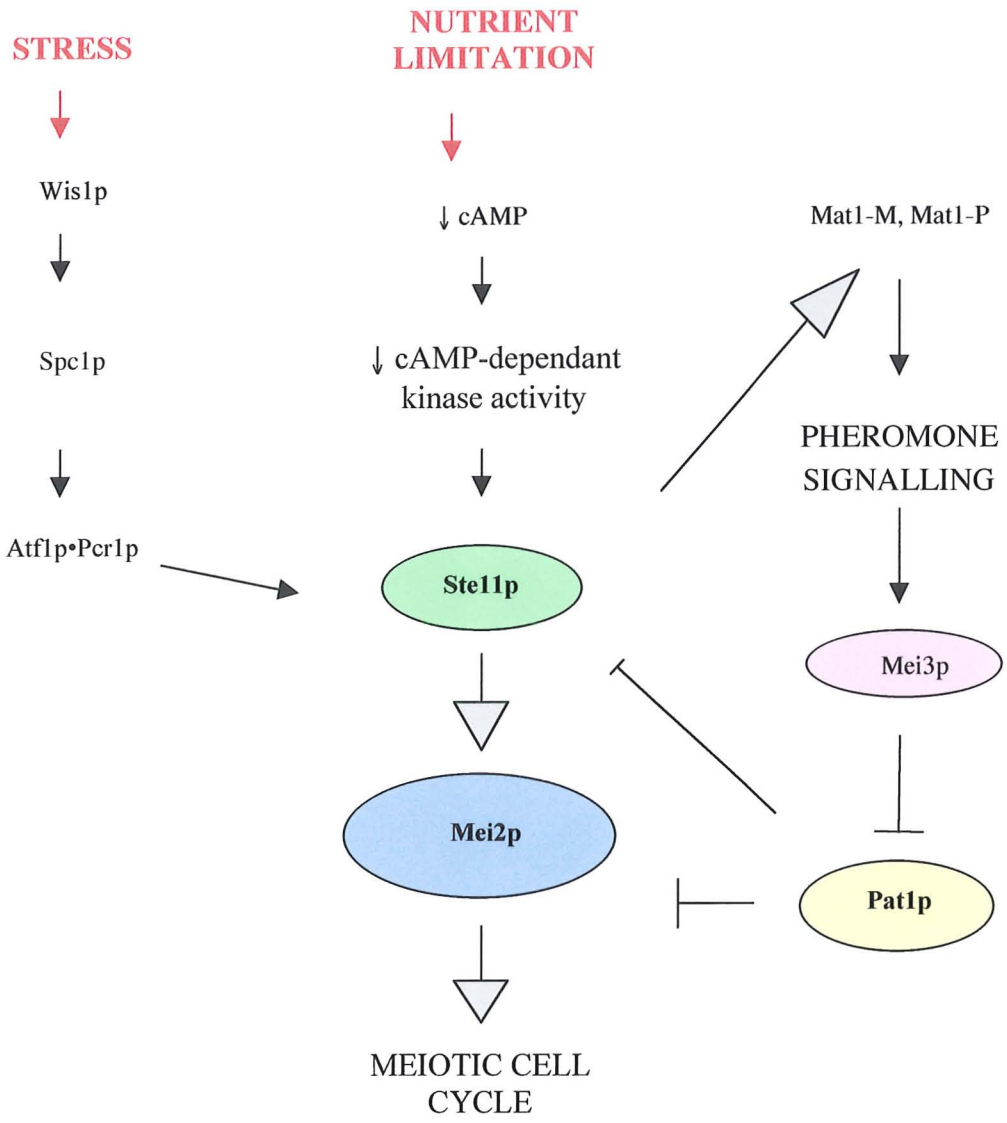


Figure 1.7 Entry into Meiosis is Triggered by Mei2p Activation. During vegetative growth, Pat1p kinase suppresses meiotic development by inhibiting the meiotic transcription factor, Ste11p, and the meiotic determinant Mei2p. Stress and nutrient limitation activate Ste11p to trigger meiotic development. Ste11p directly stimulates *mei2*$^{+}$ transcription. Ste11p also indirectly promotes meiotic development by activating the pheromone signalling response. This leads to inactivation of Pat1p kinase by Mei3p, releasing its inhibitory effects on Mei2p and Ste11p to allow entry into the meiotic cell cycle.

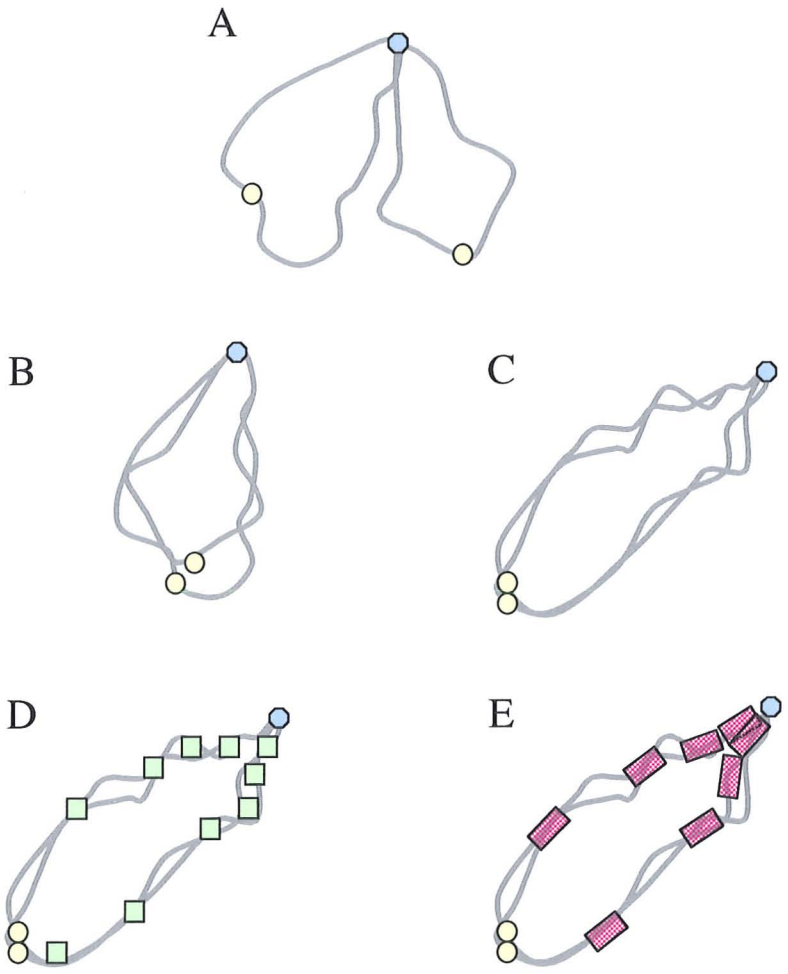


Figure 1.8 Model of Homologous Chromosome Pairing. A, Telomeres cluster and associate with the spindle pole body ○ to form a bouquet structure. **B,** The telomeres are pulled back and forth by the spindle pole body. **C,** This leads to alignment of homologous chromosomes with multiple points of contact between them. **D,** These interstitial contacts are stabilised by cohesin □ and further pairing is promoted between nearby regions of the chromosomes. **E,** Linear elements ▭ form at the sites of cohesin binding to complete synapsis. Interactions between homologs are less abundant at the centromere ○ compared to telomeric regions.

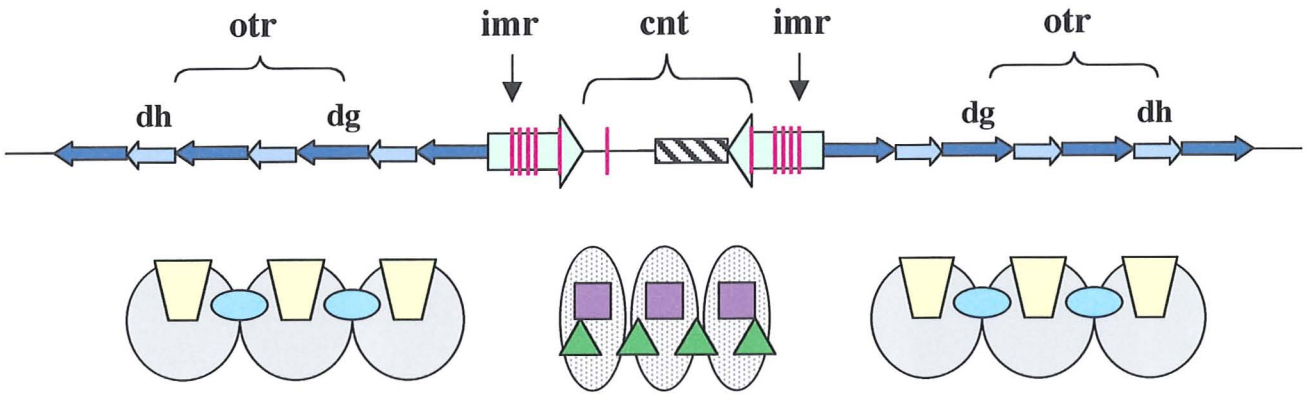


Figure 1.9 Organisation of *S. pombe* Centromeres. Centromere of chromosome III (*cen3*) is shown. **Top**: The central core (***cnt***) is distinct for each *cen* except for the highly homologous *tm* region (hatched box), which is also found in *cen1*. The central core is flanked by two inner most repeat regions (***imr***) and two outer repetitive regions (***otr***). The outer repeat regions are composed of repetitive elements ***dh*** and ***dg***. Pink lines represent tRNA-like genes present within the centromere region. **Bottom**: Proteins associated with specific centromere domains. Unique chromatin structure of central domain due to association of histone H3 variant, Cnp1p ⊙. Other proteins at *cnt* are Mis6p ■ and Mis12p ▲. Outer repeats are associated with chromo-domain proteins Swi6p ⬭ and Chp1p ▽. Both Rik1p and Clr4p are required for this association. Histones of centromere nucleosomes are hypo-acetylated ○.

S. pombe	*S. cerevisiae*	*Xenopus*	Human	*Drosophila*
Rad21p	Mcd1p/Scc1p	XRAD21	hR21	DRAD21
Psm1p	Smc1p	XSMC1	hSMC1	??
Psm3p	Smc3p	XSMC3	hSMC3	DCAP
Psc3p[a]	Scc3p	XSA1,2	STAG1,2	DSA
Rec8p[b]	Rec8p	??	hRec8	??
Rec11p[c]	Scc3p	??	STAG3	??

Table 1 Members of the Cohesin Complex

a Psc3p does not seem to be in a stable complex with Rad21p, Psm1p and Psm3p (Tomonaga *et al*, 2000)

b Rec8p replaces Rad21p in the cohesin complex in meiosis in *S. pombe* and *S. cerevisiae*

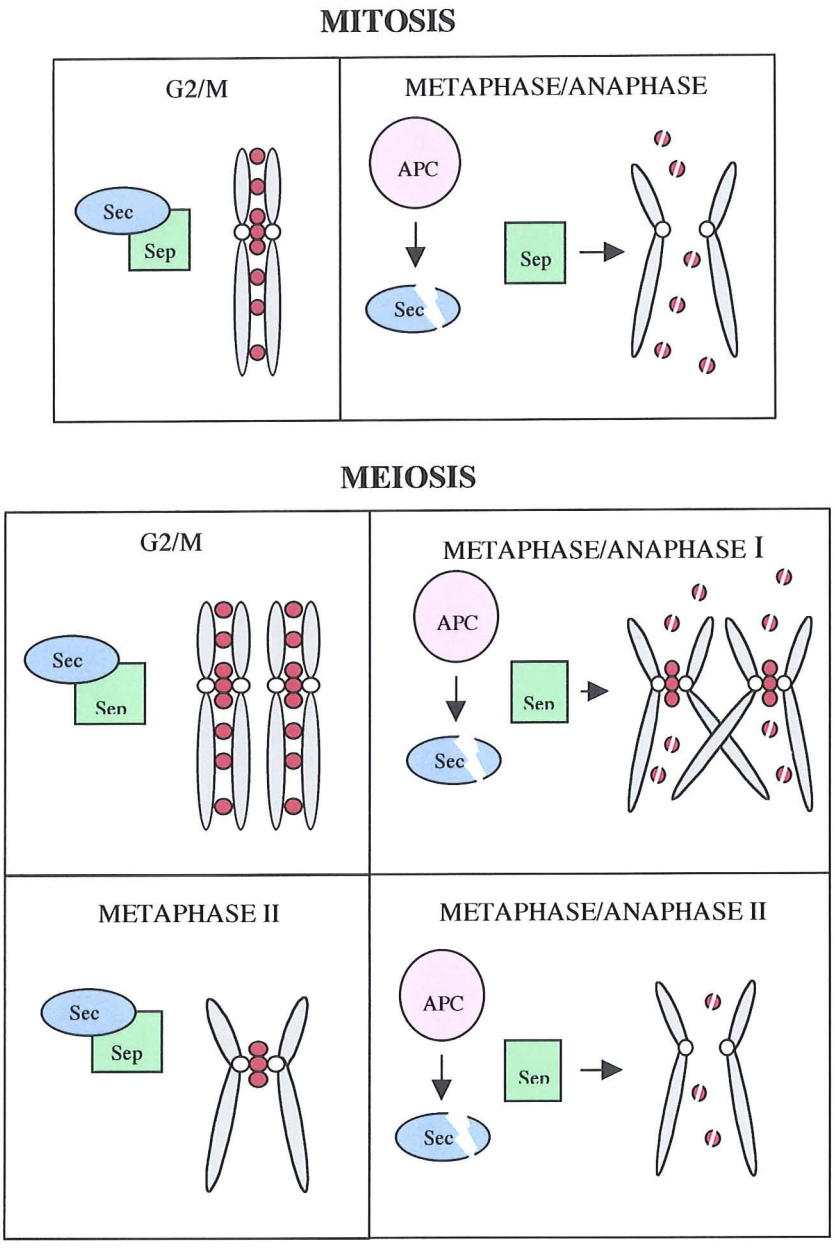


Figure 1.10 APC-Mediated Removal of Cohesin from Chromosomes during Mitosis and Meiosis. During G2/M phase, cohesin is distributed along the whole length of chromosomes. Securin (**Sec**; ESP1p) binds separin (**Sep**; PDS1p), inhibiting its proteolytic activity. During mitosis (**top**), the Anaphase Promoting Complex (**APC**) targets securin for degradation. Separin is released and triggers removal of cohesin (**red circles**) at metaphase/anaphase by the proteolytic cleavage of MCD1p. During meiosis (**bottom**) cohesin is dissolved in two stages. At metaphase/anaphase of MI, arm cohesin is cleaved by separin, allowing resolution of chiasmata and separation of homologs. Centromeric cohesin is 'protected' by an unknown mechanism. After MI, APC activity is then transiently inhibited, and securin once again binds and inhibits separin. At metaphase/anaphase II, APC is activated triggering cleavage of centromeric cohesin, allowing sister chromatid separation.

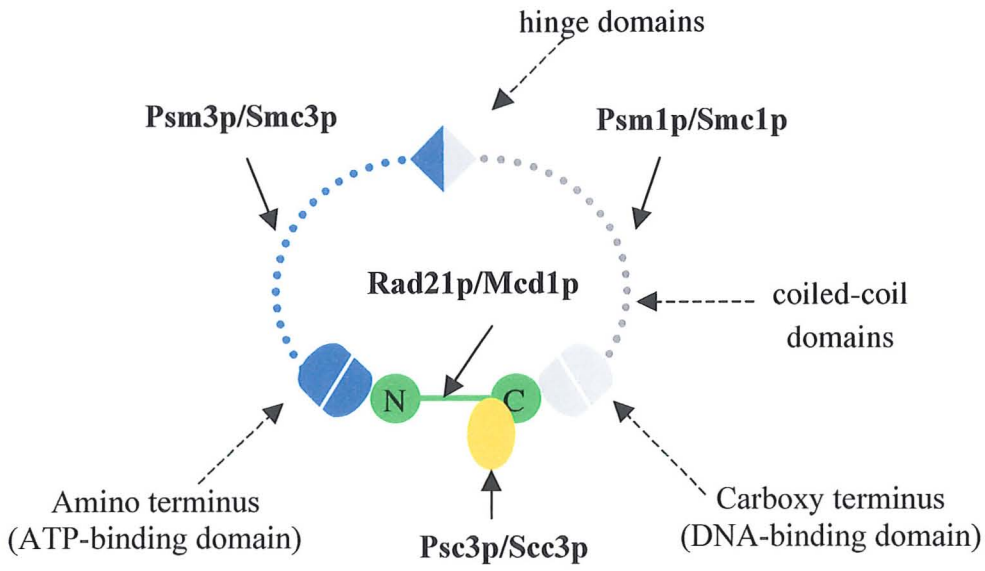


Figure 1.11 Model of the Cohesin Complex. SMC subunits Psm3p/Smc3p and Psm1p/Smc1p are proposed to associate with eachother via their hinge regions, and with themselves via their amino- and carboxy-termini. Binding of Rad21p/Mcd1p to the heads of Psm3p/Smc3p and Psm1p/Smc1p completes a ring structure that might surround double stranded DNA to elicit cohesion. N and C represent the N-and C-termini of Rad21p/Mcd1p.

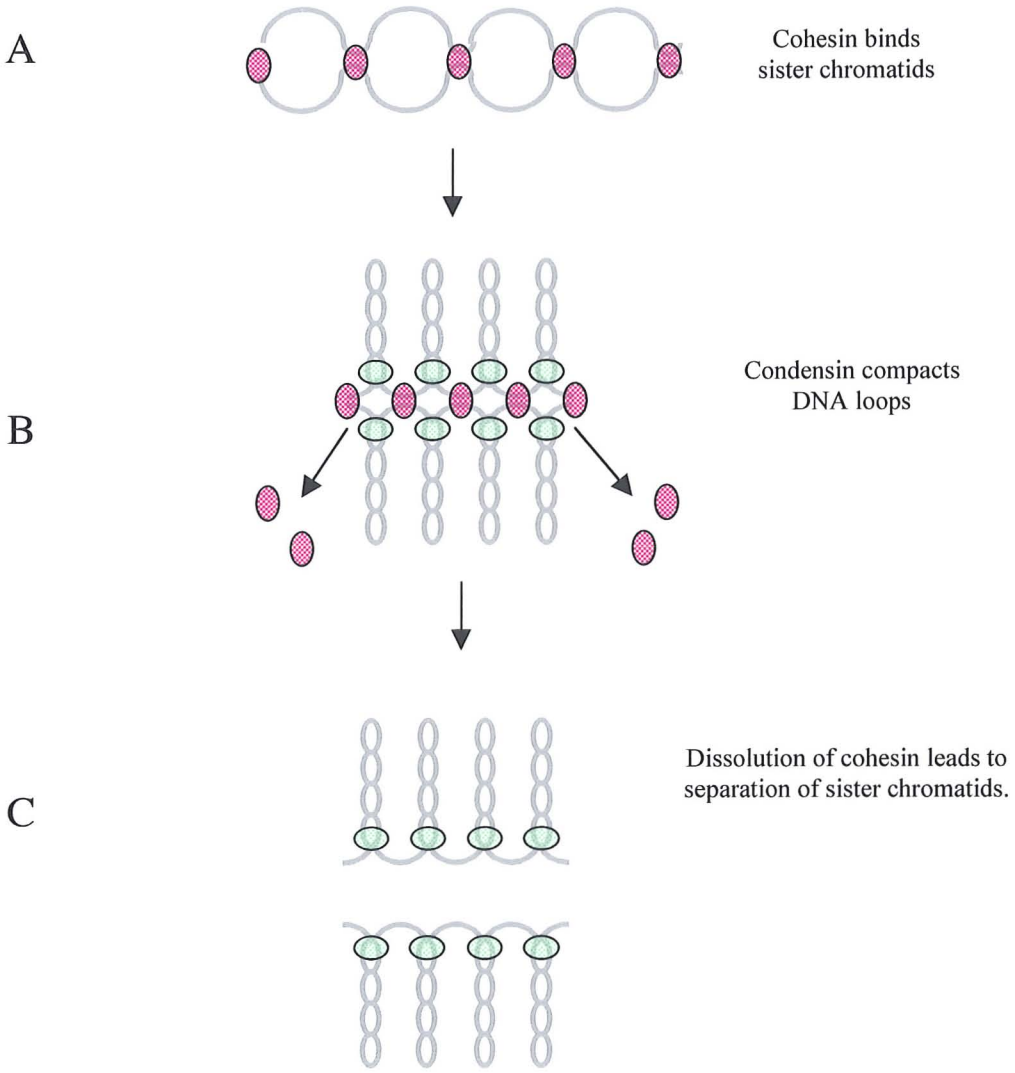


Figure 1.12 Association of Cohesin and Condensin during Mitosis. A, Cohesin ● associates with whole chromosome length, binding together sister chromatids. **B,** DNA is compacted as sites of cohesin are brought together. DNA loops protrude from central protein core, which are stabilised by condensin ○. **C,** Condensin further compacts DNA in intervening DNA loops. Dissolution of cohesin at anaphase (*S. pombe*) leads to separation of sister chromatids. DNA Removal of condensin leads to de-condensation of DNA (not shown).

Chapter 2:

Materials and Methods

2.1 BACTERIAL METHODS

Yeast strains, plasmid DNAs and primers used in this thesis are listed in Appendices I, II and III, respectively. Bacterial methods were as described in Sambrook *et al.*, 1989.

2.1.1 Bacterial Transformation

2.1.1.1 PREPARATION OF ELECTRO-COMPETENT BACTERIAL CELLS

Bacterial cells were grown up overnight, in 10 ml LB containing the appropriate antibiotic in a shaking incubator at 37°C. The next day, the culture was transferred to a flask containing 200 ml LB + antibiotic, and incubated at 37°C for a further 3-4 hours, until the culture was in exponential phase. This was indicated by an OD_{260} of 0.5-0.6, as determined by spectrophotometry.

The culture flask was placed on ice for 30 minutes. Cells were then harvested by centrifugation at 3000 rpm for 15 minutes, and the supernatant poured off. Bacterial cells were first washed by resuspending in 10 ml of ice-cold sterile dH_2O, and pelletted by centrifugation at 3000 rpm for 15 minutes. This step was repeated. The cells were then resuspended in 10 ml ice-cold 10% sterile glycerol, pelletted by centrifugation, and the supernatant poured off. This step was then also repeated. Bacterial cells were resuspended in the residual 10% glycerol, transferred as 100 µl aliquots to chilled microfuge tubes, and either used immediately or stored at -70°C.

2.1.1.2 ELECTROPORATION

LB + antibiotic plates were first prepared, the cuvettes and the cuvette holder (GenePulser/*E.coli* Pulser Cuvette; Bio-Rad, 165-2086) were put on ice for 20 minutes and SOC (Section 2.6.1.1) was thawed. Competent bacterial cells were then defrosted on ice for 10 minutes. Plasmid DNA (1-3 µl) was added to the competent cells, gently mixed and left on ice for 1 minute. This was then transferred to the chilled cuvette, inserted into cuvette holder and pulse electroporated at 2.5 KV using the Bio-Rad *E. coli* Pulser. One millilitre of SOC was added then the cells transferred to screw-cap tubes in a shaking incubator at 37°C for 1 hour.

Transformed bacterial cells were spread onto prepared LB + antibiotic plates and incubated overnight at 37°C. Colonies that grew indicated cells that had successfully taken up plasmid DNA conferring antibiotic resistance.

2.1.2 Plasmid Miniprep

10 ml of LB broth containing the appropriate antibiotic, was inoculated with bacterial cells (*E. coli* strain DH5α) from a glycerol stock, and incubated overnight at 37°C. 1.5 ml of the culture was transferred to a microfuge tube, and cells pelletted by centrifugation at 13,000 rpm for 1 minute, and the supernatant poured off. The microfuge tube was refilled with 1.5ml of bacterial culture and after a repeat spin the supernatant was poured off. The pellet was then resuspended in 150 µl of buffer P1 (Section 2.6.1.2) by vortexing. 150 µl of buffer P2 (Section 2.6.1.3) was added, the tube inverted to gently mix, and incubated at room temperature for 10 minutes. 150 µl of buffer P3 (Section 2.6.1.4) was then added and gently mixed by inverting. Following centrifugation at 13,000 rpm for 5 minutes, 400 µl of supernatant containing plasmid DNA was carefully pipetted off and transferred to a fresh microfuge tube. 1 ml of 100% ethanol was then added, plasmid DNA pelletted by centrifugation at 13,000 rpm for 5 minutes, and the supernatant poured off taking care not to lose pellet. 150 µl of 70% ethanol was then added, spun at 13,000 rpm for 1 minute, and the liquid carefully pipetted off. The DNA pellet was finally air-dried at room temperature then resuspended in 50 µl of buffer TE.

2.2 YEAST METHODS

Media used for the propagation of *S. pombe* were as described by Moreno *et al.*, (1991). Standard genetic procedures of Gutz *et al.*, (1974) and Kohli *et al.*, (1977) were followed. *S. pombe* strains used in this study are described in Appendix I.

2.2.1 Yeast Mating and Random Spore Analysis

Freshly growing cells were mixed together, using a sterile toothpick, in a drop of sterile dH_20 on malt extract plates. ME is a nutrient-limited culture medium which triggers *S. pombe* cells to exit the cell division cycle and undergo conjugation forming diploids, followed by meiosis and the release of four haploid spores. Cross plates were incubated at 25°C for 3 days, after which time completion of meiosis and sporulation was ascertained by the appearance of multiple asci.

A small aliquot of the resulting mixture of spores and cells were suspended in solution containing 20% helicase. This is a crude snail gut enzyme preparation that breaks down the ascus wall and kills vegetative cells by partially digesting the cell wall. Overnight

incubation in helicase at 25°C was therefore used to kill vegetative cells that had failed to sporulate.

After incubation the spore solution was diluted and spread onto YE plates, then cultured until single colonies grew (typically 5-7 days). Individual colonies (~200-400) were patched onto YE in a grid pattern. This was cultured for 1-3 days until patches had grown, then replica plated onto selective media to determine genotypes.

2.2.2 Identifying the *pat1-114* Allele

Cells carrying the temperature-sensitive *pat1-114* mutation were identified by culture on YE + phloxin at 25°C and 36°C. Phloxin B is a pink stain that accumulates in dead cells, which become stained dark red. In *pat1-114* mutants Pat1p is inactivated at 36°C and cells undergo meiosis. But as these cells have only a haploid DNA content, sporulation produces inviable products. *pat1-114* mutants were therefore identified as those that stained red at 36°C when compared to culture on YE + phloxin at 25°C.

2.2.3 Mating Type Determination

Freshly growing cells from the *S. pombe* strain of unknown mating type were patched twice onto an ME (malt extract) plate. Freshly growing cells from wild type strains of known mating type (h^+ GG 218 and h^- GG 217 were then patched alongside them, and the cells mixed together in a droplet of sterile dH_2O (Figure 2.1).

The plate was left to air-dry, then incubated at 25°C for 2-4 days until asci had formed. Iodine crystals dropped into the base of the ME plate and left 15 minutes for the vapour to stain the spores. The iodine crystals were then discarded and the ME plate left open for 5-15 minutes for the excess vapour to dissipate. The mature spore wall is rich in amylose-like materials and is stained brown by iodine vapour. Patches that had stained with iodine therefore represented those that had undergone conjugation and meiosis, and therefore had contained cells of opposite mating type.

2.2.4 Protoplast Fusion

This method was used to make stable diploid *S. pombe* cells from two haploids of the same mating type. The two haploid strains to be fused (strains A and B) were cultured in 10 ml EMM + supplements for 1-3 days at permissive temperature, then transferred to 100 ml EMM + supplements, and incubated overnight with shaking until the culture was in

exponential phase ($2 \times 10^6 - 1 \times 10^7$ cells/ml). The cells were pelletted by centrifugation at 3000 rpm for 5 minutes and the supernatant poured off. The cells were resuspended in 10 ml SP1 (Section 2.6.2.1) and 20 µl 2-mercaptoethanol and incubated at room temperature for 10 minutes. Cells were pelletted by centrifugation at 3000 rpm for 5 minutes, discarding the supernatant, and resuspended in 10 ml SP2 (Section 2.6.2.2). 20 mg of zymolyase 100T (Seikagaku, 120493), which hydrolyses the glucose bonds in the yeast cell wall, was then added and incubated at 35°C for $^1/_2$-1 hour (25°C if *ts* mutant), checking regularly until 50% of the cells were converted to spheroplasts. This was tested by placing a 5 µl droplet of cells onto a glass slide with 5 µl 0.5 % SDS. This lysed spheroplasted cells giving them a 'ghostly' appearance when compared to cells without SDS. 30 ml of SP3 (Section 2.6.2.3) was then added and the cells pelleted at 3000 rpm for 5 minutes. Pellets were washed twice in 30 ml SP3, discarding the supernatant, and resuspended in 1 ml of SP4 (Section 2.6.2.4).

Three microfuge tubes were set up containing: (i) 100 µl strain A, (ii) 100 µl strain B, (iii) 50 µl strain A + 50 µl strain B. 1 ml of SP5 (Section 2.6.2.5) was added to each tube, the cells gently mixed, then incubated at room temperature for 30 minutes. The cells were gently pelletted at 2000 rpm for 5 minutes and the supernatant poured off. Cells were then resuspended in 100 µl SP4. Strains A and B carried complementing *ade*$^-$ alleles, so that their successful fusion formed an *ade*$^+$ diploid. To isolate *ade*$^+$ diploids, cells were spread onto EMM + supplements + 1 M sorbitol plates lacking adenine, and incubated at permissive temperature for 7-14 days, until large white colonies had grown.

2.2.5 DAPI Staining of Ascospores

2.2.5.1 FIXING AND STAINING OF ASCOSPORES

Ascospores were gently resuspended in 0.5 ml PBS, then fixed in 95% ethanol in PBS, by gradual dehydration. First ascospores were pelletted by a pulse spin at high speed, the supernatant poured off, and resuspended in 0.5 ml 10% ethanol. Ascospores were pelleted again by a pulse spin, the supernatant poured off and resuspended in 0.5 ml of 25% ethanol. Ascospores were again pelletted by a pulse spin, and resuspended in 0.5 ml 50% ethanol. Pelleting was repeated followed by resuspension in 0.5 ml 70% ethanol. Finally, after pelleting again, asci were fixed by resuspension in 0.5 ml 95% ethanol.

To label spore DNA, diamino-2-phenylindole dihydrochloride (DAPI; Sigma, D-9542) was added to a final concentration of 1 µl/ml (0.5 µl of 1 mg/ml stock) and gently mixed. Stained ascospores were viewed immediately or stored at 4°C in the dark.

2.2.5.2 VIEWING OF DAPI STAINED ASCOSPORES

To view the DAPI labelled ascospores, glass slides and coverslips were first rinsed in 100% ethanol and air dried. The slides were coated with poly-L-lysine (Sigma, P-8920; diluted by a factor of 10 with dH_2O) and left to dry for 15 minutes. Excess poly-L-lysine was rinsed off briefly with dH_2O and the slides dried at 80°C. Five microlitres of fixed, DAPI-stained ascospores were pipetted onto the poly-L-lysine coated slides and left for 5 minutes. Five microlitres of mounting medium (Vectashield; Vector, H-1000) was placed over the ascospores, then covered with a coverslip. These were then left for 30 minutes before viewing to allow ascospores to fix to the slide.

Ascospores were visualised by differential interference contrast with fluorescence microscopy using an Olympus BX60 microscope. Images were captured using a Princeton Instrument digital camera.

2.2.6 Yeast Transformation

2.2.6.1 MAKING COMPETENT YEAST

A 100 ml *S. pombe* culture was grown to exponential phase (2-5 x 10^6 cells/ml; OD_{600}; 0.5-0.7) in liquid YE, then pelleted at 3000 rpm for 5 minutes. The cells were washed once in 5 ml of LiAc/TE (Section 2.6.2.6), pelleted at 3000 rpm for 5 minutes, then resuspended in 1 ml LiAc:TE + 1 ml 2 M sorbitol. Cells were aliquoted into 100 µl and stored at -80°C.

2.2.6.2 TRANSFORMATION

1 µg of DNA and 50 µg of carrier DNA (5 µl of 10 mg/ml stock; Section 2.6.2.8) were added to 100 µl competent yeast cells in a microfuge tube. 700 µl of PEG mix (Section 2.6.2.7) was added, the contents mixed by vortexing, then incubated at 30 °C for 30 minutes. The cells were heat shocked at 42°C for 15 seconds, then pelleted at 3000 rpm for 5 minutes. The cells were resuspended in 200 µl sterile dH_2O then plated onto selective media.

2.2.7 Viability Assay

rad21$^+$-repressible cells were cultured to mid-exponential phase (2-5 x 10^6 cells/ml) either with thiamine to repress *rad21*$^+$ transcription, or without thiamine. Cell counts were determined using a Beckton Dickinson Coulter Counter.

10 ml was taken from each culture and the cells pelleted by centrifugation at 3000 rpm for 5 minutes. Cells were then re-suspended in sterile dH_20 to a final concentration of 1000 cells/ml. 200 µl of these cell suspensions from each culture were then seeded neat or at serial dilutions (Figure 2.5) onto EMM + thiamine or EMM - thiamine. For each culture, cells were seeded at each dilution 5 times, and the mean number of colonies calculated. Plates were then incubated at 25°C for 3-5 days. The number of colonies on each plate was counted, with each colony representing growth of a single cell. This was then calculated as a percentage of the total number of cells seeded per plate, and plotted as a histogram. Viability assays were performed at least 3 times for each strain.

2.2.8 Calculating Generation Times

Cells were cultured in liquid media and cell counts taken, using a Beckton Dickinson Coulter counter, at 1 hour intervals throughout the period of exponential growth. The generation times of cells were calculated using the following equation;

$$T = \frac{\log (2^{t2-t1})}{\log (y/x)}$$

where T = generation time, x =cells/ml at time 1 (t1), y = cells/ml at time 2 (t2). For this, T was calculated from several intervals over an 8 hour culture period, and the mean value determined.

2.2.9 Spore Viability

Freshly growing cells were mixed together in a drop of sterile water on an ME plate, then incubated at 25°C for 2-3 days until lots of asci had formed. Small aliquots of asci were lifted and spread very thinly onto YE plates. Individual asci were isolated and, using the micro-manipulator, moved to new positions on the YE plate so that they were aligned in a column using the micro-manipulator grid system. Asci were incubated at 36°C for 2-5 hours to promote breakdown of the ascus coat. Using the micro-manipulator individual spores were then removed and transferred along the grid so that spores from individual asci were

arranged in separate rows. The spores were then incubated at 25°C for 3-7 days until colonies had grown. Spore viability was then calculated as the percentage of spores originally isolated that grew to form colonies.

2.3 RNA METHODS

2.3.1 Preparation of Total RNA

Each cell pellet was resuspended in 1 ml of STE (Section 2.6.3.1) and transferred to a screw-cap microfuge tube. The cells were then re-pelleted by a pulse-spin at high speed, the supernatant discarded and the pellet resuspended in 200 µl of STE. 0.3 g of acid-washed glass beads were then added followed by 600 µl of NTES (Section 2.6.3.2). 500 µl of water-saturated hot phenol at 65°C (heated in dry heat block) was added and the cells lysed by 3 x 40 second bursts at setting 4 in a Hybaid RiboLyser™. After centrifugation at 13,000 rpm for 5 minutes, the upper aqueous phase was transferred to a second screw-cap tube containing 500 µl of hot phenol and ribolysed for 1 x 40 second burst. After a 5 minute spin the upper aqueous phase was transferred to 400 µl of hot phenol and ribolysed for 1 x 40 second burst. Following a repeat spin the upper aqueous phase was transferred to 400 µl phenol:chloroform (1:1) at room temperature and vortexed After a repeat spin the upper aqueous phase was transferred to a second 400 µl phenol:chloroform and vortexed. After a repeat spin the upper aqueous phase was transferred to 300 µl phenol:chloroform. After a repeat spin the upper aqueous phase was transferred to 300 µl of chloroform and vortexed. After a repeat spin the upper aqueous phase was transferred to fresh microfuge tube, and 3 volumes of 100% ethanol and one tenth volume of 3 M sodium acetate (pH 5.2) added. The RNA was then precipitated overnight at -20°C.

The next day the RNA was pelleted by centrifugation at 13,000 rpm for 10 minutes and the supernatant discarded. The pellet was washed by adding 200 µl of 70% ethanol in RNase-free dH_2O and centrifuged for 1 minute. The ethanol was then carefully removed using a pipette. Each pellet was resuspended in 55 µl RNase-free dH_20 (Difco laboratories) and the RNA dissolved by incubating at 65°C with repeat pipetting. 5 µl of RNA was diluted in 500 µl of dH_20 to measure its concentration and purity by spectrophotometry (Section 2.4.2).

2.3.2 RNA Formaldehyde Gels

1 g agarose was dissolved in 63 ml of dH_2O and 20 ml of 5 x MNE (Section 2.6.3.3) by boiling then cooled to 60°C. 17 ml of formaldehyde was added and gently mixed, poured into a horizontal gel mould and set for 0.5-1 hour. 15 µl of RNA buffer (Section 2.6.3.4) and 1µl of 0.5 mg/ml ethidium bromide were added to each 10 µg RNA sample, incubated at 60°C for 5 minutes then loaded into the wells of the formaldehyde gel. RNA was then separated by electrophoresis at 60v for 3-4 hours in 1 x MNE.

2.3.3 Filter Hybridisation of RNA

2.3.3.1 NORTHERN BLOTTING

RNA was transferred onto GeneScreen membrane (NEN, NEF983) by capillary blotting, following manufacturers instructions. Two long pieces of Whatmann paper were soaked in 0.1 M sodium phosphate buffer (pH 6.5; Section 2.6.3.5) and placed on an elevated glass plate so that the ends formed a wick; 2 smaller pieces (slightly larger than gel size) were also soaked and placed on top. The RNA gel was also soaked in sodium phosphate buffer (pH 6.5), inverted so that the RNA was on the upper side of the gel, then placed on top of the filter papers, and gel spacers placed along each side of the gel. GeneScreen membrane was cut to gel size, soaked in 0.1 M sodium phosphate buffer (pH 6.5), then placed on top of the gel, taking care to avoid air bubbles. Three pieces of Whatmann paper were cut to slightly larger than gel size and placed on top of membrane; 20 absorbent towels were placed on top of stack and then topped with a weight as in Figure 2.2.

RNA transfer was carried out at room temperature for 16-24 hours after which the towels and Whatmann paper were discarded. The membrane was removed, marked for orientation, then washed on 0.1 M sodium phosphate buffer (pH 6.5) for 20 minutes with gentle agitation. The membrane was then air-dried on Whatmann paper, and RNA was then fixed to membrane by UV crosslinking.

2.3.3.2 HYBRIDISATION

Membranes were pre-hybridised in Hybridisation buffer (Section 2.6.3.6) for 2-4 hours at 42°C in a Techne hybridisation oven. Radio-labelled probe DNA was denatured at 94°C for 5 minutes, placed on ice for 10 minutes, then added directly to the hybridisation buffer. Hybridisation of DNA probe to the RNA on the membrane was carried out at 42°C for 16

hours. The hybridisation buffer was discarded and the membrane washed. First it was washed twice at low stringency using 50 ml of 2 x SSPE (Section 2.6.3.7) at 42°C for 5 minutes. The membrane was then washed once at high stringency in 50ml of 2 x SSPE + 0.5% SDS at 65°C for 15 minutes. The signal to background ratio was checked and, if necessary, the high stringency wash repeated, then it was finally rinsed twice in 50 ml of 0.1 x SSPE at room temperature. The membrane was wrapped in cling film and excess liquid drained, then processed for autoradiography.

2.3.3.3 STRIPPING MEMBRANES

Membranes were stripped of radio-labelled probe to allow sequential hybridisations with different probes. This was done by wash in 200 ml of Strip solution (Section 2.6.3.8) for 30 minutes at 70°C in a Techne hybridisation oven. The signal was checked and, if necessary, the strip wash repeated. Membranes were air dried on Whatmann paper and stored at room temperature.

2.4 DNA METHODS

2.4.1 Preparation of Genomic DNA

A 10 ml *S. pombe* cultures was grown at permissive temperature for 3-5 days. The cells were harvested by centrifugation at 3000 rpm for 5 minutes, the supernatant poured off, and the pellet resuspended in 0.5 ml of dH_2O. The cells were transferred to a screw-cap microfuge tube, pelleted by centrifugation at 13,000 rpm for 1 minute, and the supernatant poured off. Cells were then resuspended in the residual liquid. The following were then added to the cells: 200 µl solution A (Section 2.6.4.2), 200 µl phenol: chloroform (1:1), 0.3 g acid-washed glass beads.

A Hybaid RiboLyser™ was used to lyse the cells, giving them 3 x 40 second bursts at setting 4. 400 µl of TE was added followed by centrifugation at 13,000 rpm for 5 minutes. The aqueous layer, containing both DNA and RNA, was transferred from to a fresh 1.5 ml microfuge tube leaving behind the liquid at protein interface. 1 ml of 100% ethanol was added to the aqueous layer and gently mixed by inverting, to avoid breakage of larger DNA fragments. This was then pelleted by centrifugation at 13,000 rpm for 5 minutes, and the supernatant discarded. This was spun for an additional 2 minutes and the remaining ethanol pipetted off.

To digest the RNA in the pellet, it was gently resuspended in 400 µl of TE, Ribonuclease A (Sigma, R-5503) added to a final concentration of 50 µg/ml (2µl of a 10 mg/ml stock), and incubated at 37°C for 5 minutes. To precipitate the DNA, 8 µl of 5 M ammonium acetate was then added along with 1 ml of 100% ethanol, gently mixed by inversion, and left at -70°C for 5 minutes. The DNA was then pelleted by centrifugation at 13,000 rpm for 10 minutes, and the supernatant discarded. The DNA pellet was air-dried, gently resuspended in 100µl of TE and spun for 2 minutes at 13,000 rpm. The supernatant was then transferred to a fresh microfuge tube and the DNA stored at -20°C.

2.4.2 Quantification of Nucleic Acids by Spectrophotometry

DNA and RNA samples were diluted 100-fold in dH_2O and the concentration estimated by measuring the absorbance at 260 nm on a spectrophotometer. The following equation was then used to calculate DNA and RNA concentrations in µg/µl:

A_{260} of 1.0 = 40 µg/ml RNA *or* 50 µg/ml DNA

Therefore multiply A_{260} by dilution factor (**x 100**)
then **x 40** (RNA) or **x 50** (DNA)
÷ 1000 (to get µg/µl)

eg A_{260} of RNA sample is 0.2,

RNA A_{260} of 1.0 = 40 µg/ml

A_{260} of 0.2 = $\frac{40}{5}$ µg/ml = 8 µg/ml

x 100 dilution = 800 µg/ml

÷ 1000 = 0.8 µg/µl

A useful indication of the purity of DNA and RNA samples is provided by the ratio of absorbances at 260 nm/280 nm. Pure DNA samples have ratio of 1.8 and pure RNA samples a ratio of 2.0. Contamination of samples with phenol or protein will cause an increase in these ratios.

2.4.3 Polymerase Chain Reaction (PCR)

The following were added together, in order, in a 0.2 ml Thermo-Tube (Abgene, AB-0620): 1 μg template DNA (or 5-10 μg genomic DNA), 25 pmol each of forward and reverse primers (Appendix II), 5 μl thesit buffer (Section 2.6.4.1), 2 mM dNTPs, *Taq* Polymerase (Promega, M1861)/ *pfu* DNA Polymerase (Startagene, 600135) and the volume made up to 100 μl with dH_2O. The contents were gently mixed and placed into a MWG Primus thermal cycler, and PCR performed using the following basic programme:

1. Denaturation	94°C	5 minutes
2. Denaturation	94°C	1 minute
3. Annealing	55°C*	1 minute
4. Extension	68°C	1 minute
Steps 2-4 were repeated a further 29 times, then:		
5. Final extension	68°C	4 minutes
6.	4°C	indefinite

The annealing temperatures and extension times varied depending on the primers used and the length of the expected product. Primer annealing temperatures (Tm) were calculated using the equation: Tm = 4 x (G+C) + 2 x (A+T). The annealing temperature used was that of the primer with the lowest Tm value. One minute extension time was used for each kb of expected product i.e. an expected PCR product of 1.5 kb would be given an extension time of 1.5 minutes.

2.4.4 Gel Purification of DNA

DNA of 70 bp to 10 kb was extracted and purified from agarose gels using the QIAquick Gel Extraction Kit (Quiagen, 28704). All solutions were provided with the kit. DNA fragment was excised from the agarose gel with a scalpel and the excess agarose removed. The gel slice was weighed in a microfuge tube and 3 volumes of Buffer QG added (eg 900 μl of Buffer QG was added to 300 mg of gel). The gel slice was incubated at 50°C for 10 minutes, vortexing every 2-3 minutes to dissolve the gel completely. A QIAquick spin column was placed into a 2 ml collection tube, and the dissolved gel sample added to the column, to bind DNA to the column membrane. The column was centrifuged at 13,000 rpm

for 1 minute and the flow through discarded. To wash the column, 0.5 ml of Buffer QG was added, centrifuged at 13,000 rpm and the flow through discarded. To wash the DNA on the column, 0.75 ml of Buffer PE was left to stand for 2-3 minutes, then centrifuged at 13,000 rpm for 1 minute. The flow through was discarded and the column given a repeat spin to remove any remaining Buffer PE. The column was then placed in clean microfuge tube. To elute the DNA from the column, 50 µl of dH_20 was added *directly* to the column membrane, left to stand for 1 minute, and centrifuged for 1 minute at 13,000 rpm. The purified DNA was then stored at -20°C

2.4.5 Southern Blotting

2.4.5.1 DNA GEL

20 µg of genomic DNA (Section 2.4.1) was digested overnight with *Bgl* II at 37°C. To precipitate the DNA, 7 µl of 3 M sodium acetate (pH 5.2) and then 300 µl 100% ethanol were added, gently mixed and placed on ice for 30 minutes. The DNA by pelleted at 13,000 rpm for 10 minutes and the supernatant poured off. The spin was repeated for 2 minutes, the remaining liquid pipetted off, and the pellet resuspended in 20 µl TE.

The DNA was then loaded, with 5 µl loading buffer, onto a 0.6-2 % agarose gel in 0.5 x TBE. DNA was separated by electrophoresis at 50 v in 0.5 x TBE for 1 hour. A DNA molecular weight marker was also run on the gel. The gel was then photographed under a UV lamp, aligning the top of a ruler with the wells in gel, adjacent to DNA ladder.

2.4.5.2 DNA TRANSFER

To hydrolyse the gel, which improves DNA transfer, the gel was soaked in 0.5 M HCl (Section 2.6.4.3) for 10 minutes and rinsed 3 times in dH_2O. The gel was then soaked in southern denaturation solution (Section 2.6.4.4) twice for 20 minutes, and rinsed once in dH_2O. The gel was then soaked in southern neutralisation solution (Section 2.6.4.5) twice for 20 minutes, and rinsed in dH_2O. The gel was finally rinsed in 10 X SSC (Section 2.6.4.6) for 5 minutes, the transfer apparatus as in Figure 2.3, and transferred by capillary blotting for 18 hours.

The top of the gel was trimmed off at the wells. The membrane was marked for orientation, then rinsed 5 times in 5 x SSC. The DNA was then fixed to the membrane by baking at 80°C for 1-2 hours.

2.4.5.3 PROBING MEMBRANE

25 ml of southern hybridisation solution (Section 2.6.4.7) was heated to 42°C in a Hybaid hybridisation oven while the membrane was soaked in 2 x SSC. Pre-hybridisation was then carried out for 1-2 hours at 42°C. The radio-labelled DNA probe (Section 2.4.6) was mixed with 500 µl of salmon sperm DNA (Sigma, D-1626; 10 mg/ml stock), denatured at 94°C for 5 minutes, then placed on ice for 10 minutes. This was added directly to the southern hybridisation solution and incubated with the membrane overnight at 42°C.

2.4.5.4 WASHING MEMBRANE

The membrane was washed twice in 1 x SSC + 0.1 % SDS for 15 minutes at room temperature, followed by one wash for 30 minutes in 0.25 x SSC + 0.1 % SDS at 37°C. The signal to background ratio was checked and, if necessary, the last step repeated. The membrane was then washed twice for 30 minutes in 0.25 x SSC + 0.1 % SDS while regularly checking the signal.

The membrane was then wrapped in cling film, smoothing out any air bubbles and excess liquid blotted, then placed in an intensifying screen cassette and exposed, DNA side, to X ray film overnight at –70°C.

2.4.6 Preparation of Radio-labelled DNA Probe

Probes for northern and southern blot analysis were prepared by random hexanucleotide (N_6) primed labelling of PCR-generated DNA fragments. The following were added together in a screw-cap eppendorf tube in this order: 5 µl denatured probe DNA (incubated at 94°C for 5 minutes, then on ice for 10 minutes) made up to 11 µl with dH_2O, 2 µl of N_6 (random hexanucleotide mixture (Roche, 1 277 081)), 2µl of Klenow buffer (Roche, 1 008 404), 2 µl of dCTP (Promega, U1221) 2 µl of [α-P^{32}] dCTP (Amersham, 3000 Ci/mmol). The ingredients were mixed and incubated at 37°C for 1-4 hours.

To prepare a size-exclusion column, Sephadex G-50 solution was first made by adding 2 volumes of TE to Sephadex G-50 beads and autoclaving. The plunger from a 1 ml disposable syringe was removed and siliconised wool pushed to bottom of syringe, to create a plug. A microfuge tube was placed at the end of the syringe, and the whole column placed in a 50 ml tissue culture tube. The syringe was filled with G-50 solution, the column spun at

2000 rpm for 2 minutes and the TE flow through discarded. The syringe was re-filled with G-50 solution and spun again to remove the remaining TE. Typically ~ 0.7 ml of G-50 was left in the column, which was spun one final time to remove any residual TE.

Successfully polymerised DNA probe was separated from non-polymerised DNA by passing through a Sephadex G-50 size exclusion column. A fresh screw-cap microfuge tube was placed at bottom of column, the labelled probe DNA along with 100 µl of dH_2O was added to the column then spun at 2000rpm for 2 minutes. Small molecules were trapped in the Sephadex beads while the larger polymerised DNA molecules passed through, and were collected in the screw-cap eppendorf tube. A Geiger counter was held up to the flow-through collected in the screw-cap tube to confirm that it contained radio-labelled DNA. The DNA probe was then denatured by heating to 94°C for 5 minutes then placing on ice for 10 minutes before adding it to the hybridisation buffer.

2.4.7 DNA Ligation

2.4.7.1 PHOSPHORYLATION OF INSERT DNA

If the DNA to be inserted was a PCR product, which was to be ligated into a blunt-cut plasmid, it was first necessary to phosphorylate the insert DNA at its 5' termini. This is because the cut vector had been de-phosphorylated to prevent re-ligation. This was done using T4 polynucleotide kinase (PNK; Promega, M4101), according to manufacturer's instructions.

To ensure sufficient DNA would be harvested for subsequent DNA ligations, for each DNA to be phosphorylated, six identical reactions were set up. In order, the following ingredients were added together in microfuge tubes; 5 µl of insert DNA, 1µl T4 PNK buffer, 1 µl T4 PNK enzyme and 10 mM ATP (adenosine 5'-trophosphate disodium salt; Sigma, A-7699; 1µl of 100 mM stock). Each was made up to 10 µl with dH_2O then incubated at 37°C for 1 hour.

The reactions were pooled together, and the phosphorylated DNA purified using the QIAquickgel Extraction kit, according to manufacturer's instructions. In the final step of the manufacturer's protocol, the phosphorylated DNA was eluted in 50 µl dH_20.

2.4.7.2 LIGATION

Insert DNA was ligated into linearised plasmid using T4 DNA ligase (Promega; M1801) according to manufacturer's instructions. Plasmid DNA was first linearised by digestion with the appropriate restriction enzymes, then purified using the QIAquickgel extraction kit. In order, the following ingredients were added together in microfuge tubes; 5-7 µl insert DNA and 1-3 µl linearised plasmid DNA*, 1 µl T4 Ligase buffer, 1 µl T4 DNA Ligase. The reaction was then incubated for 3 hours at room temperature, or overnight at 15°C.

* A 1:3 molar ratio of vector: insert DNA was used for ligations. The following equation was used to calculate how much DNA to use:

$$\frac{\text{ng vector x size of insert (kb)}}{\text{sizeof vector (kb)}} \times \text{molar ratio of } \frac{\text{insert}}{\text{vector}} = \text{ng insert}$$

2.4.8 Flow Cytometry

2.4.8.1 FIXATION

Cells were pelletted by spinning at 13,000 rpm for 1 minute and the supernatant discarded. Cells were washed in 1 ml of dH_2O then fixed by gradually resuspending, drop by drop, in 70% ethanol while vortexing. Fixed cells could now be stored at 4°C.

2.4.8.2 STAINING AND PROCESSING

0.3 ml of fixed cells were pelletted by centrifugation at 13,000 rpm for 1 minute and the supernatant poured off. The cells were washed in 0.5 ml of 50 mM sodium citrate, pelletted and resuspended in 1 ml 50 mM sodium citrate. For DNA labelling, 0.1 mg/ml of RNase A (Ribonuclease A; Sigma, R-5503; 5 µl of 10 mg/ml stock) was added followed by 2 µg/ml of propidium iodide (Sigma, P-4170; 50 µl of 40 µg/ml stock), the tubes vortexed then incubated at 37°C for 2-4 hours. The cells could be processed immediately or stored at 4°C in the dark for up to 1 week.

Samples were analysed using a Becton Dickinson FACScan according to manufacturers instructions. Data was collected using CellQuest software; 10,000 cells were counted and DNA content (FL2-H) plotted against counts (Moreno *et al.*, 1991).

2.5 PROTEIN METHODS

2.5.1 Western Blotting

2.5.1.1 PREPARATION OF PROTEIN

50-80 ml of cells from log phase cultures were harvested by centrifugation at 3000 rpm for 5 minutes. The supernatants were discarded and pellets resuspended in 1 ml of Stop buffer (Section 2.6.5.1) and transferred to screw-cap eppendorf tubes. The cells were pelleted by a pulse spin at high speed, the supernatant poured off with the remainder then stored at -70°C.

The pellets were resuspended in 200 µl ice-cold lysis buffer (Section 2.6.5.2), pelletted by a pulse spin at high speed, and resuspended in 200 µl lysis buffer. 0.3 g of acid-washed glass beads were the added and the cells kept on ice. The cells were lysed using a Hybaid RiboLyser™ for 1 x 40 second burst at setting 4. Cell debris was pelletted by centrifugation at 13,000 rpm for 5 minutes at 4°C. The protein supernatants were transferred to chilled microfuge tubes then clarified by centrifugation at 13,000 rpm for 30 minutes at 4°C. The supernatants were transferred to chilled microfuge tubes, 1 µl removed for protein quantification, and stored at -70°C.

2.5.1.2 PROTEIN QUANTIFICATION

Protein assay reagent (Bio-Rad, 500-0006) is a colorimetric assay for protein concentration. The assay is based on the reaction of protein with alkaline copper tartrate solution and Folin reagent. Bio-Rad protein assay reagent concentrate was first diluted 5-fold in dH_2O. Known amounts of bovine serum albumin (BSA) were added, in different dilutions, to 1ml diluted Bio-Rad assay reagent in a disposable cuvette. The absorbance was measured at 595nm (A_{595}) by spectrophotometry, and a standard curve drawn, plotting A_{595} against mg/ml. To estimate the concentration of protein in samples, 1 µl of sample was also diluted in Bio-Rad assay reagent and the absorbance read at 595 nm. This value was plotted on the standard curve and an estimate of the protein concentration extrapolated from this.

2.5.1.3 POURING POLYACRYLAMIDE GEL

Gel electrophoresis was performed using a Mini-PROTEAN 3 Cell (Bio-Rad, 165-3301) according to manufacturers instructions. 6% Resolving gel solution (Section 2.6.5.3) was poured between two glass plates assembled in the casting frame to 1 cm below the comb teeth, overlayed with isopropanol, and left 30 minutes at room temperature to set. The isopropanol was then rinsed off with dH_2O. Stacking gel solution (Section 2.6.5.4) was prepared and poured on top of the resolving gel, taking care to avoid bubbles. The comb was inserted between the glass plates and the gel left to set at room temperature for 30 minutes. The gel cassette was then transferred to an electrophoresis tank containing 700 ml of Tank buffer (Section 2.6.5.5)

2.5.1.4 LOADING GEL AND ELECTROPHORESIS

An equal volume of sample buffer (Section 2.6.5.6) was added to 10 μg protein along with 3 μl of DTT. Each sample was boiled for 5 minutes, then loaded into the wells using gel-loading tips. 1.5 μg of protein marker (Amersham, RPN800.) was also loaded. The gel was then run at 150 volts for 1-1.5 hours.

2.5.1.5 PROTEIN TRANSFER

The gel was carefully removed from between the glass plates and transferred to a sheet of Whatmann filter paper, pre-wetted in transfer buffer (Section 2.6.5.7). The gel was then marked for orientation. Wetted nitrocellulose membrane (Hybond ECL; Amersham, RPN78D) was placed over the gel, then wetted filter paper placed over the nitrocellulose membrane. Each filter paper was then covered with fibre pads completing the sandwich as in Figure 2.4. The gel cassette was closed, placed in a transfer tank containing a Bio-ice cooling unit, and covered with 400 ml of Transfer buffer. Protein transfer was carried out at 50 volts for 1-1.5 hours. To check electrophoretic transfer of protein, the membrane was rinsed in 10 ml of Ponceau S (Sigma, P-7170), then rinsed in dH_20. The positions of protein marker bands were marked on membrane.

2.5.1.6 INCUBATION WITH ANTIBODIES AND DETECTION

The membrane was transferred to a 50 ml tissue culture tube and incubated in 10ml of blocking solution (Section 2.6.5.8) for 1 hour at room temperature. The blocking solution

was poured off, then the membrane incubated with 10ml of primary antibody solution (Section 2.6.5.9) for 2 hours at room temperature, or overnight at 4°C. The membrane was then washed 4 times, each for 15 minutes, in wash solution (Section 2.6.5.10) at room temperature. Incubation with the secondary antibody solution (Section 2.6.5.11) was then carried out at room temperature for 2 hours. This was followed by 2 washes, each for 30 minutes, in wash solution, and a brief rinse in high salt wash (Section 2.6.5.12).

2.5.1.7 DETECTION BY ELECTROCHEMILUMINESCENCE

Western blots were analysed by electrochemiluminescence (ECL) using ECL™ Western Blotting Detection Reagents (Amersham Pharmacia, RPN2209) according to manufacturer's instructions; equal volumes of solutions 1 and 2 were mixed, poured onto the protein side of the membrane, and incubated for 1 minute. Excess reagent was drained off, the membrane wrapped in cling film and any air pockets smoothed out. The membrane was then placed in a film cassette, protein side up. In the dark room a sheet of hyperfilm ECL™ (Amersham Pharmacia, RPN3103K) was placed on top of the membrane and exposed for 15 seconds-4 minutes. The film was then developed in an automatic X ray developer (Kodak X-Omat 2000).

2.6 MAKING CONSTRUCTS

2.6.1 *rad21*P:*rec8*$^+$ Constructs

2.6.1.1 GENERATION OF *rad21*P AND *rec8*$^+$ ORF DNA BY PCR

To place the *rec8*$^+$ gene under the control of the *rad21*$^+$ promoter, the entire *rec8*$^+$ open reading frame and *rad21*$^+$ promoter region were amplified by PCR (Section 2.4.3), using wild type *S. pombe* genomic DNA (from GG 217) as a template. Restriction sites were incorporated to enable manipulation of *rad21*P and *rec8*$^+$ DNAs in the cloning vectors pBCKS+, pJK148 and pSP1.

The 1.5 kb untranslated region upstream of the *rad21*$^+$ open reading frame was generated using primers GO 146 and GO 147, at an annealing temperature of 48°C and 1.5 minute extension time. These primers added a *Not* I restriction site at the 5' end, and a *Sma* I site at the 3' end, of the *rad21*P DNA. Primers GO 152 and GO 153 were used to amplify the entire 2 kb open reading frame of *rec8*$^+$, at an annealing temperature of 54°C, and extension time of 2 minutes, with *Sma* I and *Xho* I restriction sites at the 5' and 3' ends,

respectively. The additional restriction sites were incorporated to enable manipulation of *rad21*P and *rec8*$^+$ DNAs in the cloning vectors pBCKS+, pJK148 and pSP1. All primers are described in Appendix II.

2.6.1.2 SUB-CLONING *rad21*P AND *rec8*$^+$ORF DNAs INTO pBCKS+

pBCKS+ is a chloramphenicol-resistant *E. coli* plasmid that contains the *lacZ* gene interrupted by a multiple cloning site (MCS). *lacZ* encodes β-galactosidase, an enzyme that hydrolyses the chromogenic substrate X-β-gal to a blue end product. When DNA is inserted into the MCS, the *lacZ* gene is disrupted and no longer transcribed. Therefore, when transformed into *E. coli*, and incubated with X-β-gal, no blue product is detected.

pBCKS+ was digested with *Hinc* II which leaves blunt cohesive ends, enabling ligation of blunt-ended PCR products into the plasmid. *rad21*P DNA was phosphorylated using T4 Polynucleotide kinase (Section 2.4.7.1), then ligated into *Hinc* II-digested pBCKS+ to give the construct pBCKS+/ *rad21*P (GB 149; Figure 2.6A).

pBCKS+ was also digested with the restriction enzyme *Eco*R V, which leaves blunt 5' and 3' ends. *rec8*$^+$ was phosphorylated and ligated into the linearised pBCKS+ creating the construct pBCKS+/ *rec8*$^+$ (GB 157; Figure 2.6A).

Ligation products were transformed into *E. coli* strain DH5α and recombinant plasmids isolated by blue/white selection on LB + chloramphenicol plates containing X-β-gal. Plasmid DNA was then purified and accurate ligation confirmed by diagnostic restriction digests. Insert DNAs were sequenced by MWG to confirm that the *rad21*P and *rec8*$^+$ DNAs contained no mutations.

GB 149 (pBCKS+/ *rad21*P) was linearised at the 3' end of the *rad21*P DNA with the *Sma* I restriction enzyme. The *rec8*$^+$ ORF DNA was cut also from GB 157 (pBCKS+/ *rec8*$^+$) also using *Sma* I, and ligated onto the 3' end of *rad21*P. This created the construct pBCKS+/ *rad21*P: *rec8*$^+$ (GB 171; Appendix III; Figure 2.6B). Successful integration was confirmed using diagnostic restriction digests.

2.6.1.3 CLONING *rad21*P: *rec8*$^+$ INTO YEAST VECTOR pSP1

The *rad21*P: *rec8*$^+$ DNA was digested from GB 171 (pBCKS+/ *rad21*P: *rec8*$^+$) and cloned into pSP1 (Cottarel *et al.*, 1993). This is a low copy yeast plasmid, carrying the *S. cerevisiae LEU2* gene which complements *S. pombe leu1*$^+$ prototrophs, permitting growth on –leucine

media. pSP1 can also be propagated in *E. coli* conferring ampicillin resistance on the host organism.

GB 171 was digested with *Not* I and *Xho* I releasing the *rad21*P: *rec8*$^+$ DNA. pSP1 was also linearised within the MCS by digestion with *Not* I and *Xho* I. The *rad21*P: *rec8*$^+$ DNA was then ligated into the linearised vector, generating the construct pSP1/ *rad21*P: *rec8*$^+$ (GB 183; Appendix III; Figure 2.6C). Ligation product was then transformed into *E. coli* and transformants selected by ampicillin resistance. Plasmid DNA was then purified (Section 2.1.2) and successful ligation confirmed using diagnostic restriction digests.

2.6.1.4 CLONING *rad21*P: *rec8*$^+$ INTO *S. POMBE* INTEGRATING VECTOR pJK148

The *S. pombe* plasmid pJK148 (Keeney & Boeke, 1994) was used to integrate *rad21*P: *rec8*$^+$ into *rad21*$^+$-repressible cells in single copy. As in pBCKS+, the MCS in this plasmid also interrupts the *lacZ* gene, allowing selection of recombinants by blue/white selection. This plasmid also carries the *S. pombe leu1*$^+$ gene, so linearision of pJK148 within this sequence allows its integration into the chromosomal *leu1*$^+$ locus by homologous recombination.

The *rad21*P: *rec8*$^+$ DNA was removed from pSP1 (GB 183) by digestion with *Not* I and *Sal* I. pJK148 was linearised with *Not* I and *Sal* I and the *rad21*P: *rec8*$^+$ DNA inserted into the MCS by DNA ligation (Section 2.4.7), creating the construct pJK148/ *rad21*P: *rec8*$^+$ (GB 190; Appendix III). Ligation product was transformed into *E. coli* and cultured overnight on LB + ampicillin plates containing X-β-gal. Cells that had taken up the *rad21*P: *rec8*$^+$ DNA were isolated by blue/white selection. These cells were propagated and plasmid DNA purified, then accurate uptake of *rad21*P: *rec8*$^+$ DNA confirmed by diagnostic restriction digests.

2.6.2 *rec8*P: rad21$^+$ Constructs

2.6.2.1 GENERATION OF *rec8*P AND *rad21*$^+$ ORF DNAs BY PCR

The 1.5 kb untranslated region upstream of the *rec8*$^+$ open reading frame was amplified by PCR. Using genomic DNA from a wild type *S. pombe* strain (GG 217) as a template, PCR was performed using primers GO 150 and GO 151, creating the *rec8*P DNA with a *Xho* I restriction site at its 5' end and a *Sma* I site at its 3' end. PCR was performed at an annealing temperature of 50°C and with a 1.5 minutes extension time (Section 2.4.3).

Primers GO 148 and GO 149 were used to amplify the 2 kb *rad21*$^+$ open reading frame with *Sma* I and *Not* I restriction sites at the 5' and 3' ends, respectively, by PCR. Genomic DNA prepared from a wild type *S. pombe* strain (GG 217) was used as a template, and PCR was performed at an annealing temperature of 54°C, and an extension time of 2 minutes.

2.6.2.2 SUB-CLONING *rec8*P AND *rad21*$^+$ DNAs INTO pBCKS+

Manipulation of *rec8*P and *rad21*$^+$ DNAs was carried out using the bacterial vector pBCKS+. Because this is a high copy vector it was used to generate large quantities of insert DNA. Furthermore, pBCKS+ carries the *lacZ* gene interrupted by a MCS, which enables isolation of recombinant pBCKS+ by blue/white selection.

pBCKS+ was linearised by digestion with the restriction enzyme *Eco*R V, which leaves blunt cohesive ends at the restriction site. The double stranded *rec8*P PCR product was phosphorylated at its 5' termini using T4 Polynucleotide Kinase (PNK; Section 2.4.7.1), enabling its subsequent ligation into the *Eco*R Vsite of pBCKS+. This created the construct GB 125 (pBCKS+/ *rec8*P; Figure 2.7A).

pBCKS+ was digested with *Sma* I which also leaves blunt cohesive ends. The *rad21*$^+$ ORF PCR product was phosphorylated with PNK and ligated into pBCKS+ creating GB 124 (pBCKS+/ *rad21*$^+$; Figure 2.7A).

Ligation products were transformed into *E. coli* by electroporation, and colonies that had taken up pBCKS+ selected by overnight growth on LB + chloramphenicol plates. Colonies carrying recombinant pBCKS+ i.e. with *rec8*P or *rad21*$^+$ ORF DNA inserted into the MCS, were identified by blue/white selection.

Positive colonies were transferred separately to 10 ml LB + chloramphenicol and cells propagated by overnight culture at 37°C. Cells from each culture were harvested by centrifugation, and plasmid DNA purified (Section 2.1.2). Diagnostic restriction digests confirmed successful ligation of each insert DNA into pBCKS+. The absence of mutations in *rad21*$^+$ and rec8P DNAs, which can be caused by errors during polymerisation, was confirmed by DNA sequencing.

GB 125 was linearised by digestion with *Sma* I cutting the *rec8*P insert at its 3' end. The *rad21*$^+$ORF DNA was removed from GB 124 by digestion with *Sma* I, then ligated onto the free 3' end of the *rec8*P DNA in GB 125. This created the construct GB 168 (pBCKS+/ *rec8*P: *rad21*$^+$; Figure 2.7B). Ligation product was transformed into *E. coli* by

electroporation and cultured overnight on LB + chloramphenicol plates.The next day 10-20 colonies were transferred to 10 ml LB + chloramphenicol, and cells propagated by overnight incubation. Plasmid DNA was purified and successful ligation confirmed using diagnostic restriction digests.

2.6.2.3 CLONING *rec8*P: *rad21*$^+$ INTO YEAST VECTOR pSP1

The yeast vector pSP1 was used to introduce the *rec8*P: *rad21*$^+$ DNA into *rec8*Δ *S. pombe* strains (Cottarel *et al.*, 1993). This vector is replicated in low copy in *S. pombe* (~3 copies per cell), so was used to elicit ectopic expression of *rad21*$^+$ during meiosis at physiological levels.

pSP1 was linearised by digestion with *Xho* I and *Not* I. The *rec8*P: *rad21*$^+$ DNA was cut from GB 168 with *Xho* I and *Not* I, then inserted into linearised pSP1 by DNA ligation (Section 2.4.7). Ligation product was transformed into competent *E. coli* cells by electroporation (Section 2.1.1.2), then cells cultured on LB + ampicillin plates overnight. Colonies that grew represented cells that had taken up circular pSP1 plasmid DNA, conferring ampicillin resistance on *E. coli* cells. 10-20 colonies were transferred to 10 ml LB + ampicillin and cultured overnight. The next day, plasmid DNA was prepared and successful ligation of the *rec8*P: *rad21*$^+$ insert into pSP1 confirmed by diagnostic restriction digests. This construct was named GB 179 (Appendix III).

2.6.2.4 CLONING *rec8*P: *rad21*$^+$ DNA INTO *S. POMBE* VECTOR pJK148

In addition to using pSP1, the *S. pombe* vector pJK148 (Keeney & Boeke, 1994) was also used to elicit *rad21*$^+$ expression during *rec8*D meiosis, at physiological levels. As described above, pJK148 has been widely used to introduce DNA into the *S. pombe* genome by homologous recombination.

The *rec8*P: *rad21*$^+$ DNA was cut from GB 168 with *Xho* I and *Not* I. pJK148 was digested with *Sal* I and *Not* I, producing cohesive ends that were compatible with *Xho* I/ *Not* I cut *rec8*P:*rad21*$^+$. *rec8*P: *rad21*$^+$ was then inserted into pJK148 by DNA ligation (Section 2.4.7), creating the construct pJK148/ *rec8*P: *rad21*$^+$ (GB189). Ligation product was then transformed into *E. coli* cells by electroporation. Cells that had taken up plasmid DNA were isolated by growth on LB + ampicillin plates, and transferred to 10 ml LB + ampicillin for overnight culture. Plasmid DNA was purified and accurate ligation of insert DNA confirmed by diagnostic restriction digests.

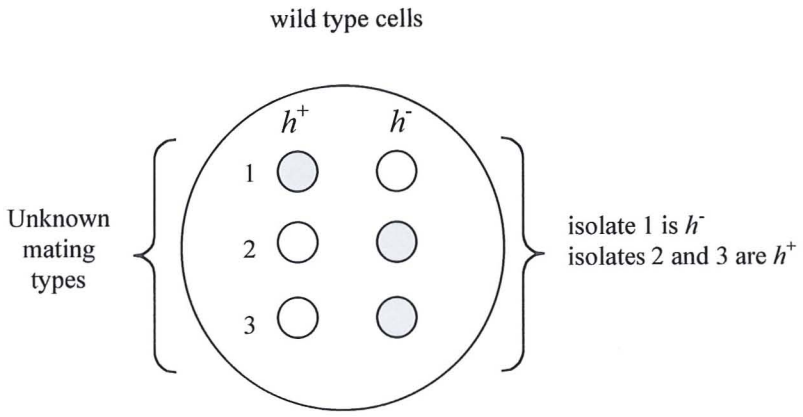


Figure 2.1 Determination of *S. pombe* Mating Types. The mating types of fission yeast isolates were determined by mating with h^+ and h^- strains. Patches that then stained brown with iodine vapour contained cells of opposite mating type that had undergone conjugation and sporulation.

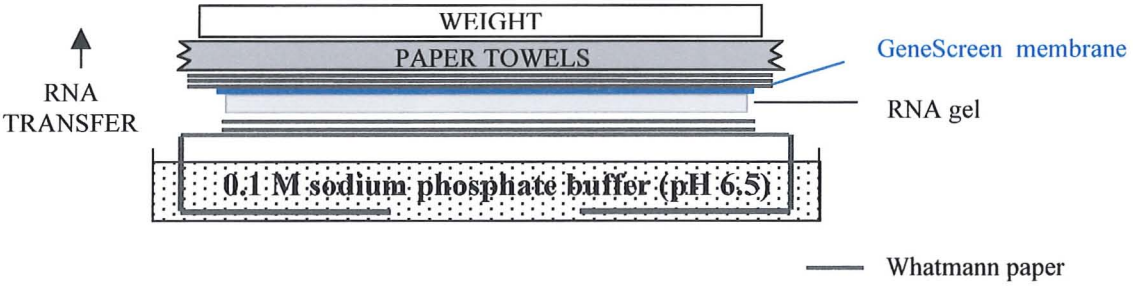


Figure 2.2 RNA Transfer for Northern Blot Analysis. RNA samples were separated by electrophoresis on a formaldehyde gel then transferred to a nitrocellulose membrane for northern blot analysis.

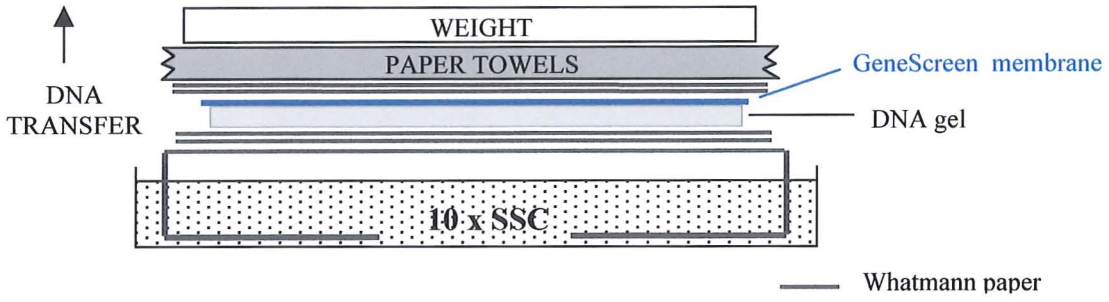


Figure 2.3 DNA Transfer for Southern Blot Analysis. DNA samples were separated by electrophoresis on a formaldehyde gel then transferred to a nitrocellulose membrane for southern blot analysis.

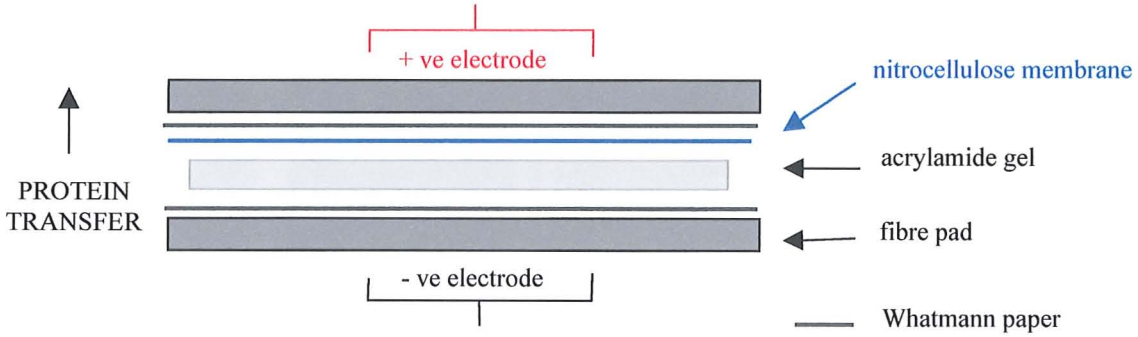
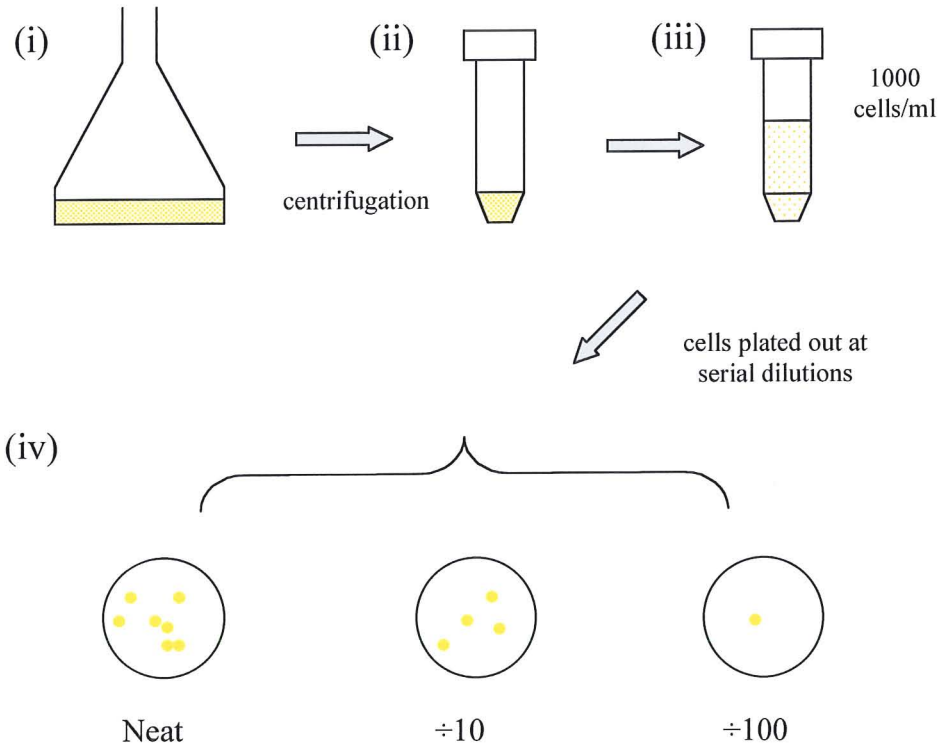


Figure 2.4 Protein Transfer for Western Blot Analysis. Protein samples were separated on a polyacrylamide gel by electrophoresis, then transferred to a nitrocellulose membrane, at 50 V for 1.5 hours, for western blot analysis.

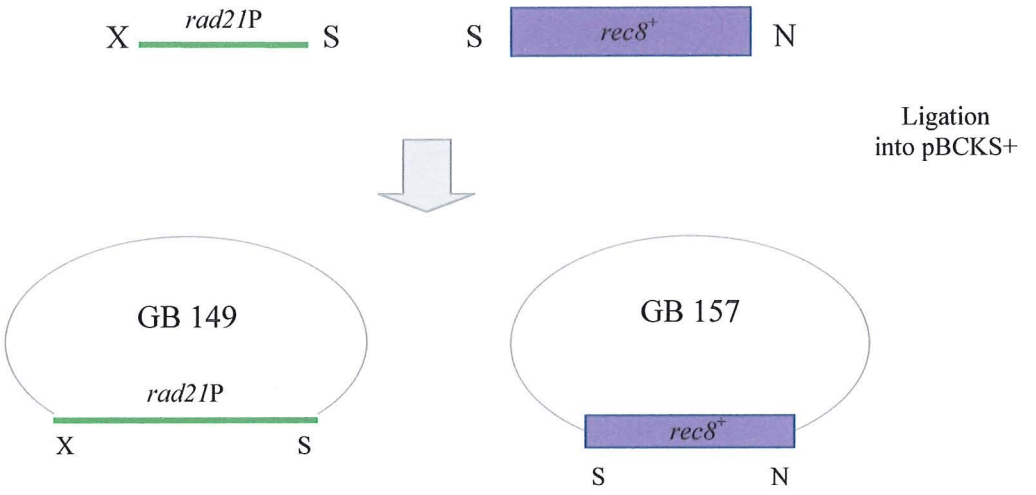


(v)

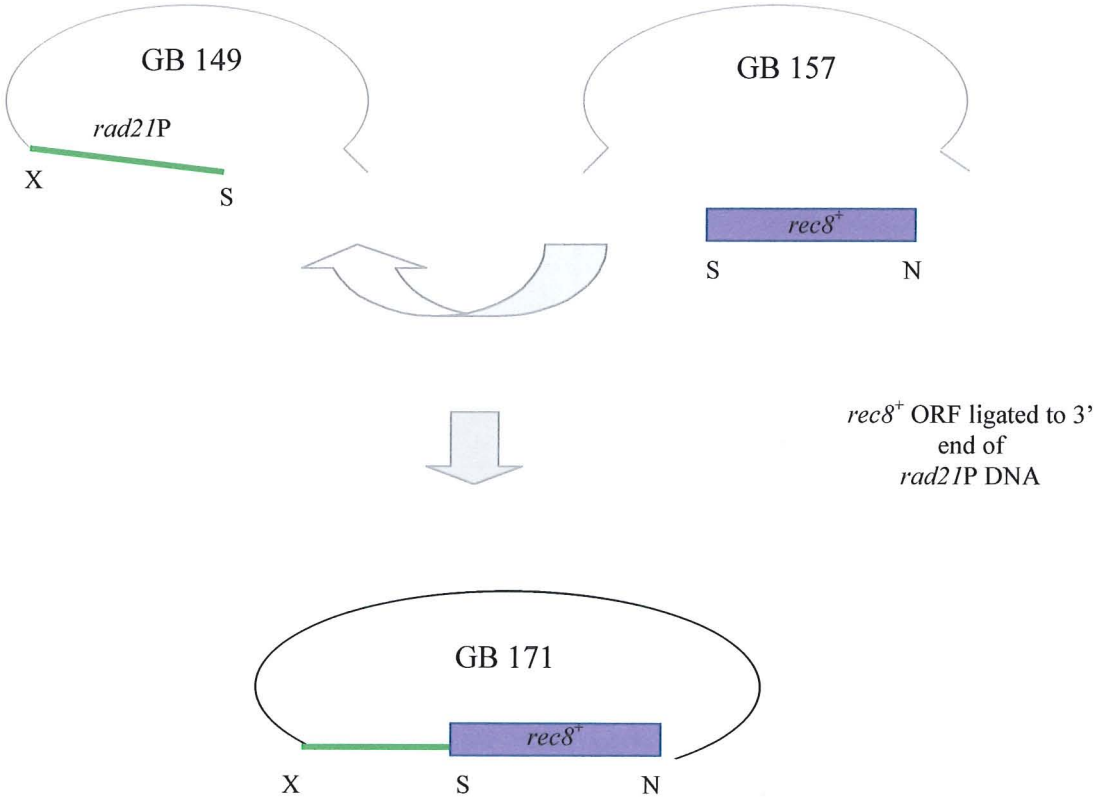
DILUTION	No. colonies expected
Neat	200
÷10	20
÷100	2

Figure 2.5 Viability assay. (i) *rad21*$^{+}$-repressible cells were cultured to exponential phase in liquid EMM + or - thiamine. **(ii)** Cells were pelletted by centrifugation at 3000 rpm for 5 minutes, then resuspended to a final concentration of 1000 cells/ml **(iii)**. **(iv)** Cells were then seeded onto EMM + or - thiamine at serial dilutions and incubated at 25°C for 3-5 days until colonies grew. **(v)** The number of colonies expected per plate if 100% viability is shown.

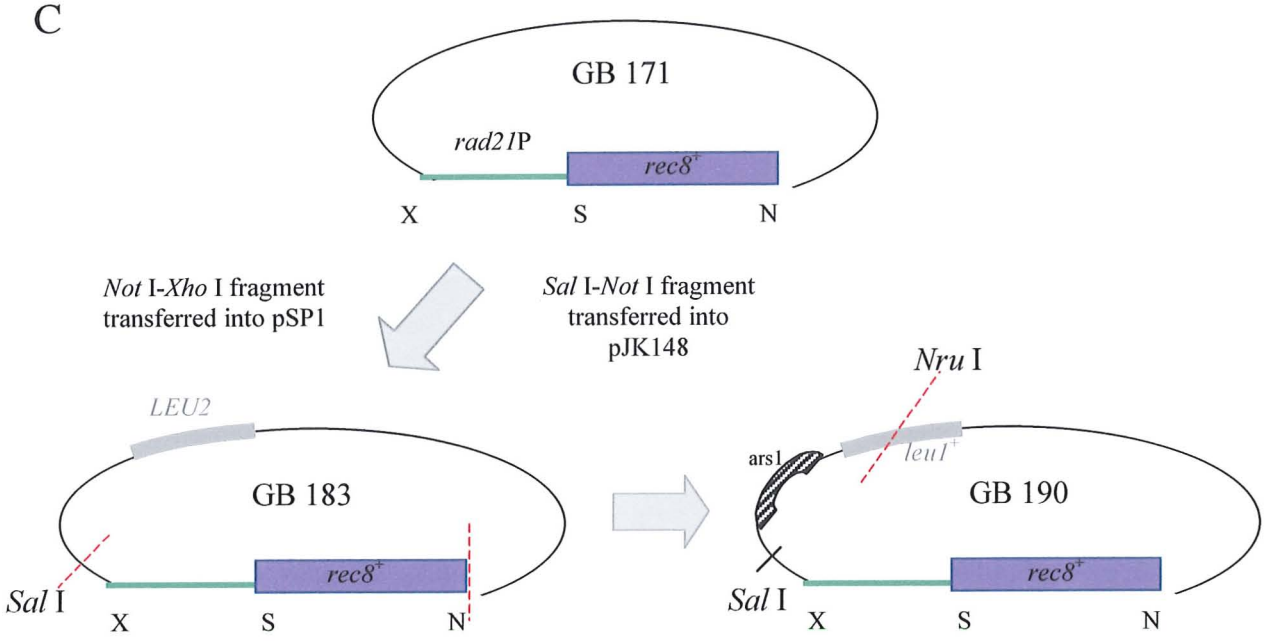
A


X
rad21P
S
S
rec8+
N
Ligation
into pBCKS+
GB 149
rad21P
X
S
GB 157
rec8+
S
N
rec8+ cut from
GB 157,
rad21P DNA
digested at 3' end

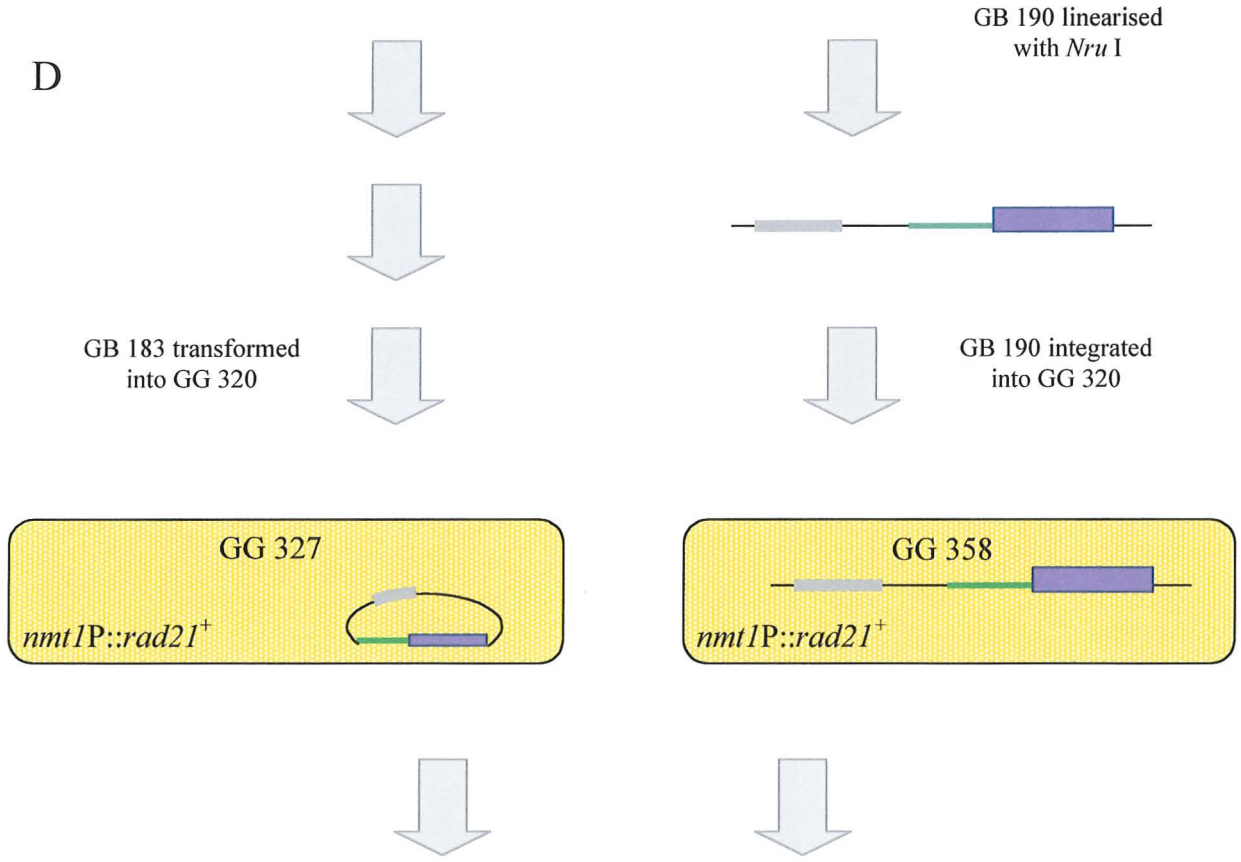
B


GB 149
rad21P
X
S
GB 157
rec8+
S
N
rec8+ ORF ligated to 3'
end of
rad21P DNA
GB 171
rec8+
X
S
N

C



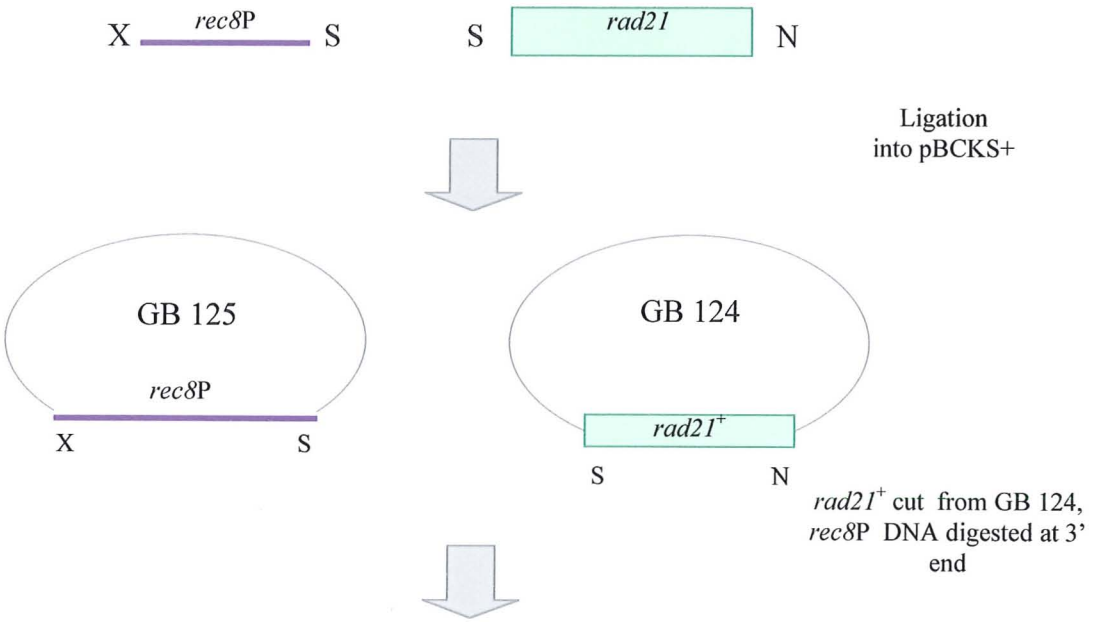
D



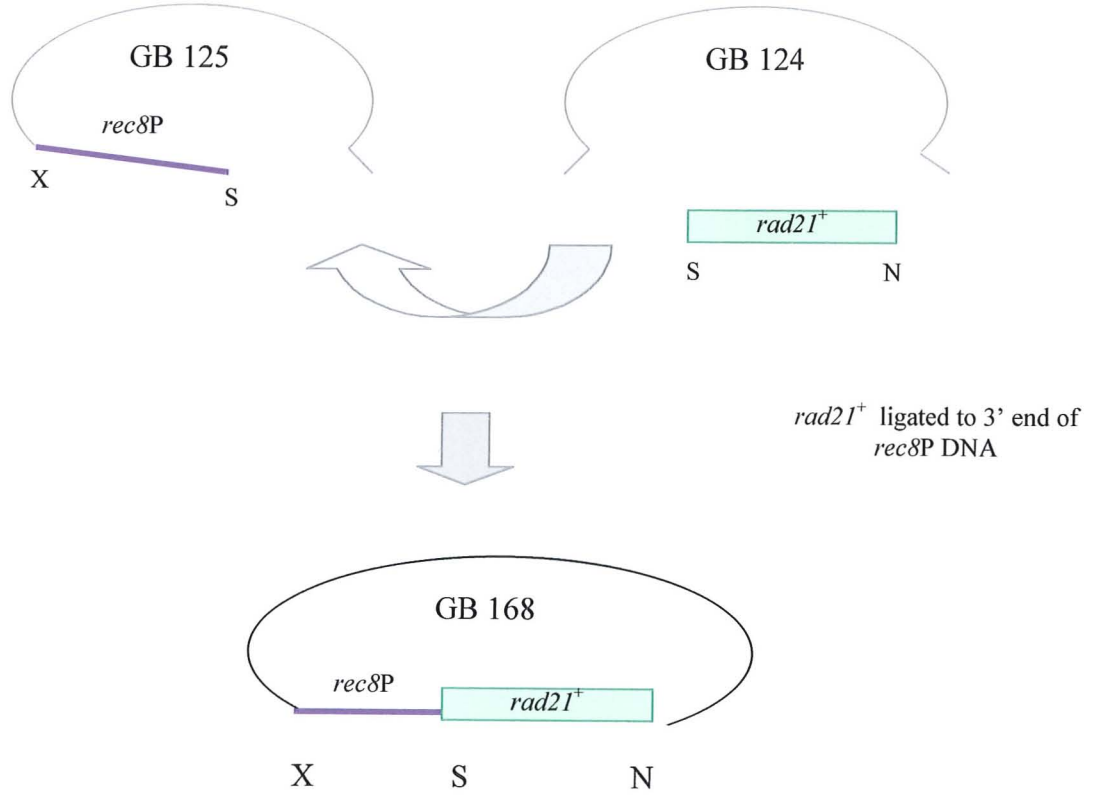
rec8^{+} expressed during mitotic cell cycle

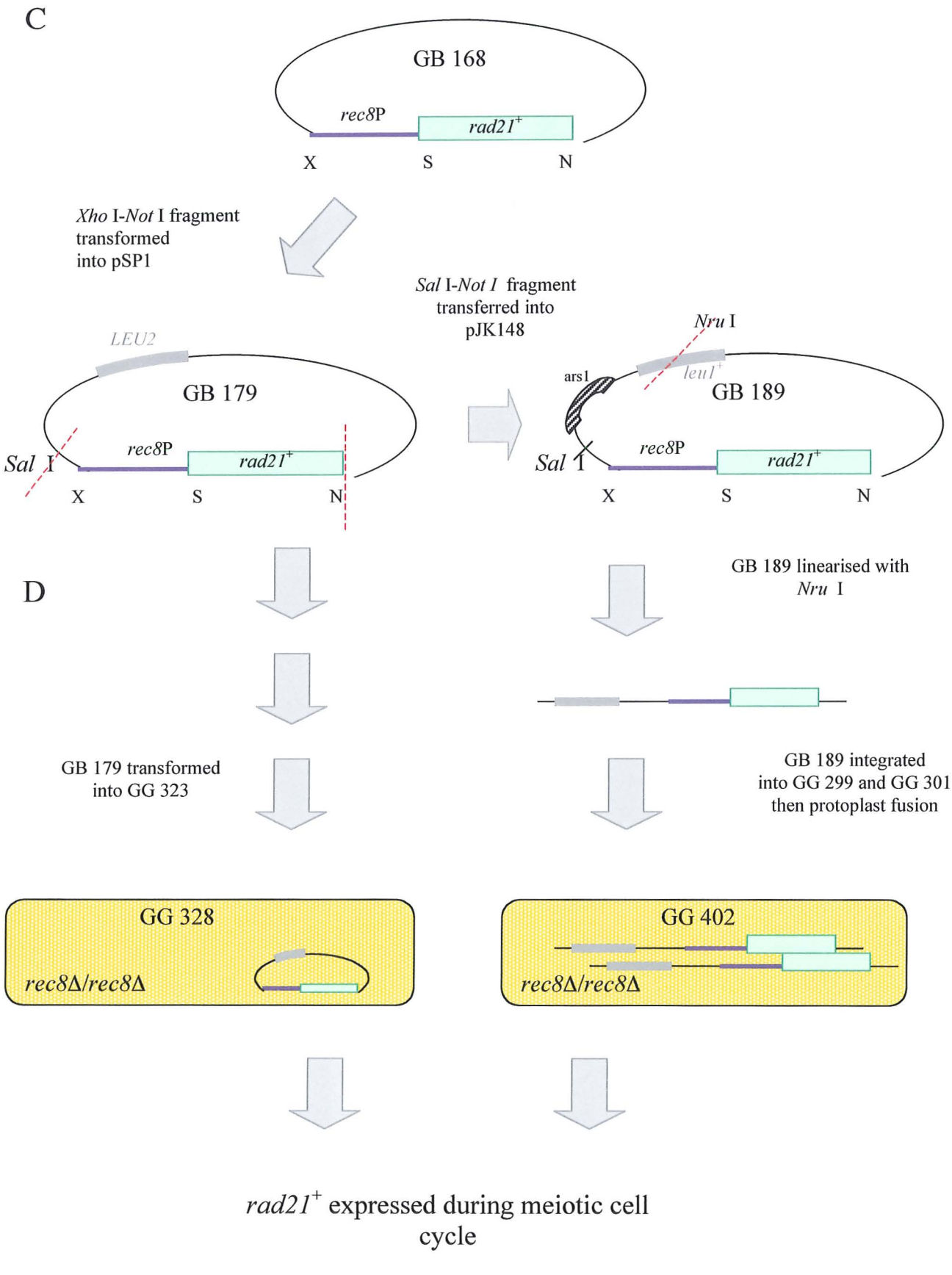
Figure 2.6 Creating *rad21*P: *rec8*$^+$ ORF Constructs. A, *rad21*P and *rec8*$^+$ ORF DNAs were generated by PCR. Primers for both DNAs were designed to incorporate specific restriction sites at the 5' and 3' termini, enabling their manipulation in cloning vectors; X: *Xho* I, S: *Sma* I, N: *Not* I. *rad21*P was ligated into *E. coli* plasmid pBCKS+ creating GB 149. *rec8*$^+$ ORF DNA was ligated into pBCKS+ to create GB 157. **B,** GB 149 was linearised by digestion with *Sma* I. GB 157 was digested with *Sma* I to release the *rec8*$^+$ ORF DNA. *rec8*$^+$ ORF DNA was ligated onto the 3' end of *rad21*P in GB 149, creating GB 171. **C,** *rad21*P: *rec8*$^+$ ORF DNA was removed from GB 171 by digestion with *Not* I and *Xho* I, then transformed into pSP1 creating GB 183. *rad21*P: *rec8*$^+$ was digested from pSP1 by digestion with *Sal* I and *Not* I, then ligated into pJK148 to create GB 190. **D,** GB 183 was transformed into GG 320, to give the *rad21*Δ: *rec8*$^+$ strain GG 327. GB 190 was linearised at the unique restriction site *Nru* I and integrated into the *leu1-32* loci of G 320, producing the *rad21*Δ *rad21*P: *rec8*$^+$ strain GG 358.

A


X
rec8P
S
S
rad21
N
Ligation
into pBCKS+
GB 125
rec8P
X
S
GB 124
rad21+
S
N
rad21+ cut from GB 124,
rec8P DNA digested at 3'
end

B


GB 125
rec8P
X
S
GB 124
rad21+
S
N
rad21+ ligated to 3' end of
rec8P DNA
GB 168
rec8P
rad21+
X
S
N



rad21^{+} expressed during meiotic cell cycle

Figure 2.7 Creating *rec8*P: *rad21*$^+$ ORF Constructs. A, *rec8*P and *rad21*$^+$ ORF DNAs were generated by PCR. Primers for both DNAs were designed to incorporate specific restriction sites at the 5' and 3' termini, enabling their manipulation in cloning vectors; X: *Xho* I, S: *Sma* I, N: *Not* I. *rec8*P was ligated into *E. coli* plasmid pBCKS+ creating GB 125. *rad21*$^+$ ORF DNA was ligated into pBCKS+ to create GB 124. **B,** GB 125 was linearised by digestion with *Sma* I. GB 124 was digested with *Sma* I to release the *rad21*$^+$ ORF DNA. *rad21*$^+$ ORF DNA was ligated onto the 3' end of *rec8*P in GB 125, creating GB 168.**C,** *rec8*P: *rad21*$^+$ ORF DNA was removed from GB 168 by digestion with *Not* I and *Xho* I, then transformed into pSP1 creating GB 179. *rec8*P: *rad21*$^+$ was removed from pSP1 by digestion with *Sal* I and *Not* I, then ligated into pJK148, which contains an *ars* element (hatched area), to create GB 189. **D,** GB 179 was transformed into GG 323, to give the *rec8*Δ: *rad21*$^+$ diploid strain GG 328. GB 189 was linearised at the unique restriction site *Nru* I and integrated into the *leu1-32* locus of GG 299 and GG 301, producing GG348 and GG349 respectively. Protoplast fusion of GG 348 and GG 349 created *rec8*Δ *rec8*P: *rad21*$^+$ diploid strain GG 402.

2.6 SOLUTIONS

All solutions were stored at room temperature unless otherwise indicated.

2.6.1 Bacterial Methods

2.6.1.1 SOC MEDIUM

2% tryptone T (Oxoid, LP0043)	20 g
0.5% yeast extract (DIFCO, 0127-17)	5 g
10 mM NaCl	0.5 g
2.5 mM KCl	2.5 ml of 1 M stock

pH to 7 with 5 M NaOH, add dH_20 to 975 ml then autoclave.

10 mM $MgCl_2$	5 ml of 2 M stock
20 mM glucose	20 ml of 1 M stock

Store at -20°C.

2.6.1.2 P1

50 mM Tris Cl (pH 8)	25 ml of 1 M stock
10 mM EDTA	10 ml of 0.5 M stock
100 µg/ml Rnase A*	50 mg
dH_2O	up to 500 ml

* Ribonuclease A (Sigma, R-5503)

Store at 4°C.

2.6.1.3 P2

0.2 M NaOH	4 g
1% (w/v) SDS	5 g
dH_2O	up to 500 ml

2.6.1.4 P3

2.55 M KOAc (pH 4.8)	125 1 g
dH_2O	up to 500 ml

2.6.2 Yeast Methods

2.6.2.1 SP1

1.2 M sorbitol	109.32 g
50 mM sodium citrate	7.4 g
50 mM sodium phosphate	3.5 g
40 mM EDTA	7.4 g
dH_2O	to 500 ml

pH to 5.6 then autoclave.

2.6.2.2 SP2

1.2 M sorbitol	109.32 g
50 mM sodium citrate	7.4 g
50 mM sodium phosphate	3.5 g
dH_2O	to 500 ml

pH to 5.6 then autoclave.

2.6.2.3 SP3

1.2 M sorbitol	109.32 g
10 mM Tris Cl	5 ml of 1M stock
dH_2O	to 500 ml

pH to 7.2 then autoclave.

2.6.2.4 SP4

1.2 M sorbitol	109.32 g
10 mM Tris Cl	5 ml of 1M stock
10 mM $CaCl_2$	0.74 g
dH_2O	to 500 ml

pH to 7.6 then autoclave.

2.6.2.5 SP5

20% PEG*	100 g
10 mM Tris Cl	5 ml of 1M stock
10 mM $CaCl_2$	0.74 g
dH_2O	to 500 ml

pH to 7.6 then filter sterilise.

* polyethylene glycol 3350 (Sigma, P-3640)

2.6.2.6 LiAc/TE

100 mM LiAc	1.02 g
TE	up to 100 ml

2.6.2.7 PEG MIX*

40% PEG 3350	4 g
100 mM LiAC/TE	7ml
TE	up to 10 ml

* This was made up fresh each time.

2.6.2.8 CARRIER DNA

Herring testes DNA (Sigma, D-1626; 10 mg/ml stock) was denatured at 94°C for 5 minutes, then placed on ice for 10 minutes.

2.6.3 RNA Methods

2.6.3.1 STE

0.32 M sucrose

20 mM Tris Cl (pH 7.5)

10 mM EDTA (pH 8)

Make up to 100 ml with dH_2O then autoclave.

2.6.3.2 NTES

100 mM NaCl

5 mM EDTA (pH 8)

50 mM Tris Cl (pH 7.5)

Make up to 100 ml with dH_2O then autoclave.

2.6.3.3 MNE BUFFER (x 5)

120 mM MOPS	13.08 g
25 mM NaOAc	1.03 g
5 mM EDTA	0.19 g
dH_2O	to 500 ml

Adjust pH to 7 with NaOH then store at 4°C in the dark.

2.6.3.4 RNA BUFFER

formaldehyde	600 µl
formamide	200 µl
MNE buffer	240 µl of 5 x stock
dH_2O	160 µl

2.6.3.5 0.1 M SODIUM PHOSPHATE BUFFER (pH 6.5)

Na_2HPO_4	61 ml of 1 M stock
NaH_2PO_4	39 ml of 1 M stock

Make up to 1 litre with dH_20.

2.6.3.6 HYBRIDISATION BUFFER

formamide	10 ml
P buffer[a]	4 ml
50% (w/v) dextran sulphate	4 ml

Incubate at 42°C for 10 minutes, add 1.16g NaCl, then incubate at 42°C for a further 10 minutes. Add 200 µl denatured salmon sperm DNA[b] and mix thoroughly.

a. P buffer

1% (w/v) bovine serum albumin

1% (w/v) ficoll

250 mM TrisCl (pH 7.5)

0.5% (w/v) tetra-sodium pyrophosphate

5% (w/v) SDS

b. Salmon Sperm DNA

Deoxyribonucleic acid (Sigma, D-1626) was dissolved at a concentration of 10 mg/ml in dH_2O, mixed gently for 3-4 hours at room temperature, then sonicated until a runny consistency. Prior to use, DNA was denatured at 94°C for 5 minutes then chilled on ice for 10 minutes.

2.6.3.7 20 x SSPE

3 M NaCl

20 mM $NaH_2PO_4.dH_2O$

200 mM EDTA

Adjust pH to 7.4 with NaOH and make volume up to 1 litre with dH_2O.

2.6.3.8 STRIP SOLUTION

5 mM Tris Cl	5 ml of 1 M stock
0.2 mM EDTA	400 µl of 0.5 M stock
0.05% (w/v) sodium pyrophosphate	500 mg
0.002% (w/v) polyvinyl-pyrrolidone	20 mg
0.002% (w/v) bovine serum albumin	20 mg
0.002% (w/v) ficoll	20 mg

Make volume up to 1 litre with dH_2O.

2.6.4 DNA Methods

2.6.4.1 THESIT BUFFER

300mM TrisCl (pH 8.5)

20mM $MgCl_2$

50mM β-Mercaptoethanol

0.1% Thesit

Make volume up with dH_20

2.6.4.2 SOLUTION A

2 % Triton X-100	10 ml
1 % SDS	50 ml of 10 % stock
100 mM NaCl	50 ml of 1 M stock
100 mM Tris Cl (pH 8)	50 ml of 1 M stock
1 mM EDTA	500 µl of 1 M stock
dH_2O	to 500 ml

2.6.4.3 0.5M HCl*

dH_2O	954 ml
conc. HCl	46 ml

* add acid to water.

2.6.4.4 SOUTHERN DENATURATION SOLUTION

1.5 M NaCl	87.66 g
0.5 M NaOH	20 g
dH_20	to 1 litre

2.6.4.5 SOUTHERN NEUTRALISATION SOLUTION

3 M NaCl	175.32 g
0.5 M Tris Cl (pH 7.4)	500 ml of 1 M stock
dH_20	to 1 litre

2.6.4.6 20 x SSC

3 M NaCl	175.32 g
0.3 M sodium citrate	88.23 g
dH_20	to 1 litre

2.6.4.7 SOUTHERN HYBRIDISATION SOLUTION

50 % formamide	50 ml
10 % dextran sulphate	10 g
0.5 % SDS	5 ml of 10 % stock
Ficoll 400	20 mg
BSA*	20 mg
0.75 M NaCl	4.4 g
40 mM Na_2HPO_4	4 ml of 1 M stock
5 mM EDTA	1 ml of 0.5 M stock
dH_2O	to 100 ml

* bovine albumin, Sigma; A-7638.

2.7.5 Protein Methods

2.7.5.1 STOP BUFFER

150 mM NaCl

50 mM NaF

10 mM EDTA

1 mM NaN_3

pH to 8 then make volume up to 100 ml with dH_2O.

2.7.5.2 LYSIS BUFFER

50 mM KCl	50 µl of 1 M stock
50 mM Tris Cl	50 µl of 1 M stock
25 % glycerol	0.5 ml of 50 % stock

2 mM DTT[a]	10 µl of 1 M stock
0.1 % Triton X-100	10 µl of 10 % stock
5 µg/ml chymostatin	5 µl of 1 mg/ml stock
5 µg/ml pepstatin	5 µl of 1 mg/ml stock
5 µg/ml antipain	5 µl of 1 mg/ml stock
5 µg/ml leupeptin	5 µl of 1 mg/ml stock
5 µg/ml aprotonin	5 µl of 1 mg/ml stock
dH_2O	353 µl
0.2 mM PMSF[b]	2 µl of 100 mM stock

a. DTT: dithiothreitol (Sigma, D-9760); 1 M solution in dH_2O; store at 4°C.

b. PMSF: phenylmethanesulfonylfluoride (Sigma, P-7626); 100 mM solution in isopropanol. *Store at 4°C.*

2.7.5.3 RESOLVING GEL SOLUTION (6%)

acrylamide *	5.4 ml
resolving buffer[a]	18 ml
10% ammonium persulfate (AMP)	360 µl
TEMED	36 µl

*Acrylamide:Bis-acrylamide 40% solution

a. Resolving buffer

1.5 M Tris	18 g
0.4%SDS	0.4 g

Dissolve in 50 ml of dH_20, adjust pH to 8.8 with 5 M HCl, and make volume up to 100 ml with dH_20.

2.7.5.4 STACKING GEL SOLUTION (4.5%)

Acrylamide*	1.8 ml
Stacking buffer[b]	8.2 ml
10% AMP	120 µl
TEMED	12 µl
dH_2O	to 12 ml

* Acrylamide:Bis-acrylamide 40% solution

b. Stacking buffer

500 mM Tris	6 g

0.4% SDS	0.4 g

Dissolve in 85 ml dH_20, adjust pH to 6.8 with 5 M HCl, and make volume up to 100 ml with dH_20.

2.7.5.5 TANK BUFFER (x 5)

0.12 M Tris	15 g
1 M Glycine	72 g
0.5% SDS	5 g
dH_2O	to 1 litre

2.7.5.6 SAMPLE BUFFER

62.5 mM Tris	0.76 g
2% SDS	2 g
10% sucrose	10 g
dH_2O	to 100 ml

Add Pyronin Y (Sigma; P6653-5G) to give pink colour.

2.7.5.7 TRANSFER BUFFER (x 10)

0.1 M Tris	12.1 g
0.8 M glycine	57.6 g
0.08% SDS	0.8 g
dH_2O	up to 1 litre

2.7.5.8 BLOCKING SOLUTION

20 mM Tris (pH 7.2)	2 ml of 1 M stock
15 mM NaCl	0.5 ml of 3 M stock
5% non-fat milk	5 g
0.2% Tween 20	200 µl
dH_2O	to 100 ml

2.7.5.9 PRIMARY ANTIBODY SOLUTION

20 mM Tris (pH 7.2)	200 µl of 1 M stock
1% non-fat milk	0.1 g
0.1% Tween 20	10 µl
$^1/_{1000}$ dilution 1° antibody[a]	10 µl
dH_2O	to 10 ml

a. rabbit anti-Rad21p polyclonal antibody was a kind gift from Professor Suresh Subramani (*J. Biol. Chem.* (1995) **270** p7703).

2.7.5.10 WASH SOLUTION

20 mM Tris (pH 7.2)	200 µl of 1 M stock
15 mM NaCl	50 µl of 3 M stock
1% non-fat milk	100 µl
dH_2O	to 10 ml

2.7.5.11 SECONDARY ANTIBODY SOLUTION

20 mM Tris (pH 7.2)	200 µl of 1 M stock
150 mM NaCl	0.5 ml of 3 M stock
1% non-fat milk	0.1 g
$^1/_{3000}$ dilution 2° antibody[b]	3.3 µl
dH_2O	to 10 ml

b. Peroxidase-Conjugated Rabbit anti-mouse Immunoglobulins (DAKO, P0161)

2.7.5.12 HIGH SALT WASH

20 mM Tris Cl pH 7.2)	200 µl of 1 M stock
150 mM NaCl	500 µl of 3 M stock
dH_2O	to 10 ml

Chapter 3:

Expression of *rad21*$^{+}$ and *rec8*$^{+}$ Cohesins During the *S. pombe* Mitotic and Meiotic Cell Cycles

3.1 REGULATION OF Rad21p AND Rec8p COHESINS

As described in the Chapter 1, *rad21*$^+$ and *rec8*$^+$ encode cohesins, proteins that bind chromosomes during S phase and are essential for the association of sister chromatids in fission yeast (Watanabe & Nurse, 1999). Defective sister chromatid cohesion leads to abnormal chromosome segregation during M phase, and results in the generation of aneuploid products. Rad21p and Rec8p each have several additional roles during the cell cycle including DNA double strand break repair (Birkenbihl & Subramani, 1992) and meiotic homologous recombination (Molnar *et al.*, 1995; Parisi *et al.*, 1999), respectively. It has been suggested that these functions are indirect consequences of their primary role in sister chromatid cohesion.

Rad21p and Rec8p proteins contain regions of sequence homology in their N and C-termini (27% and 35% respectively; Watanabe & Nurse, 1999), that are also conserved between species (Losada *et al.*, 1998; Parisi *et al.*, 1999; Guacci *et al.*, 1997; Bhatt *et al.*, 1997; McKay *et al.*, 1996; Figure 3.1), indicating that these regions are important for cohesin function. However, functional domains are yet to be characterised for these proteins and the precise functions of these conserved domains remains to be elucidated.

Both Rad21p and Rec8p are phosphoproteins, with the hypo-phosphorylated forms appearing during S phase, becoming multiply phosphorylated with progression through the cell cycle (Birkenbihl & Subramani, 1995; Parisi *et al.*, 1999). The phosphorylation status of cohesins is thought to regulate their activity, for example by altering their susceptibility to cleavage by proteolytic enzymes (Rogers *et al.*, 2002; Kaitna *et al.*, 2002; Alexandru *et al.*, 2001).

Although *rad21*$^+$ is periodically transcribed, levels of chromatin-bound Rad21p are relatively constant throughout the cell cycle, with cleavage of only 5% being required for dissolution of sister chromatid cohesion during anaphase (Tomonaga *et al.*, 2000). Cleavage of both Rad21p and Rec8p requires APC activity, and both proteins contain highly conserved Separin cleavage sites (Uhlmann *et al*, 1999; Buonomo *et al.*, 2000), indicating they are both regulated during M phase by the APC-securin-separin pathway.

Transcription of both *rad21*$^+$ and *rec8*$^+$ genes is cell cycle-regulated. Both cohesin genes contain MCB elements in their promoter regions (Figures 3.2, 3.3) indicating that the DSC1 transcription factor might regulate their periodic expression (Section 1.4.1.2). The differences in position and arrangement of MCB elements in *rad21*$^+$ and *rec8*$^+$ promoters may reflect cell cycle-specific differences in their transcriptional regulation.

To summarise, *S. pombe* has two genes that have related function and appear to be regulated by the same mechanisms. This study aims to address the question of why fission yeast utilises different cohesin proteins and to examine life cycle-specific differences in their functions. The *rad21*$^+$ gene is expressed during G1/S phase of the mitotic cell cycle (Birkenbihl & Subramani, 1995). *rec8*$^+$ expression is triggered upon induction of meiosis (Lin *et al.*, 1992) while Rad21p has also been reported to have a meiotic role (Watanabe & Nurse, 1999). From current data the precise cell cycle and life cycle-specific patterns of transcriptional regulation of *rad21*$^+$ and *rec8*$^+$ is unclear. Therefore, the first stage of this study was to determine the expression profiles of *rad21*$^+$ and *rec8*$^+$ genes during the mitotic and meiotic life cycles. All *S. pombe* strains and primers are listed in Appendices I and II, respectively.

3.2 *rad21*$^+$ IS PERIODICALLY EXPRESSED DURING *cdc25-22* INDUCED SYNCHRONOUS MITOSIS

To determine the expression of *rad21*$^+$ and *rec8*$^+$ in the mitotic cell cycle, synchronous mitosis was performed using the renaturable temperature sensitive *cdc25-22* mutant (GG 95). *cdc25*$^+$ encodes a tyrosine phosphatase that de-phosphorylates Cdc2p at the G2/M border to trigger entry into mitosis (Millar *et al.*, 1991; for review, see Millar and Russell, 1992). *cdc25-22* cells are able to grow and divide as wild type at permissive temperature, but arrest in late G2 when incubated at restrictive temperature (36°C; Fantes, 1981).

cdc25-22 cells were cultured in minimal media and grown to early log phase (1-5 x 10^6 cells/ml) at permissive temperature (25°C). Cell density was monitored using a Coulter Counter. The culture temperature was shifted to 36°C for 4 hours, giving enough time for all cells, no matter what position in the cell cycle, to arrest cells at the G2/M border. The temperature was then rapidly returned to 25°C, releasing the G2 block so that cells would synchronously enter mitosis. 1-3x10^8 cells were harvested at 15 minute intervals. For a meiotic control sample, cells were harvested 3 hours after *pat1-114* induction of synchronous meiosis.

Total RNA was prepared and subjected to northern blot analysis (Sections 2.3.1-2.3.3), using radio-labelled *rad21*$^+$, *rec8*$^+$ and *cdc22*$^+$ DNA probes (Section 2.4.6; Figures 3.2, 3.3).

To monitor septation a small aliquot of each sample was also fixed in 10% formaldehyde and viewed by microscopy. The percentage of cells with septa (the early formation of new cell wall that splits the parent cell into two daughter cells) was plotted

against time (Figure 3.4A). In an exponentially growing culture of *S. pombe* cells, G1 is so short that when septa form, the cell is already in S phase. A peak in septation therefore serves as a convenient S phase marker. A peak in septation was seen at 105 minutes in 80% of cells, indicating a high degree of synchrony in cell cycle progression.

cdc22$^+$ encodes the catalytic subunit of ribonucleotide reductase that is required for DNA replication and whose expression is cell cycle-regulated, serving as a G1/S phase marker (Fernandez-Sarabia *et al.*, 1993). Periodic expression of *cdc22*$^+$ was confirmed with a peak in transcript levels seen at 45 minutes, just before S phase (Figure 3.4B). *rad21*$^+$ was periodically transcribed in parallel with *cdc22*$^+$, with maximum mRNA levels also seen at G1/S, dropping to almost undetectable levels in G2 and M phases. When probed with radio-labelled *rec8*$^+$ DNA, weak bands were seen that were smaller in size to the transcript detected in the meiotic control sample (Figure 3.4B, sample *). Equal loading of RNA was confirmed by ethidium bromide labelling of ribosomal RNA (rRNA). Messenger RNA levels were quantified by densitrometry using the NIH Image program, accounting for minor differences in RNA loading (Figure 3.4C). The positions of ribosomal RNA bands were used to confirm approximately the expected transcript sizes (*cdc22*$^+$: 3.3 kb, *rad21*$^+$: 2.6 kb, *rec8*$^+$: 1.7 kb).

3.3 *rec8*$^+$ EXPRESSION IS INDUCED DURING *pat1-114* SYNCHRONOUS MEIOSIS

In wild type *S. pombe* cells, meiosis is triggered from G1 phase in response to nitrogen starvation and mating pheromones from cells of opposite mating type. Also, because once a cell passes Start it is committed to complete that cell cycle, entry into meiosis can only be triggered from G1 (for review, see Hayles & Nurse, 1992). To induce synchronous entry into meiosis, a diploid *pat1-114* mutant (GG 177) was used to mimic natural induction of meiosis as closely as possible. To maximise synchrony, a diploid strain was used so that the conjugation step could be bypassed. *S. pombe* are highly unstable as diploids, so this was overcome by using a diploid strain that was homozygous for mating type (h^-/h^-). This strain was a gift from G. R. Smith (Seattle, USA) and arose by spontaneous diploidisation. Pat1p is a tyrosine kinase that functions during G1 phase to inhibit entry into meiosis by inhibiting Ste11p and Mei3p (Section 1.5; Figure 1.7; Nurse *et al.*, 1985; Willer *et al.*, 1995; Watanabe *et al.*, 1997; Wang *et al.*, 1998). Incubation of the *pat1-114* mutant at restrictive temperature (36°C) during G1 leads to inactivation of Pat1p triggering synchronous entry into the meiotic cell cycle (Bahler *et al.*, 1991).

Cells were cultured to early log phase (1-5 x 10^6 cells/ml) overnight in minimal media at permissive temperature (25°C). Cells were then harvested, washed and transferred to nitrogen-free media (EMM-N). Cells were then incubated overnight at 25°C to arrest cells in G1 phase. Synchronous entry into pre-meiotic S phase was triggered by rapidly switching the culture to 36°C. 0.1 M NH_4Cl was added to enhance cell cycle synchrony. Cell samples were collected at 15 minute intervals and total RNA extracted for northern blot analysis, probing for *cdc22*$^+$, *rad21*$^+$ and *rec8*$^+$ mRNAs (Figure 3.5B). mRNA levels were quantified by densitrometry using the NIH Image program, with ethidium bromide staining of ribosomal RNA used to normalise for loading differences (Figure 3.5C)

cdc22$^+$ mRNA was detected 45 minutes after meiotic induction, with maximum transcript levels seen at 105 minutes, just prior to the onset of DNA replication. By 120 minutes *cdc22*$^+$ expression had started to decline. *rec8*$^+$ transcript was detected a little later than *cdc22*$^+$, at 60 minutes, and at 120 minutes expression remained at maximal levels. No *rad21*$^+$ signal was detected in meiotic RNA throughout the experiment.

Samples were also prepared for flow cytometric analysis (Section 2.4.8) using the fluorescent dye, propidium iodide, to monitor their DNA content. A doubling of DNA content from 2C to 4C was taken to indicate progression of pre-meiotic DNA replication. This was observed between 120-180 minutes following *pat1-114* meiotic induction (Figure 3.5A).

3.4 SUMMARY

These data demonstrate that during *cdc25-22* induced synchronous mitosis the cohesin *rad21*$^+$ was periodically expressed, with mRNA levels peaking during G1/S phase. This is consistent with published findings (Birkenbihl & Subramani, 1995). Following induction of meiosis, however, *rad21*$^+$ transcript was absent, indicating that *rad21*$^+$ expression was repressed. *rec8*$^+$ transcript was observed during pre-meiotic S phase but was completely absent during the mitotic cell cycle, confirming that expression of this cohesin is meiotic-specific (Lin *et al.*, 1992). The finding that *rad21*$^+$ expression was mitotic-specific is significant because it has previously been suggested that, as well as being essential during the mitotic cell cycle, Rad21p has a meiotic role (Watanabe & Nurse, 1999). That Rad21p expression is so rapidly and completely downregulated upon meiotic induction indicates that, not only is it not required during meiosis, but also that its presence at this time may actually be damaging.

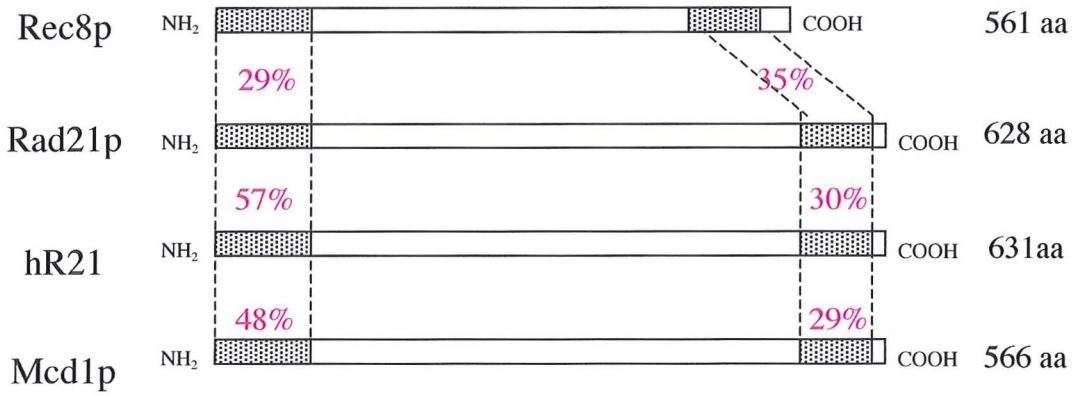


Figure 3.1. Sequence Conservation Between Rad21p and Rec8p. *S. pombe* Rec8p and Rad21p share 29% and 35% homology in the amino and carboxy-terminal thirds respectively. These regions are also conserved in evolution: *S. cerevisiae* Mcd1p and human hR21 share significant sequence homology in the amino and carboxy-termini when aligned with *S. pombe* Rad21p. However, functional domains are yet to be characterised for these proteins and the precise functions of these conserved domains remains to be elucidated.

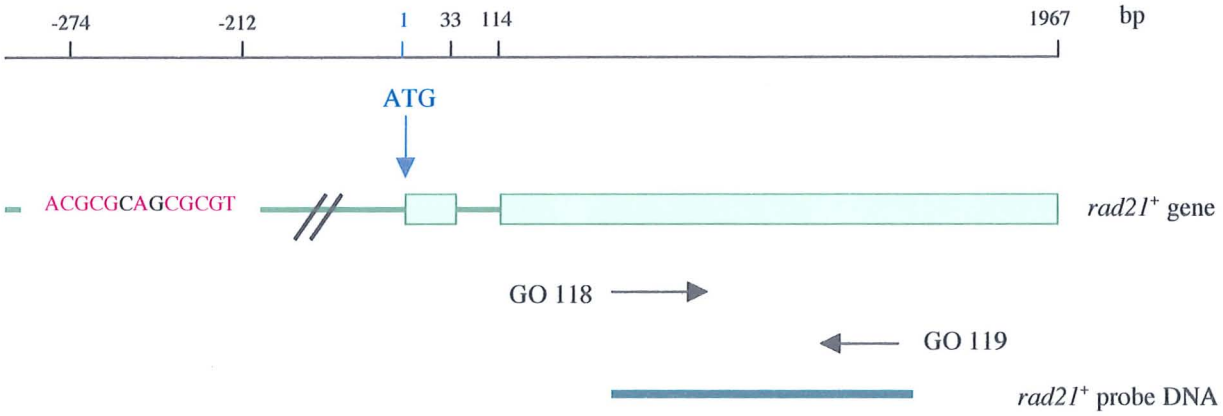


Figure 3.2. The *S. pombe rad21*$^+$ Locus on Chromosome III. The *rad21*$^+$ gene is made up of two exons (□) with one intron, and encodes an mRNA of 2.6 kb. MCB sequences occur as a doublet (ACGCGCAGCGCGT) in the *rad21*$^+$ promoter region 274 bp upstream of the ATG. Primers GO 118 and GO 119 were used to generate a 1kb fragment of *rad21*$^+$ open reading frame (▬) for northern hybridisation.

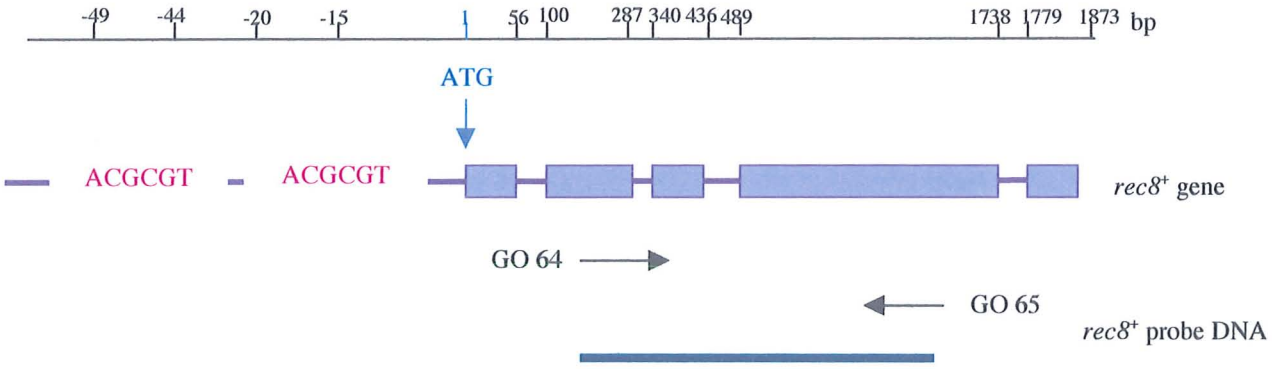
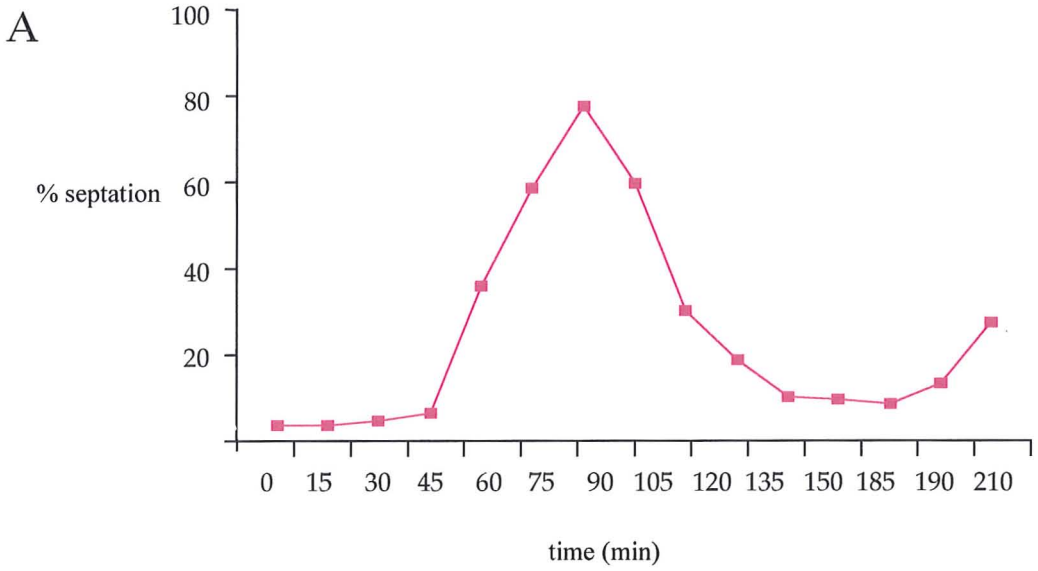


Figure 3.3. The *S. pombe rec8*$^+$ Locus on Chromosome II. The *rec8*$^+$ gene is made up of five exons (▭) with four introns and encodes a 1.7 kb mRNA. Two single consensus MCB sequences (ACGCGT) are found 49 bp and 20 bp upstream of the ATG (position 1). Primers GO 64 and GO 65 were used to generate a 1 kb fragment of *rec8*$^+$ DNA (▬) for northern blot hybridisation.


A
100
80
% septation
60
40
20
0 15 30 45 60 75 90 105 120 135 150 185 190 210
time (min)

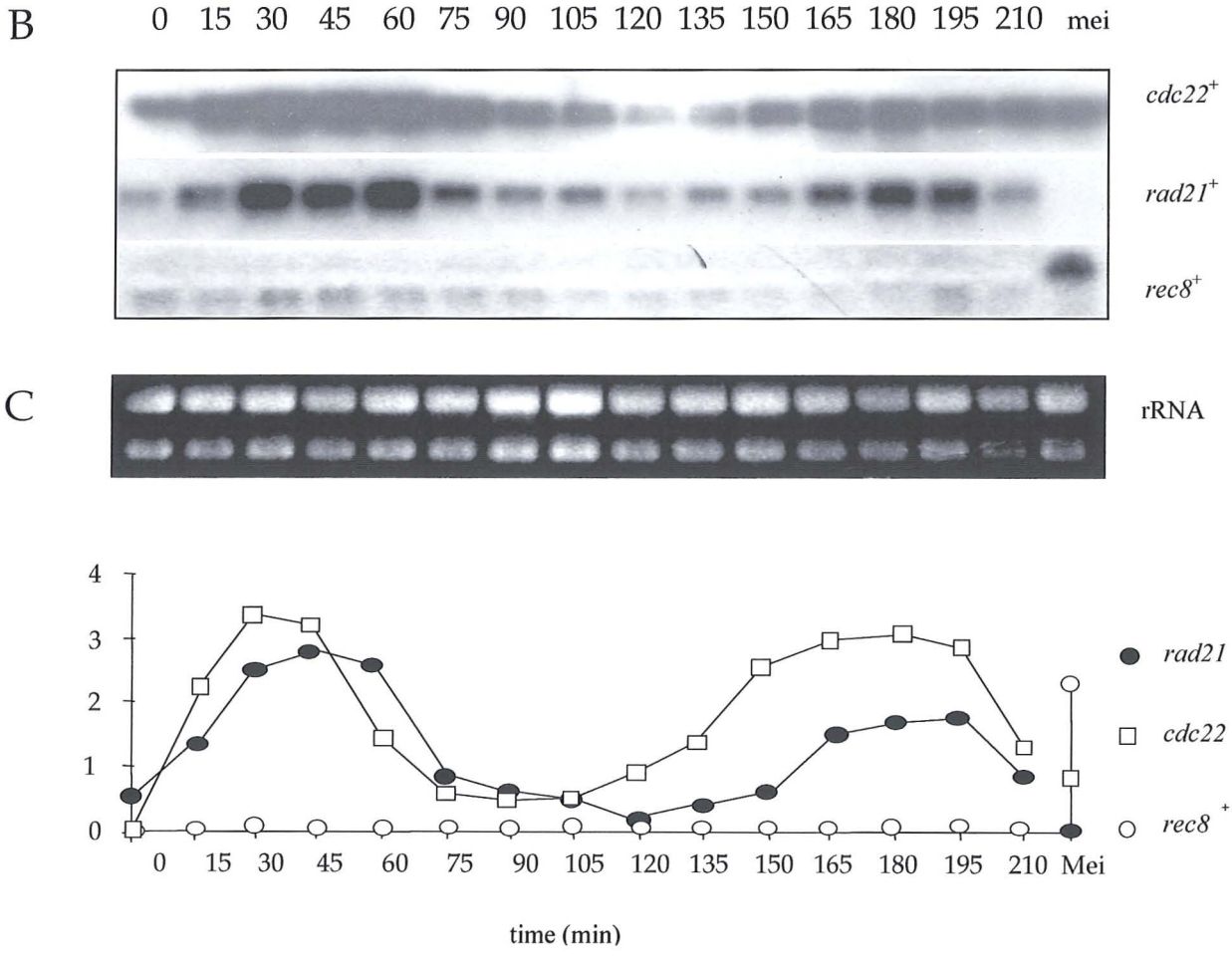
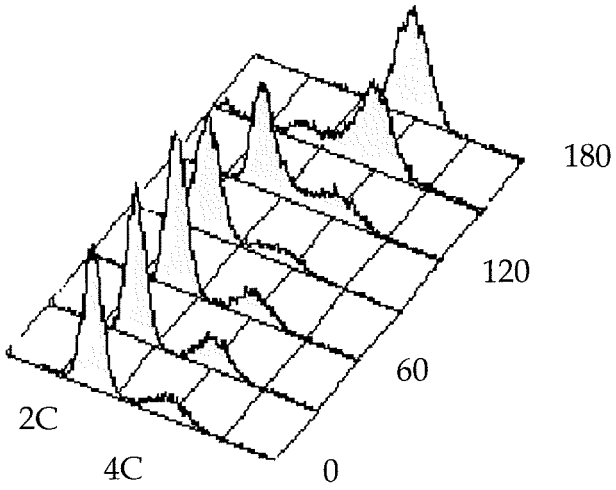

B
0 15 30 45 60 75 90 105 120 135 150 165 180 195 210 mei
cdc22+
rad21+
rec8+
C
rRNA
4
3
2
1
0
0 15 30 45 60 75 90 105 120 135 150 165 180 195 210 Mei
rad21+
cdc22+
rec8+
time (min)

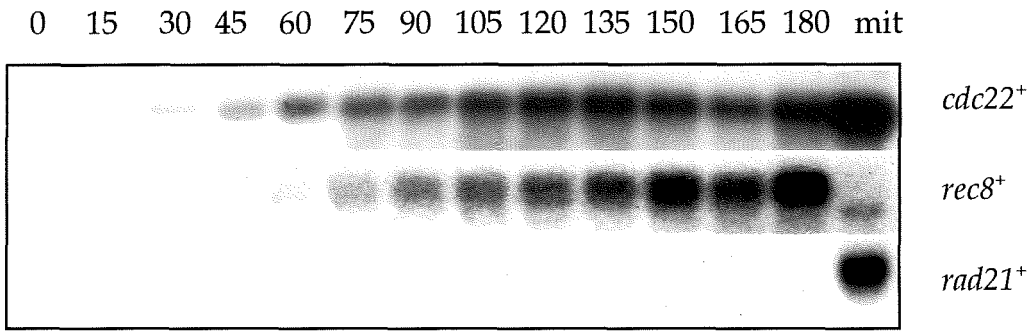
Figure 3.4 *rad21*$^{+}$ is Periodically Transcribed during the Mitotic Cell Cycvle. *cdc25-22* cells (GG 95) were arrested at G2 by incubation at restrictive temperature (36°C). Synchronous mitosis was then triggered by switching to permissive temperature (25°C). Cells were collected at 15 minute intervals. **A,** Cells were fixed in formaldehyde and viewed by microscopy to monitor septation. A peak in septation at 90 minutes represents 80% of cells in S phase. **B,** 10 μg total RNA was separated on a formaldehyde gel, transferred to a nylon membrane and hybridised with ^{32}P-labelled DNA probes. A meiotic RNA sample, taken 3 hours after *pat1-114* meiotic induction was loaded as a control (**mei**). **C,** Equal loading of RNA was confirmed by ethidium bromide labelling of ribosomal RNA (rRNA). Transcript levels were quantified by densitrometry using NIH image software; units given are arbitrary.

A

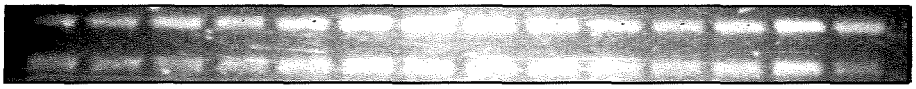


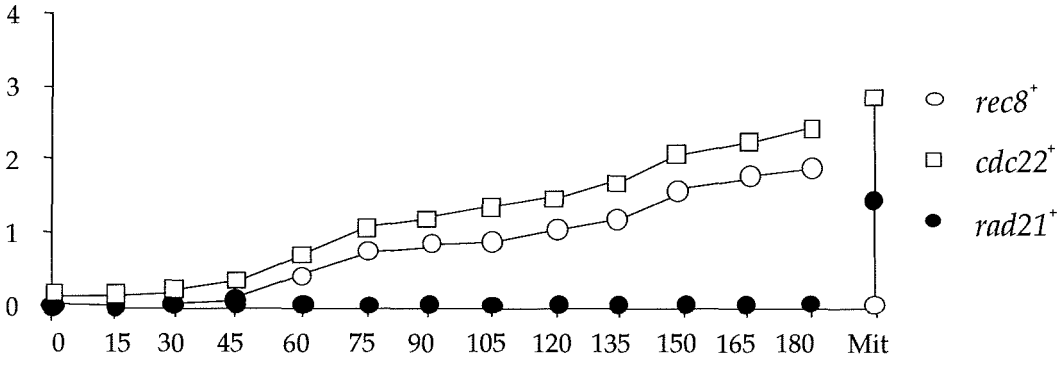
B

time (min)



C





time (min)

Figure 3.5 *rec8*$^+$ is Expressed Following Induction of Meiosis. *pat1-114* cells (GG 177) were arrested in G1 by nitrogen starvation for 16 hours at 25°C. By transferring the culture to restrictive temperature (36°C) synchronous entry into meiosis was induced. Cell samples were harvested at 15 minute intervals. **A,** To monitor S phase progression cells were stained with propidium iodide for analysis by flow cytometry. **B,** 10 μg total RNA was separated on a formaldehyde gel, transferred to a nylon membrane and probed for *cdc22*$^+$, *rad21*$^+$ and *rec8*$^+$ transcripts. A mitotic RNA sample, taken at 45 minutes after *cdc25-22* block and release, was loaded as a control (**mit**). **C,** Equal loading of RNA was confirmed by ethidium bromide labelling of ribosomal RNA (rRNA). Transcript levels were quantified using NIH image software; units given are arbitrary.

Chapter 4:

Reciprocal Exchange of *rad21*$^{+}$ and *rec8*$^{+}$ Cohesin Expression

4.1 BACKGROUND

As shown in the previous chapter, *S. pombe* expresses alternative life cycle-specific cohesin genes: *rec8*$^+$ is transcribed exclusively during the meiotic cell cycle, while *rad21*$^+$ is mitotic-specific, being repressed upon induction of meiosis. This is an significant finding because, a literature search reveals that, other the DSC1 transcription factor co-activator Rep2p, no genes that are expressed during the mitotic cell cycle in *S. pombe* are known to be repressed during the meiotic cell cycle. What makes this situation especially interesting is that *rad21*$^+$ encodes a protein that is essential for viability, yet appears not to be required during sexual development.

4.2 AIM OF STUDY

The aim of this study was to examine why expression of the pair of *S. pombe* cohesin genes, *rec8*$^+$ and *rad21*$^+$, is regulated in this unusual way. Two *S. pombe* strains were created in which the promoter regions of *rad21*$^+$ and *rec8*$^+$ were interchanged, so that life cycle-specific cohesin expression was switched (Figure 4.1). DNA constructs were generated in which the *rad21*$^+$ open reading frame was fused at its 5' end to the *rec8*$^+$ promoter region, while the *rec8*$^+$ open reading frame was fused at its 5' end to the *rad21*$^+$ promoter. These constructs were then introduced into *S. pombe* strains in which endogenous Rad21p and Rec8p were eliminated. As a result, the *rec8*$^+$ gene, now under *rad21*$^+$ promoter control, was expressed in place of *rad21*$^+$ during the cell division cycle. Conversely, when meiosis was triggered *rad21*$^+$, now under regulation by the *rec8*$^+$ promoter, was expressed in place of *rec8*$^+$. *S. pombe* strains, primers and constructs described in this Chapter are described in Appendices I, II and III, respectively.

4.3 EXCHANGING Rad21p FOR THE MEIOTIC COHESIN Rec8p

4.3.1 Creating *rad21*$^+$ Repressible Strain

Because *rad21*$^+$ is an essential gene, rather than permanently inactivating the gene, for example by disrupting or deleting it, the *rad21*$^+$ gene was placed under control of the repressible *nmt1*$^+$ promoter (*nmt1*P) so that its expression could be regulated. This promoter regulates the *S. pombe nmt1*$^+$ gene, which encodes an intermediate in the biochemical pathway that synthesises the amino acid thiamine (Maundrell, 1990). In culture conditions where thiamine is lacking, the *nmt1*$^+$ gene is expressed and stimulates thiamine biosynthesis.

In media supplemented with thiamine, where cells therefore no longer need to synthesise thiamine, the *nmt1*$^+$ promoter completely and rapidly represses transcription of *nmt1*$^+$. These features of the *nmt1*$^+$ promoter have been widely exploited in *S. pombe* to regulate expression of heterologous genes placed under its control.

To create a *rad21*$^+$ repressible strain, a *nmt1*P::*rad21*$^+$ DNA construct was used (GB 92; Birkenbihl & Subramani, 1995). This construct was made by replacing the 1.5 kb of DNA immediately upstream of the *rad21*$^+$ open reading frame with the *S. pombe ura4*$^+$ gene and *nmt1*$^+$promoter. Integration of *nmt1*P::*rad21*$^+$ into the chromosomal *rad21*$^+$ locus, replacing the native *rad21*$^+$ promoter was possible because the construct incorporates part of *rad21*$^+$ 5' untranslated region and part of the 5' region of the *rad21*$^+$ open reading frame, enabling homologous recombination to occur (Figure 4.3).

The *nmt1*P::*rad21*$^+$ DNA was integrated into a *S. pombe cdc25-22* mutant (GG 95), using the lithium acetate method of transformation (Section 2.2.6; Okazaki *et al.*, 1990). Successful integrants were selected by growth on minimal media lacking uracil. Three positive colonies were isolated, and accurate integration of *nmt1*P::*rad21*$^+$ DNA confirmed by PCR and Southern blot analysis.

4.3.2 Confirming Accurate Integration of *nmt1*P::*rad21*$^+$ Construct

4.3.2.1 INTEGRATION AT THE CORRECT LOCUS

To confirm that the *nmt1*P::*rad21*$^+$ construct had integrated at the correct locus in the genome, PCR was performed using primers designed to amplify the *rad21*$^+$ gene, the forward primer annealing within the *nmt1*$^+$ promoter region of the *nmt1*P::*rad21*$^+$ DNA, and the reverse primer annealing to the 3' end of the *rad21*$^+$ ORF, outside the integrated DNA region (Figure 4.2). Only when the *nmt1*P::*rad21*$^+$ DNA has integrated at the correct locus, would the forward end reverse primers anneal close enough to generate a PCR product.

Cells from the three positive colonies isolated were cultured for 3-4 days then harvested and genomic DNA purified (Section 2.4.1). PCR was then performed to amplify the *nmt*P::*rad21*$^+$ locus. For each of the three positive isolates a single band of 1.5 kb was seen, confirming that *nmt1*P::*rad21*$^+$ had integrated correctly at the *rad21*$^+$ locus (Figure 4.2B).

4.3.2.2 SINGLE INTEGRATION INTO THE GENOME

To confirm that *nmt1*P::*rad21*$^+$ had integrated only once into the genome, Southern blot analysis was performed (Section 2.4.5). Cells from one positive colony (GG 320) were cultured for 3-4 days then harvested and genomic DNA purified (Section 2.4.1). Genomic DNA was then digested overnight with the restriction enzyme *Bgl* II. *Bgl* II restriction sites occur twice within the *rad21*$^+$ locus, once in the 5' untranslated region, and once within the 3' end of the *rad21*$^+$ ORF. In the wild type *rad21*$^+$ locus this would produce a DNA fragment with the expected size of 4.3 kb, while in cells with *nmt1*P::*rad21*$^+$ integrated at the *rad21*$^+$ locus, it would produce a larger 5.7 kb fragment (Figure 4.3A). Multiple integrations would be expected to produce additional larger DNA fragments of >10 kb.

Southern blot hybridisation was then performed using *rad21*$^+$ probe DNA (Figure 3.2; Section 2.4.6). In lane 1, containing *Bgl* II-digested genomic DNA from GG 95 (*rad21*$^+$), the *rad21*$^+$ DNA probe hybridised to a single band of 4.3 kb, as expected. In lane 2 a single larger band of 5.7 kb was detected, confirming that *nmt1*P::*rad21*$^+$ had integrated only once into the genome (Figure 4.3B).

4.3.2.3 TRANSCRIPTIONAL REPRESSION OF *rad21*$^+$ BY *nmt1*$^+$ PROMOTER

To determine how efficiently the *nmt1*$^+$ promoter was able to repress *rad21*$^+$ transcription in the *rad21*$^+$-repressible strain (GG 320), western blot analysis was performed (Section 2.5.1) to monitor depletion of Rad21p protein.

GG 320 was first cultured to early exponential phase, 10 μM thiamine added then cell samples taken at time points 0-6 hours. Western blot analysis was performed, incubating sequentially with the primary antibody, polyclonal rabbit anti-Rad21p (Birkenbihl & Subramani, 1995), then HRP-conjugated secondary antibody, mouse anti-rabbit immunoglobulin.

At time 0 several bands were detected, ranging from 92-100 kDa (Figure 4.4; Birkenbihl & Subramani, 1995). This range in size was probably too large to represent Rad21p in different degrees of phosphorylation, which could be tested by treating the protein samples with a broad range kinase inhibitor such as staurosporine. Instead the smaller band may represent a truncated form of Rad21p, which has been cleaved due to Rad21p being overexpressed. In support of this the smaller band was not detected in the wild type sample. This western blot demonstrates that in the absence of thiamine there was no repression of

rad21$^+$ transcription by the *nmt1*$^+$ promoter. After addition of thiamine to the culture, levels of Rad21p decreased rapidly, so that after 2 hours Rad21p was barely detectable. These data demonstrate that in *rad21*$^+$-repressible cells (GG 320), Rad21p protein was rapidly depleted after the addition of thiamine. They also show that under these conditions the *nmt1*$^+$ promoter was able to rapidly repress transcription of the *rad21*$^+$gene.

4.3.3 Placing the *rec8*$^+$ Gene under *rad21*$^+$ Promoter Control

To place the *rec8*$^+$ gene under the control of the *rad21*$^+$ promoter, the entire *rec8*$^+$ open reading frame and *rad21*$^+$ promoter region were amplified by PCR (Section 2.6.1.1), and confirmed by DNA sequencing. Restriction sites were incorporated to enable ligation of *rad21*P and *rec8*$^+$ DNAs into cloning vectors. The PCR products were ligated together in the *E. coli* plasmid, pBCKS+ (Section 2.6.1.2) The *rad21*P:*rec8*$^+$ double construct was then cloned into one of two fisson yeast plasmids. pSP1, a multi-copy plasmid, creating pSP1/*rad21*P:*rec8*$^+$ (GB 183; Section 2.6.1.3), and the integrating plasmid, pJK148, creating pJK148/*rad21*P:*rec8*$^+$ (GB 190; Section 2.6.1.4; Appendix III). Alternative yeast plasmids were used to determine whether, if *rec8*$^+$ was able to complement *rad21*$^+$, it could do so only when in multiple copy (ie overexpressed), or if it could complement when expressed in single copy.

4.3.4 Introducing *rad21*P: *rec8*$^+$ into *rad21*$^+$-Repressible Cells

pJK148/ *rad21*P: *rec8*$^+$ (GB 190) was linearised by digestion with *Nru* I, then integrated into the *leu1*$^+$ locus of the *rad21*$^+$-repressible strain, GG 320 (Section 2.2.4), creating GG 358 (Appendix I). pSP1/ *rad21*P: *rec8*$^+$ (GB 183) was transformed into GG 320 creating GG 327.

4.3.5 Cohesin Expression in *rad21*$^+$-Repressible Strains

4.3.5.1 INDUCTION OF SYNCHRONOUS MITOSIS

To confirm that the *rad21*$^+$-repressible strains carrying *rad21*P: *rec8*$^+$ DNA, both in the low copy plasmid pSP1 (GG 327) and integrated in single copy (GG 358), could now express *rec8*$^+$ during the mitotic cell cycle in place of *rad21*$^+$, synchronous mitosis was induced by a transient thermal inactivation of the temperature sensitive *cdc25-22* mutant as described in Section 3.1, and cell samples were taken at 15 minute intervals. Samples were also taken

from each time point and fixed in 10% formaldehyde so that septation could be monitored by microscopy.

4.3.5.2 ANALYSIS OF COHESIN EXPRESSION DURING SYNCHRONOUS MITOSIS

Total RNA was prepared for northern blot analysis (Section 2.3.3.1-2.3.3.2). Control samples were also loaded alongside: wild type samples, taken at 45 and 90 minutes of *cdc25-22* synchronous mitosis, representing the peak (P) and trough (T) in *rad21*$^+$ expression, respectively. As a meiotic control, RNA taken 3 hours after *pat-114* meiotic induction (Section 3.3), was included (M). Finally, asynchronous samples of *rad21*$^+$-repressible cells (GG 327 or GG 358), cultured without thiamine, were also loaded. Membranes were then hybridised with radio-labelled *rad21*$^+$, *rec8*$^+$and *cdc22*$^+$ DNA probes (Section 2.4.6).

4.3.5.3 *rad21*-REPRESSIBLE, rad21P: *rec8*$^+$ PLASMID STRAIN

For the GG 327 synchronous mitotic cell cycle, a peak in septation at 90 minutes represented 80% of cells in S phase, and demonstrated a high degree of synchrony for the *cdc25-22* block and release (Figure 4.5).

cdc22$^+$ was periodically expressed, with maximum transcript seen at 45 minutes, corresponding to late G1/ S phase. In the *rad21*$^+$-repressible cells synchronous mitosis, in which the cells were cultured with thiamine, *rad21*$^+$ expression was barely detectable. However, in the control sample of *rad21*$^+$-repressible cells cultured without thiamine, the level *rad21*$^+$ expression was very high, so that most of the *rad21*$^+$ probe was sequestered away from the mitotic control samples (Figure 4.5B).

When probed for *rec8*$^+$mRNA, the levels were found to be periodic in pattern that mirrored *cdc22*$^+$ transcript levels. Peak *rec8*$^+$ transcript levels were seen at 45 minutes and trough levels at 105-135 minutes. This profile is the same as that seen for *rad21*$^+$ in a wild type synchronous mitosis (Figure 3.4). In GG 327 synchronous mitosis, *rec8*$^+$ probe hybridised to three bands with the highest intensity band at ~3 kb.

4.3.5.4 *rad21*-REPRESSIBLE, *rad21*P: *rec8*$^+$ INTEGRANT STRAIN

cdc25-22 synchronous mitosis was also induced in *S. pombe* strain GG 358 (*rad21*$^+$-repressible cells with *rad21*P: *rec8*$^+$integrated in single copy) and northern blot analysis performed to examine *rad21*$^+$ and *rec8*$^+$ expression (Figure 4.6).

cdc22$^+$ transcript levels peaked at 45 minutes, just prior to S phase, represented by a peak in septation at 90 minutes (Figure 4.7A). Expression of *rec8*$^+$ was also cell cycle-regulated, with maximum transcript levels during G1/S phase at 45 minutes. In GG 358 RNA, the *rec8*$^+$ probe hybridised to several transcripts. The larger, more defined band was ~2.5 kb, which is significantly larger than the 1.3 kb meiotic *rec8*$^+$ transcript (mei). *rec8*$^+$ hybridisation was also detected as a smear of ~3 kb (Figure 4.6B).

In the asynchronous sample of GG 358 cells cultured without thiamine (*nmt1*$^+$ promoter active), *rad21*$^+$ transcript levels were more than tenfold higher than the peak wild type mitotic RNA sample (P). However, in the presence of thiamine (*nmt1*$^+$ promoter repressed), *rad21*$^+$ expression was barely detectable, with transcript levels significantly lower than in the wild type trough mitotic sample (T; Figure 4.6B).

4.3.6 Replacement of *rad21*$^+$ with *rec8*$^+$ During Synchronous Mitosis

Summarising, in both *rad21*$^+$-repressible strains (GG 327 and GG 358), when cultured with thiamine, the *nmt*1$^+$ promoter was inactive, and *rad21*$^+$ transcription repressed. When cultured without thiamine, the *nmt1*$^+$ promoter was active, and *rad21*$^+$ was expressed. In the absence of inhibition by the *nmt1*$^+$ promoter, *rad21*$^+$ was constitutively over-expressed. Furthermore, *rec8*$^+$, under *rad21*$^+$ promoter control in these cells, was expressed in place of *rad21*$^+$, and at levels comparable to those seen for *rad21*$^+$ in wild type mitotic induction (Figure 3.2). These strains could therefore potentially serve as useful tools to investigate life cycle-specific cohesin function.

4.4 EXCHANGING THE MEIOTIC COHESIN Rec8p FOR Rad21p

4.4.1 Placing the *rad21*$^+$ Gene under *rec8*$^+$ Promoter Control

To create *S. pombe* strains that would express the mitotic cohesin *rad21*$^+$ in place of *rec8*$^+$ during meiosis, the *rad21*$^+$ gene was placed under *rec8*$^+$ promoter control, and introduced into *rec8*Δ strains. The *rec8*$^+$ promoter region (1.5 kb untranslated region upstream of the *rec8*$^+$ ORF) and the *rad21*$^+$ ORF were amplified by PCR (Section 2.4.2.1) then ligated together in the *E. coli* plasmid pBCKS+ (Section 2.6.2.2). The *rec8*P:*rad21*$^+$ double construct was then cloned separately into the yeast plasmids pSP1 (Section 2.6.2.3) and pJK148 (Section 2.6.2.4) creating pSP1/*rec8*P:*rad21*$^+$ (GB 179) and pJK148/*rec8*P:*rad21*$^+$ (GB 189).

4.4.2 Making *rec8*Δ Strains

To create *S. pombe rec8*$^-$ mutants strains that could be used to induce synchronous meiosis, the temperature-sensitive *pat1-114* mutation, as well as complementing *ade*$^-$ alleles were introduced into *rec8*::*ura4*$^+$disruption (*rec8*Δ) strains. This was achieved by performing a series of genetic crosses followed by random spore analysis.

4.4.2.1 INTRODUCING *pat1-114* ALLELE INTO *rec8Δ* STRAINS

The *rec8*Δ mutant *h*$^-$ *rec8*::*ura4*$^+$ *ura4-D18 leu1-32* (GG 278) was first crossed with *S. pombe* strain *h*$^+$ *pat1-114 his1-102 leu1-32 ura4-294* (GG 190; Section 2.2.1) to introduce the *pat1-114* allele into the *rec8*::*ura4*$^+$background (Appendix I). Clones carrying the temperature-sensitive *pat1-114* mutation (Section 2.2.2) as well as the *ura4*$^+$ and *leu1-32* alleles were isolated their mating types determined (Section 2.2.3), creating GG 292 (*h*$^-$) and GG 297 (*h*$^+$).

4.4.2.2 INTRODUCING COMPLEMENTING *ade6*$^-$ ALLELES

The meiotic experiments in this study were performed in diploids, so that they would mimic wild type meiosis as closely as possible. However *S. pombe* cells are very unstable as diploids, and immediately undergo meiosis therefore returning to the stable haploid state. Any population of diploid *S. pombe* cells might therefore also contain a haploid fraction resulting from spontaneous meioses.

One way to avoid contamination with haploids was to introduce the complementing *ade6-M210* and *ade6-M216* alleles into diploid strains. *ade6*$^+$ encodes an intermediate in the adenine biosynthesis pathway. When both alleles are present in diploid strains they complement each other, a wild type *ade6*$^+$ product is translated, and adenine biosynthesis takes place within the cell. However, if only one allele is present i.e. in haploid *ade6*$^-$ cells, the *ade6*$^+$ product is non-functional and adenine must be added exogenously. Growth in culture media lacking adenine was therefore be used to select for diploids.

ade6-M210 and *ade6-M216* alleles were introduced into *rec8*Δ haploids by crossing GG 297 with GG 164 (*ade6-M216*) and GG 292 with GG 165 (*ade6-M210*). Colonies of *pat1-114*, *rec8*::*ura4*$^+$, *leu1-32* cells that now also carried either the *ade6-M216* or *ade6-M210* alleles were isolated and their mating types determined, creating GG 299 (*h*$^-$ *pat1-114*, *rec8*::*ura4*$^+$, *ura4-D18*, *leu1-32*, *ade6-M216*) and GG 301 (*h*$^-$ *pat1-114*, *rec8*::*ura4*$^+$, *ura4-D18*, *leu1-32*, *ade6-M216*)

The mating types of positive isolates were then determined as described in Section *2.2.3. The resulting *S. pombe* strains were called GG 299 (*h*$^-$ *pat1-114*, *rec8*::*ura4*$^+$, *ura4-D18*, *leu1-32*, *ade6-M216*) and GG 301 (*h*$^-$ *pat1-114*, *rec8*::*ura4*$^+$, *ura4-D18*, *leu1-32*, *ade6-M216*)

4.4.3 Making *rec8*Δ *rec8*P: *rad21*$^+$ Diploids

4.4.3.1 *rec8*Δ *rec8P*: *rad21*$^+$ PLASMID STRAIN

*4.4.3.1.1 Protoplast Fusion of rec8*Δ *Haploids*

Typically, to create diploid *S. pombe* strains, cells are starved of nitrogen, which triggers conjugation between cells of opposite mating type followed by meiosis and sporulation. If these cells are transferred to rich medium at the diploid zygote stage, they can be maintained as diploids. However, as described above, one problem when working with *S. pombe* diploids is that they are unstable, undergoing spontaneous meiosis at a high frequency.

Another way to overcome this problem is to create diploid strains that are homozygous for mating type. However, haploid cells of the same mating type will not conjugate under normal mating conditions. Instead, to create diploids that are homozygous for mating type, haploid cells were brought into union using a technique known as 'protoplast fusion' (Section 2.2.4).

Protoplast fusion was used to create a diploid *rec8*Δ strain from haploids GG 299 (*h⁻ pat1-114, rec8::ura4⁺, ura4-D18, leu1-32, ade6-M216*) and GG 301 (*h⁻ pat1-114, rec8::ura4⁺, ura4-D18,leu1-32, ade6-M210*). *S. pombe* cell walls were first digested using zymolyase, an enzyme preparation containing lysozyme. Cells were then incubated with polyethylene glycol (PEG) to promote fusion of cell protoplasms. This was followed by incubation for 7-14 days on media supplemented with sorbitol, to promote recovery and reconstitution of new cell walls.

Successful protoplast fusion between GG 299 and GG 301, resulted in the generation of a diploid strain carrying complementing *ade6-M210* and *ade6-M216* alleles. These diploids were isolated by culture on EMM lacking adenine. When cultured without adenine, *ade⁻* cells grew only slowly, forming small pink colonies, due to the accumulation of an intermediate in the adenine biosynthesis pathway. *ade⁺* colonies were identified as large white colonies growing on EMM plates lacking adenine. These colonies were lifted and patched onto YE and their genotypes determined by replica plating.

Because adenine prototrophs may have resulted from meiotic recombination occurring between the *ade6-M210 and ade6-M216* alleles, diploidy was confirmed by microscopy. Haploid cells are smaller in both length and diameter than diploids, so ploidy was ascertained by comparing the sizes of the *ade⁺* colonies with haploid parental strains GG 164 and GG 165. The resulting *h⁻/h⁻, pat1-114/pat1-114, rec8::ura4⁺/rec8::ura4⁺, ade6-M210/ade6-M216* diploid strain was called GG 323.

4.4.3.1.2 Introducing rec8P: rad21⁺ DNA Into rec8Δ Diploid

pSP1/ rec8P: *rad21⁺* (GB 179) was transformed into competent *rec8*Δ diploid cells, GG 323 (Section 2.2.6) creating GG 328, in which meiotic *rec8⁺* expression was now replaced with the mitotic cohesin *rad21⁺*. This was later confirmed by northern blot analysis. pSP1 carries the *S. pombe leu1⁺* gene, so cells that had successfully taken up GB 179 DNA were identified by growth in the absence of leucine.

4.4.3.2 *rec8Δ rec8P: rad21⁺* INTEGRANT STRAIN

In addition to using pSP1 as a vector to confer meiotic expression of *rad21⁺*, the *rec8*P: *rad21⁺* DNA was introduced into *rec8*Δ cells using the plasmid vector pJK148. This *S. pombe* vector was used to integrate the *rec8*P: *rad21⁺* DNA into the genome of *rec8*Δ cells in single copy, thereby ensuring meiotic *rad21⁺* expression at physiological levels. pJK148

carries the *S. pombe leu1*$^+$ gene, which enables its integration into the chromosomal *leu1*$^+$ locus by homologous recombination.

The *rec8*P: *rad21*$^+$ DNA (GB 189) was first integrated into *rec8*Δ haploids, GG 299 and GG 301 (Section 2.2.6), creating *S. pombe* strains GG 348 and GG 349. Protoplast fusion of GG 348 and GG 349 (Section 2.2.4) then generated a *rec8*Δ *rec8*P:*rad21*$^+$ integrant diploid strain, GG 402. Replica plating and microscopy confirmed the genotypes and ploidy of positive isolates.

4.4.3.3 CREATING ISOGENIC *rec8*$^+$ DIPLOIDS

For the meiotic experiments with *rec8*Δ *rec8*P: *rad21*$^+$ strains, it was also necessary to have *rec8*$^+$ counterparts. These control strains were made genotypically as similar as possible (isogenic) to GG 328 and GG 402, except that they carried the wild type *rec8*$^+$ allele.

4.4.3.3.1 rec8$^+$ *Diploid Plasmid Strain*

Mating of the haploid strains *h*$^-$ *pat1-114, leu1-32* (GG 335) and *h*$^+$*ade6-M210* (GG 165) followed by random spore analysis (Section 2.2.1) were used to introduce the *ade6-M210* allele into a *pat1-114, leu1-32* background. The mating types of positive colonies were determined as described in Section 2.2.3. This cross generated the *S. pombe* strains GG 339 (*h*$^-$) and GG 340 (*h*$^+$).

To introduce the *ade6-M216* allele into a *pat1-114, leu1-32* background, GG 336 (*h*$^+$ *pat1-114, leu1-32*) was crossed with GG 164 (*h*$^-$ *ade6-M216*) followed by random spore analysis, creating *S. pombe* strains GG 341 (*h*$^+$) and GG 342 (*h*$^-$).

A stable *rec8*$^+$ diploid was generated from GG339 and GG342 by protoplast fusion and named GG375 (*h*$^-$/*h*$^-$ *pat1-114/pat1-114, leu1-32/leu1-32, ade6-M210/ade6-M216*).

So that this *rec8*$^+$ diploid strain was isogenic to the *rec8*Δ *rec8P*: *rad21*$^+$ plasmid strain (GG 328), pSP1 (GB 84) was then transformed into GG 375 (Section 2.2.6), creating GG 408.

4.4.3.3.2 rec8$^+$ *Diploid Integrant Strain*

The integrating plasmid pJK148 (GB 186) was linearised by digestion with *Nru* I which cuts within the *leu1*$^+$ sequence. It was then integrated, by homologous recombination, into the *leu1-32* loci of both GG 342 (*h*$^-$) and GG 339 (*h*$^-$) using the lithium acetate method. Cells that had successfully taken up pJK148 DNA were isolated by growth on minimal media

lacking leucine creating GG 347 and GG 359. Protoplast fusion between GG 347 and GG 359 resulted in the formation of a stable diploid *rec8*$^+$ integrant strain GG 401 that was isogenic to the diploid *rec8*Δ *rad21*$^+$ integrant strain, GG 402.

4.4.4 Cohesin Expression in *rec8*Δ *rec8P*: *rad21*$^+$ Cells

To confirm that *rad21*$^+$ was expressed in *rec8*Δ *rec8P*: *rad21*$^+$ strains instead of *rec8*$^+$, synchronous meiosis was induced and northern blot analysis performed.

4.4.4.1 INDUCTION OF SYNCHRONOUS MITOSIS

Synchronous meiosis was induced in *rec8*Δ *rec8P*: *rad21*$^+$ strains GG 328 and GG 402 by thermal inactivation of Pat1p, as described in Section 3.3, each time point were also fixed in 70% ethanol then labelled with 2 μg/ml propidium iodide for flow cytometric analysis (Section 2.4.8). To analyse meiotic cohesin expression in these cells, northern blots were hybridised with radio-labelled *rad21*$^+$, *rec8*$^+$ and *cdc22*$^+$ DNA probes (Section 2.4.6).

4.4.4.2 *rec8*Δ *rec8*P: *rad21*$^+$ PLASMID STRAIN

At time zero a single peak in DNA content showed that cells in this culture had 2C DNA contents (Figure 4.9A). This is because they were arrested in late G1. With progression of DNA replication during pre-meiotic S phase, the peak in DNA content started to shift from 2C to 4C. This started at ~ 120 minutes, and by 180 minutes less than half of the cells had completed replication. This is in contrast to the wild type meiotic induction (Figure 3.5), in which DNA replication was completed by 180 minutes.

cdc22$^+$ mRNA first appeared at 45 minutes and peaked at 135 minutes, just after the onset of DNA replication (Figure 4.9B). No *rec8*$^+$ expression was detected throughout the 3 hour time course in *rec8*Δ *rec8*P: *rad21*$^+$ cells. *rad21*$^+$ transcript was absent from the wild type meiotic RNA sample (Figure 3.5B, Figure 4.9B 'mei'). However, the *rec8*Δ *rec8*P: *rad21*$^+$ plasmid strain expressed *rad21*$^+$ from 60 minutes in a pattern reflecting that of *rec8*$^+$ during a synchronous wild type meiosis (Figure 3.5B).

4.4.4.3 *rec8Δ rec8*P: *rad21*$^+$ INTEGRANT STRAIN

In *rec8Δ rec8P*: *rad21*$^+$ cells DNA replication, seen by flow cytometric analysis as a shift in DNA content from 2C to 4C, took place between 120 and 180 minutes (Figure 4.11A), the same as seen during the synchronous wild type meiosis (Figure 3.5A).

No *rec8*$^+$ transcript was detected during pre-meiotic S phase in the *rec8Δ rec8*P: *rad21*$^+$ integrant strain. Instead *rad21*$^+$ was expressed from 75 minutes through to 180 minutes, with transcript levels similar to that seen for *rec8*$^+$ during the synchronous wild type meiotic induction (Figure 3.5B, Figure 4.11B 'mei').) In the *rec8Δ* rec8P: *rad21*$^+$ integrant strain *cdc22*$^+$ was expressed from late G1 (45 minutes) to late S phase (150 minutes).

4.4.4.4 S PHASE DELAY CAUSED BY pSP1 PLASMID

S phase progression was delayed in the *rec8Δ rec8*P: *rad21*$^+$ plasmid strain, with replication only halfway completed by 180 miutes after meiotic induction. This is in contrast to that seen during meiotic induction for wild type *S. pombe* (Figure 3.5A) and the *rec8Δ rec8*P: *rad21*$^+$ integrant strain, in which replication had been completed by 180 minutes.

To see if this delay was caused by the presence of pSP1 plasmid in these cells, S phase progression was monitored and compared between an isogenic wild type strain (GG 375), the *rec8*D: *rec8P*: *rad21*$^+$ plasmid strain (GG 328), and the wild type isogenic strain GG 409, carrying pSP1 plasmid. *pat1-114* synchronous meiosis was triggered and cells collected at 1 hour intervals, fixed in 70% ethanol, and labelled with 2 mg/ml propidium iodide. The distribution of DNA contents at each time point were then analysed by flow cytometry.

In the wild type strain DNA replication took place between 120 and 180 minutes, as seen previously (Figure 4.10A, Figure 3.5A). In the *rec8Δ rad21*$^+$ plasmid strain (GG 328), replication began around 120 minutes but was still not completed at 180 minutes (Figure 4.10B). However, this was also seen for the wild type isogenic strain, which carries the wild type *rec8*$^+$ allele as well as pSP1 (Figure 4.10C). This suggests that the delay in S phase progression seen for the *rec8Δ rec8*P: *rad21*$^+$ plasmid strain (GG 328) was not caused by replacing Rec8p with the mitotic cohesin Rad21p during pre-meiotic S phase. Instead it was due to the presence of pSP1 plasmid in these cells.

4.4.5 Replacement of Rec8p with Rad21p during Synchronous Meiosis

To summarise, *S. pombe* strains were made in which expression of the meiotic-specific cohesin *rec8*$^+$ was exchanged for the mitotic-specific cohesin *rad21*$^+$. This was achieved by introducing into cells, either in low copy plasmid or integrated in single copy, a DNA construct in which the *rec8*$^+$ promoter region was placed upstream of the *rad21*$^+$open reading frame.

To confirm the switch in cohesin expression, synchronous meiosis was induced and cohesin expression analysed. The progression of pre-meiotic DNA replication was also analysed by flow cytometry.

In the *rec8*Δ *rec8*P: *rad21*$^+$ plasmid strain (GG 328) DNA replication was slower than in a wild type meiotic induction. The shift in DNA content from 2C to 4C started 120 minutes after induction of meiosis from G1 phase, as in the wild type strain, but by 180 minutes more than half of the cells still hadn't completed replication. This was shown to be caused by the presence of pSP1 plasmid, rather than exchanging Rec8p for the mitotic cohesin Rad21p during pre-meiotic S phase. In support of this, in the *rec8*Δ *rec8*P: *rad21*$^+$ integrant strain (GG 402) no alteration in the timing of DNA replication was seen.

rec8$^+$ expression was not detected throughout the 3 hour meiotic inductions, confirming that the *rec8*::*ura4*$^+$ disruption was effective, preventing transcription of the meiotic cohesin during pre-meiotic S phase, when it would normally be induced. In both *rec8*Δ *rad21*$^+$ strains, expression of the mitotic cohesin, *rad21*$^+$, was induced in place of *rec8*$^+$. Also, because *rad21*$^+$ was under *rec8*$^+$ promoter control, *rad21*$^+$ was expressed at levels comparable to that seen for *rec8*$^+$ during a wild type meiotic induction. These strains could therefore potentially serve as useful tools for investigating the meiotic and mitotic-specific roles of Rad21p and Rec8p cohesins, and to examine the overlap in their functions.

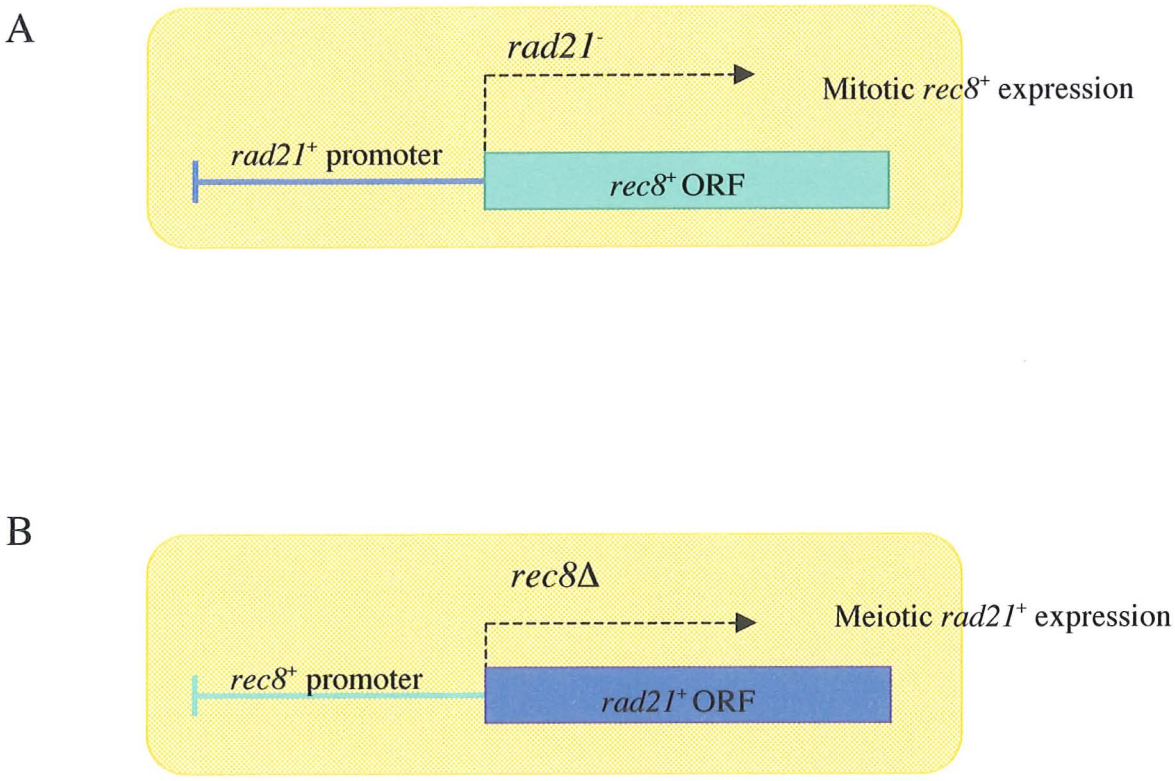


Figure 4.1 Ectopic Cohesin Expression elicited by Promoter Exchange. A, In a fission yeast strain where endogenous Rad21p was depleted (*rad21*$^{-}$), the *rec8*$^{+}$ open reading frame (ORF) was placed under *rad21*$^{+}$ promoter control, so that during the mitotic cell cycle *rec8*$^{+}$ was expressed in place of *rad21*$^{+}$. **B,** Conversely, in cells with a chromosomal disruption of the *rec8*$^{+}$ gene (*rec8*Δ), the *rad21*$^{+}$ ORF was placed under *rec8*$^{+}$ promoter control to bring about meiotic expression of *rad21*$^{+}$.

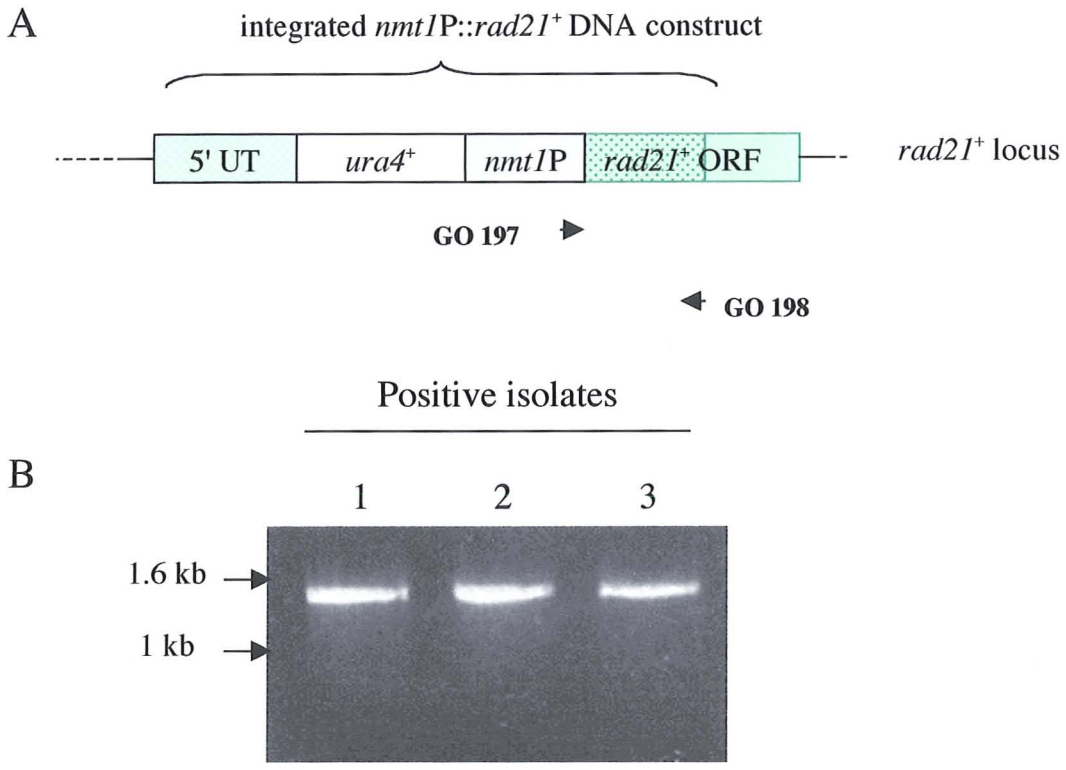


Figure 4.2 Confirmation of *nmt1*P::*rad21*$^+$ Integration by PCR. A, To confirm that the *nmt1*P::*rad21*$^+$ construct had integrated at the correct locus in the genome, PCR was performed using primers designed to amplify the *rad21*$^+$ gene, the forward primer (GO 197) annealing within the *nmt1*$^+$ promoter region of the *nmt1*P::*rad21*$^+$ construct, and the reverse primer (GO 198) annealing to the 3' end of the *rad21*$^+$. When the *nmt1*P::*rad21*$^+$ construct has integrated at the correct locus, PCR generates a 1.5 kb product. **B,** Three positive isolates were cultured to saturation, cells harvested and genomic DNA purified (Section 2.4.1). Genomic DNA from each isolate was then used as template for PCR with primers GO 197 and GO 198 (Appendix II). PCR product was then separated by gel electrophoresis. DNA was visualised by ethidium bromide staining under UV light.

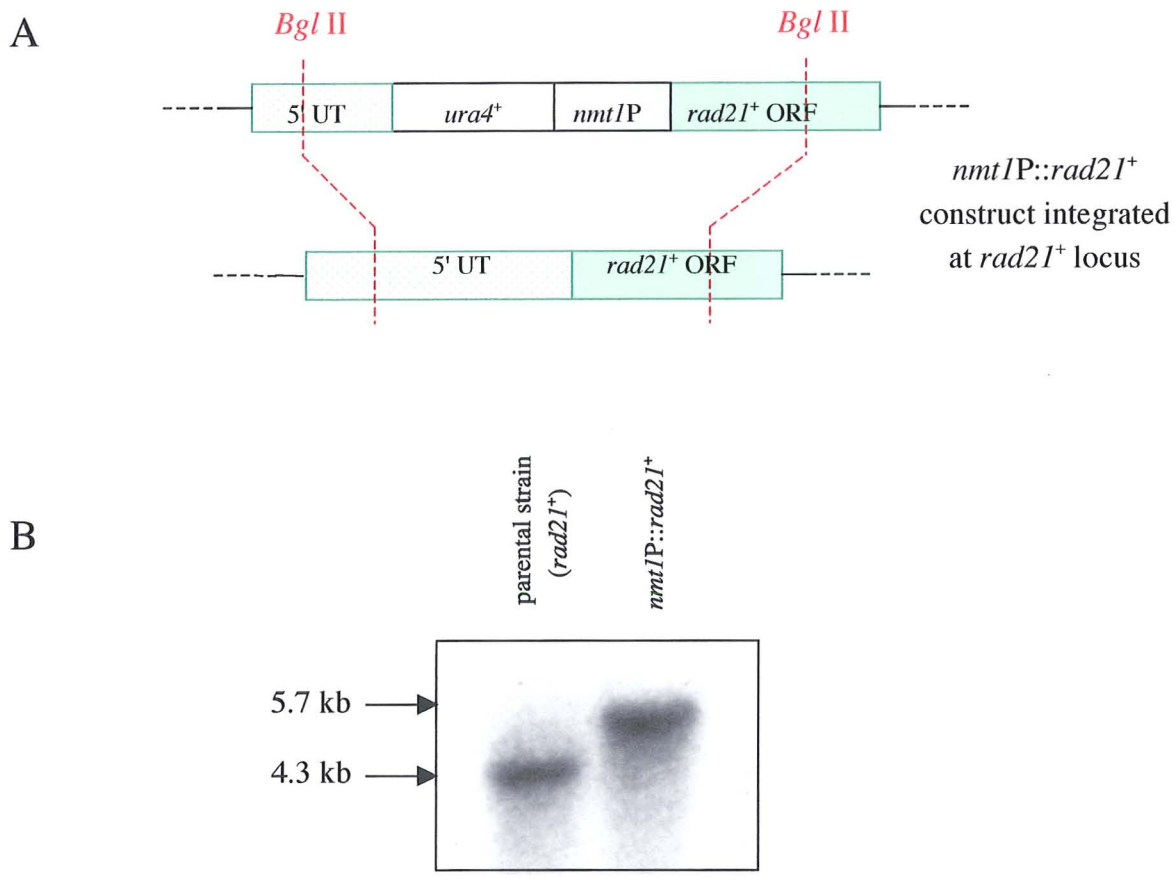


Figure 4.3 Confirmation of *nmt1*P::*rad21*$^+$ Integration by Southern Blot Analysis. A, There are two *Bgl* II restriction sites within the *rad21*$^+$ gene, in the 5' untranslated region and within the 3' end of the *rad21*$^+$ ORF. In the *rad21*$^+$ parental strain (GG 95) this produces a DNA fragment with the expected size of 4.3 kb, while with *nmt1*P::*rad21*$^+$ integrated at the *rad21*$^+$ locus, it produces a larger 5.7 kb fragment. **B,** A positive colony was cultured to exponential phase of growth and genomic DNA harvested (Section 2.4.1). 20 µg genomic DNA was digested with *Bgl* II for 16 hours, then separated by gel electrophoresis (*nmt1*P::*rad21*$^+$). 20 µg of *Bgl* II-digested *rad21*$^+$ genomic DNA, from parental strain GG 95 (Appendix I), was run on the same gel for size comparison (*rad21*$^+$). DNA was then transferred to a nylon membrane for Southern blot analysis (materials and methods). The membrane was incubated overnight with denatured ^{32}P-labelled *rad21*$^+$ DNA probe (Figure 3.2), and hybridisation detected by autoradiography.

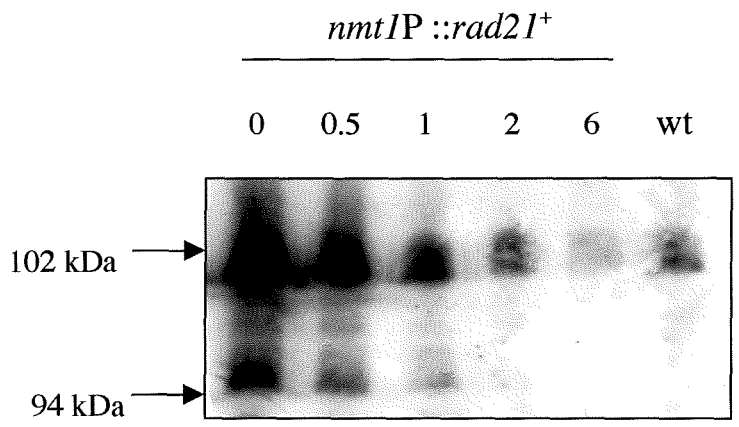


Figure 4.4 Rad21p was Rapidly Depleted from *nmt1*P ::*rad21*$^+$ Cells. *nmt1*P ::*rad21*$^+$ cells (GG 320) were cultured to early exponential phase (1-5x10^6 cells/ml) at 25°C in EMM + leucine. Thiamine was then added to a final concentration of 10 μM and cell samples taken at time points 0-6 hours for Western blot analysis. Cells were lysed and protein purified (Section 2.5.1), then 20 μg of protein from each time point was separated by electrophoresis on a denaturing SDS polyacrylamide gel. A protein sample taken from an asynchronous wild type culture (wt; GG 95) was run on the same gel as a positive control. Protein was transferred onto a nitrocellulose membrane, then incubated sequentially with the primary antibody, polyclonal rabbit anti-Rad21p (Birkenbihl and Subramani, 1995), then secondary antibody, mouse anti-rabbit immunoglobulin conjugated to horseradish peroxidase (HRP). Positive immunoreaction was then detected by chemiluminescence and visualised by autoradiography.

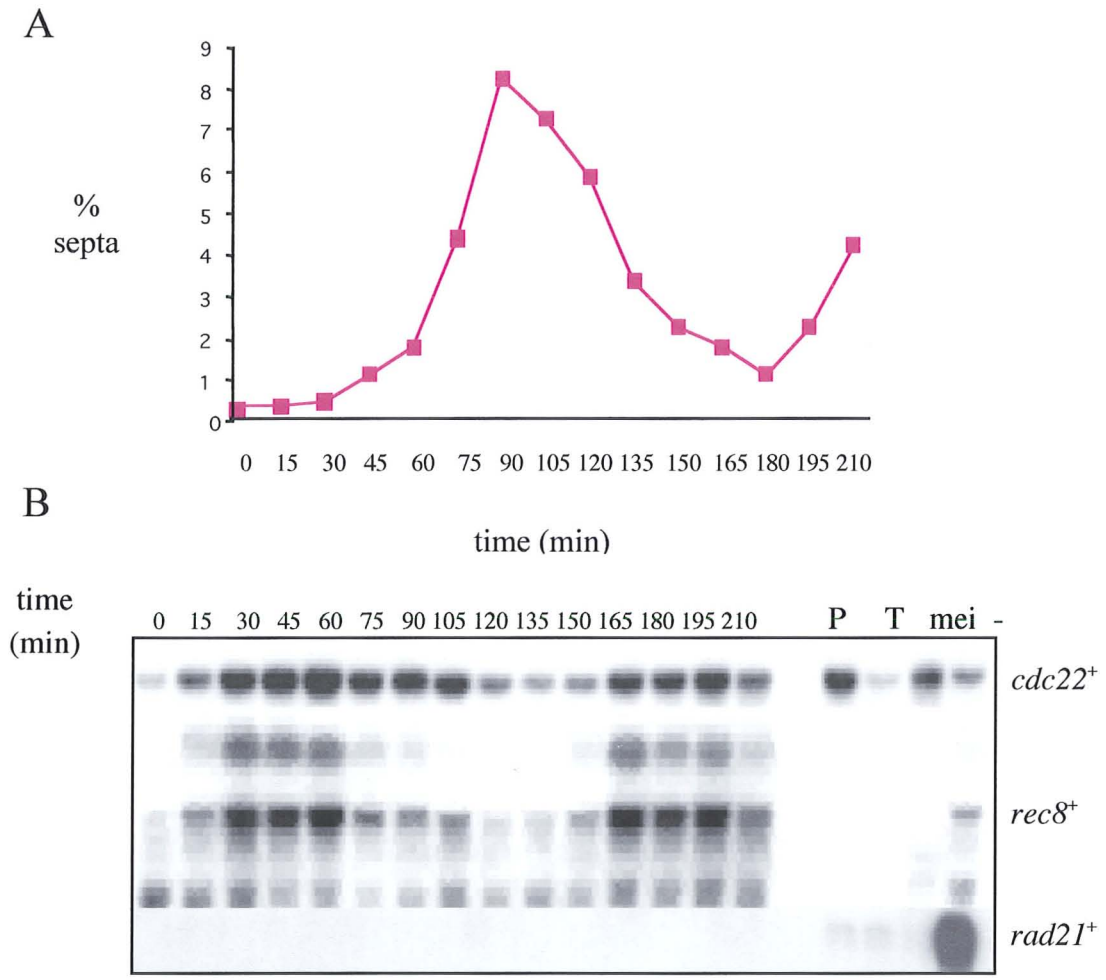


Figure 4.5 *rec8*$^+$ was Expressed in place of *rad21*$^+$ in *rad21*$^+$-Repressible Cells (Plasmid Strain). Synchronous mitosis was triggered by *cdc25-22* induced G2 block and release, and cell samples taken at 15 minute intervals. **A,** Cells were fixed in 10% formaldehyde and septation monitored by microscopy. **B,** RNA was extracted for northern blot analysis. 10 µg total RNA from each time point (0-210 minutes) was separated on a formadehyde gel by electrophoresis and transferred to a nylon membrane. Control RNA samples were also loaded; synchronous wild type mitotic *rad21*$^+$ peak (**P**) and trough (**T**), *pat1-114* synchronous wild type meiotic *rec8*$^+$ peak (**mei**), and GG 327 asynchronous cultured without thiamine (**-**). Membranes were probed for *cdc22*$^+$, *rec8*$^+$ and *rad21*$^+$ mRNAs.

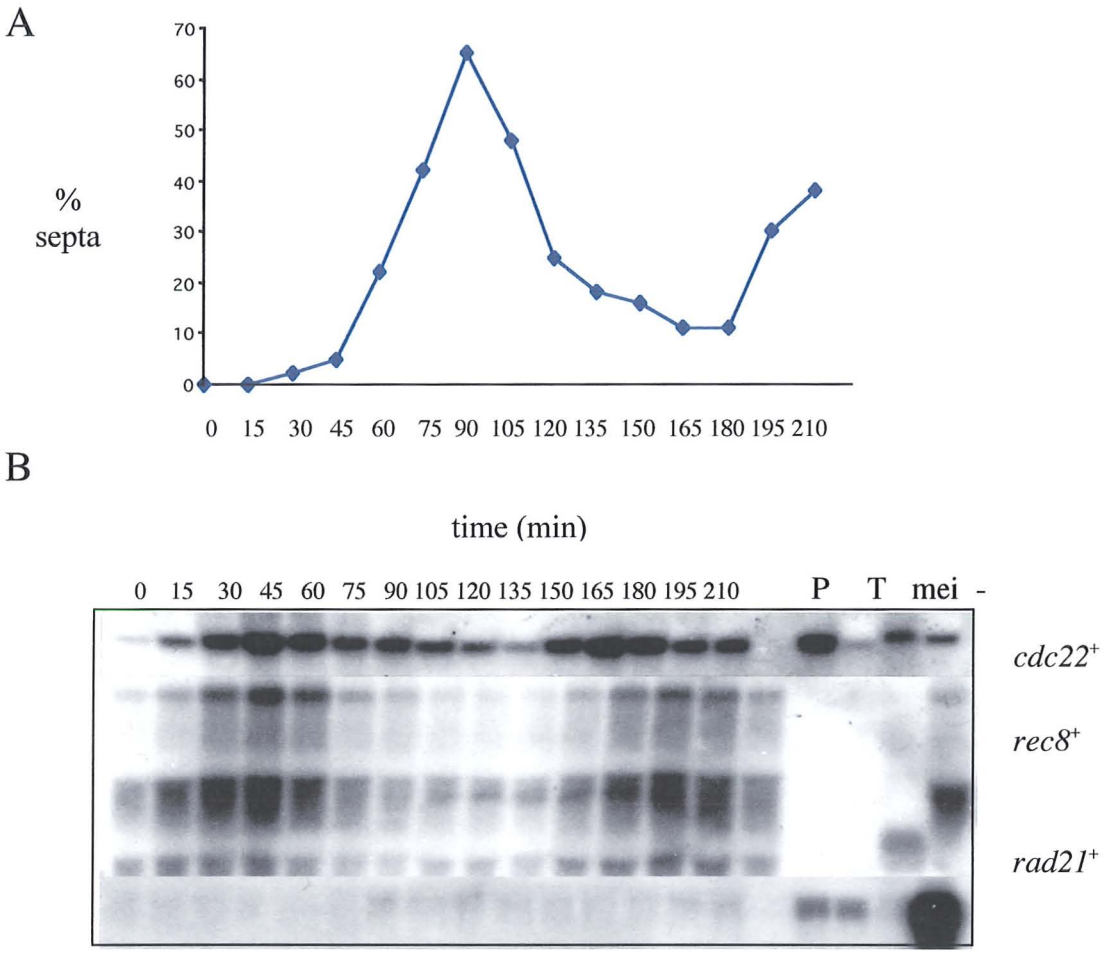
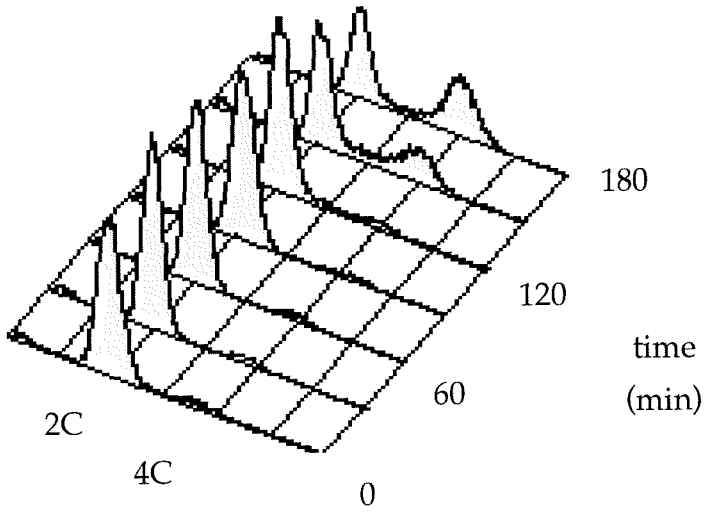


Figure 4.6 *rec8*$^+$ was Expressed in place of *rad21*$^+$ in *rad21*$^+$-Repressible Cells (Integrant Strain). Synchronous mitosis was triggered by *cdc25-22* induced G2 block and release, and cell samples taken at 15 minute intervals. **A,** Cells were fixed in 10% formaldehyde and septation monitored by microscopy. **B,** RNA was extracted for Northen blot analysis. 10 µg total RNA from each time point (0-210 minutes) was separated on a formaldehyde gel by electrophoresis and transferred to a nylon membrane. Control RNA samples were also loaded; synchronous wild type mitotic *rad21*$^+$ peak (**P**) and trough (**T**), *pat1-114* synchronous wild type meiotic *rec8*$^+$ peak (**mei**), and GG358 asynchronous cultured without thiamine (**-**). Membranes were probed for *cdc22*$^+$, *rec8*$^+$ and *rad21*$^+$ mRNAs.

A



B

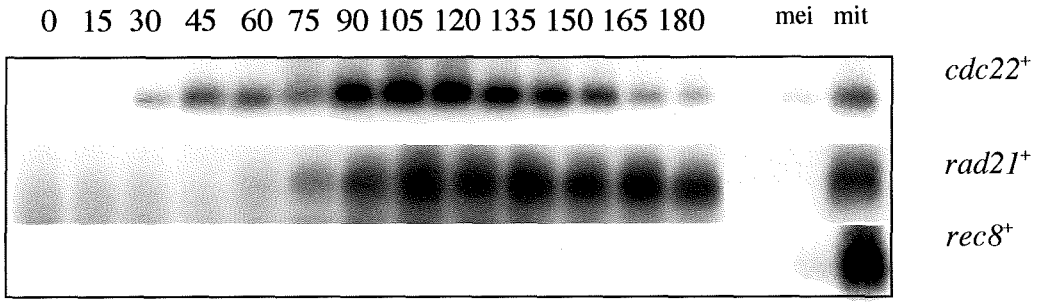
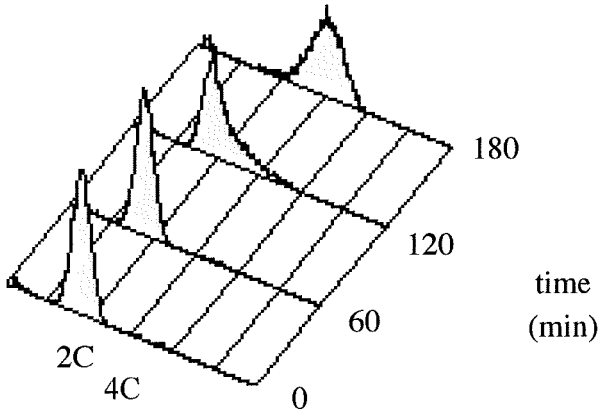


Figure 4.7 *rad21*$^+$ was Expressed in place of *rec8*$^+$ Following Induction of Meiosis (Plasmid Strain). *rec8*Δ: *rad21*$^+$ cells (GG 328) were cultured to early exponential phase (2-5 x 10^5 cells/ml) in EMM at 25°C, and transferred overnight to nitrogen-free EMM to induce G1 arrest. 0.1 M NH_4Cl was added as a nitrogen source then *pat1-114* synchronous meiosis was then triggered by transferring the culture to restrictive temperature (36°C). Cell samples were then collected at 15 minute intervals. **A**, To monitor DNA replication 1 x 10^5 cells from each time point were pelletted, fixed in 70% ethanol and labelled with 2 μg/ml propidium iodide. DNA distribution was then analysed by flow cytometry. **B**, 1 x 10^8 cells from each time point were pelletted and RNA extracted for Northern blot analysis. 10 μg total RNA from each time point was separated by electrophoresis on a formaldehyde gel then probed with ^{32}P-labelled DNA probes for *cdc22*$^+$, *rec8*$^+$ and *rad21*$^+$ transcripts.

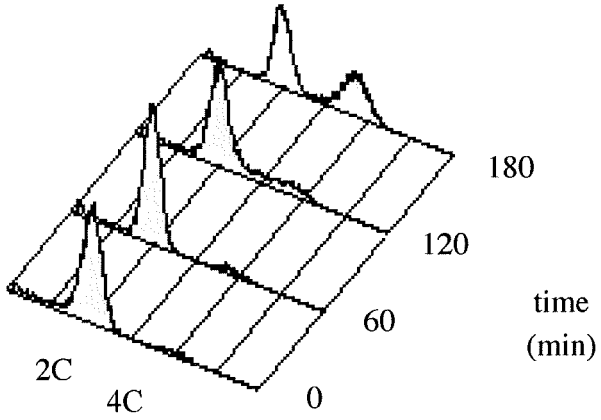
A

rec8$^+$
(GG 375)



B

*rec8*Δ
pSP1/*rec8*P: *rad21*$^+$
(GG 328)



C

rec8$^+$
pSP1
(GG 408)

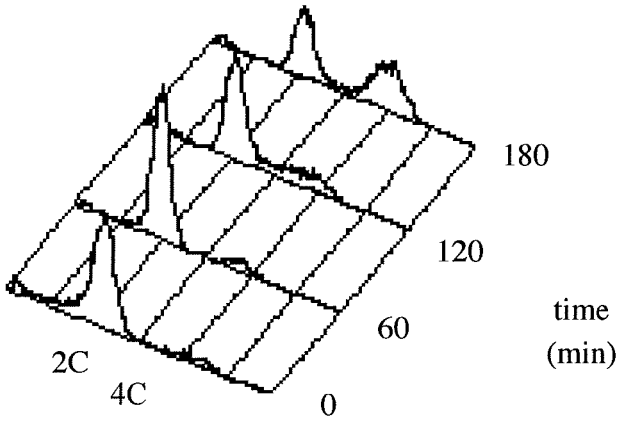


Figure 4.8 pSP1 Plasmid Causes S Phase Delay. Synchronous meiosis was induced by thermal inactivation of Pat1p. Samples (1-5 x 10^5 cells) were collected at 1 hour intervals and fixed in 70% ethanol. To follow S phase progression cells were labelled with 2 μg/ml propidium iodide, and the DNA distribution for each sample analysed by flow cytometry (Section 2.4.8). **A,** Wild type isogenic strain, no plasmid (GG 375). **B,** *rec8*Δ cells with pSP1/ *rec8*P: *rad21*$^+$(GG 328). **C,** Wild type with pSP1 plasmid (GG 408).

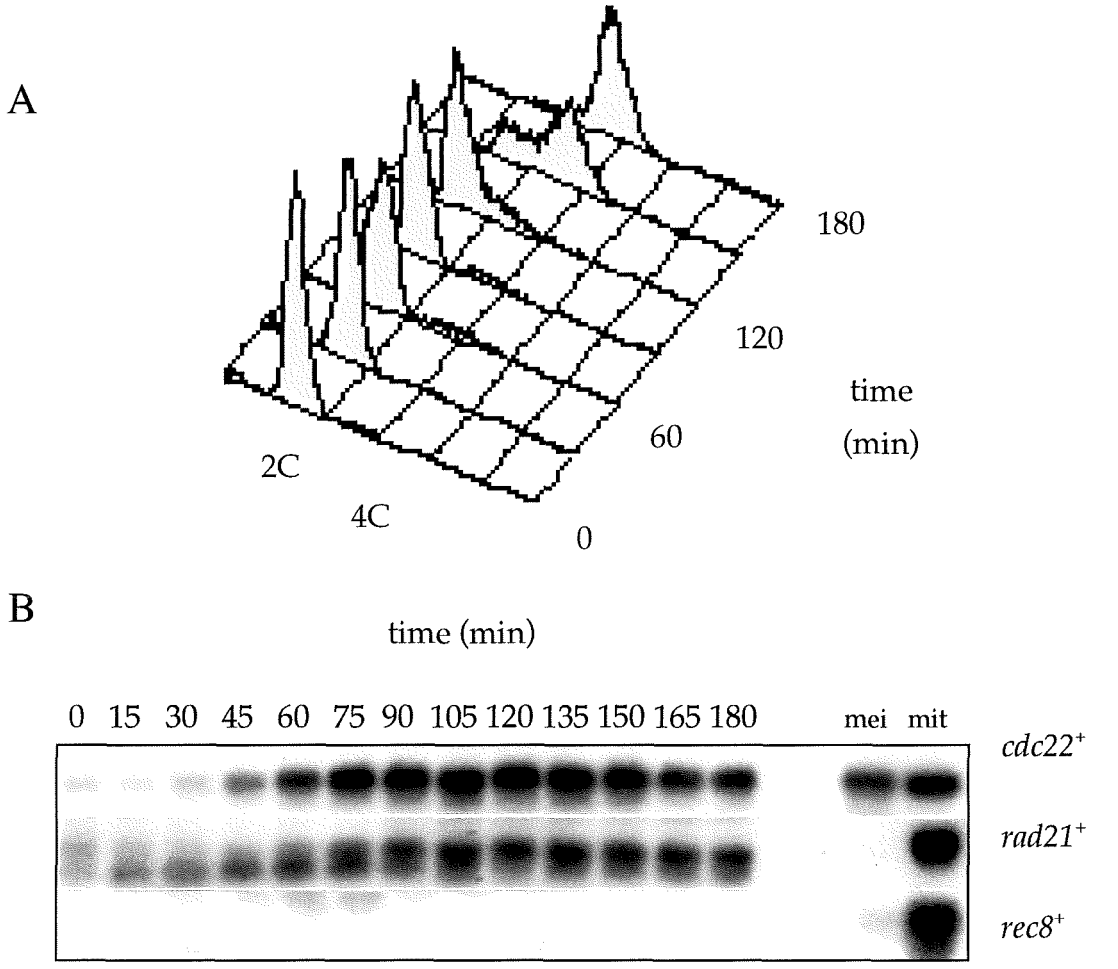


Figure 4.9 Synchronous meiotic induction in *rec8*Δ *rec8*P : *rad21*$^+$ integrant cells. *rec8*Δ *rec8*P : *rad21*$^+$ cells were cultured to early exponential phase (2-5 x 10^5 cells/ml) at 25°C in EMM, then transferred to nitrogen-free EMM (EMM-N) overnight, to arrest cells in G1 phase. *pat1-114* synchronous meiosis was then triggered by switching the culture to 36°C with 0.1M NH_4Cl added as a nitrogen source. Samples were then taken at 15 minute intervals for FACS analysis and northern blot analysis. **A**, 1 x 10^5 cells from each time point were pelletted and fixed in 70% ethanol. Cells were then labelled with 2 μg/ml propidium iodide and the progression of DNA replication measured by flow cytometry. **B**, 1 x 10^8 cells were also collected at each time point and RNA extracted for northern blot analysis. 10 μg total RNA from each sample was separated on a formaldehyde gel by electrophoresis then transferred to a nylon filter. Filters were then hybridised with ^{32}P-labelled DNA probes to detect *cdc22*$^+$, *rad21*$^+$ and *rec8*$^+$ transcripts.

Chapter 5:

Ectopically Expressed *rec8*$^{+}$ Partially Complements Rad21p Function in Mitosis

5.1 INTRODUCTION

Initial characterisations of *S. pombe* Rad21p revealed an important role for this peptide in DNA double strand break repair (Birkenbihl and Subramani, 1992, 1995). Homologous genes were soon identified in other species including *S. cerevisiae* Mcd1p, which was found to share sequence similarity with Smc1p and Smc3p, components of a multi-protein complex termed 'Condensin' required for chromosome condensation (Guacci *et al.*, 1997; Toth *et al.*, 1999; Michaelis *et al.*, 1997). Characterisation of *S. cerevisiae* Mcd1p mutants that underwent premature separation of sister chromatids during mitosis, led to the proposition of a role for Mcd1p/ Rad21p in chromosome cohesion. It is now known that Rad21p associates with other cohesins, forming part of a multi-subunit complex called Cohesin (Tomonaga et al., 2000). Rad21p also interacts genetically and physically with several other molecules involved in DNA replication, and kinetochore function (Hartsuiker *et al.*, 2001; Takeda *et al.*, 2001; Toyoda *et al.*, 2002). Indeed, it is now becoming increasingly clear that Rad21p plays a central role in the regulation of chromosome dynamics and is required for several different processes occurring during different phases of the mitotic cell cycle.

Rec8p, a meiotic homologue of Mcd1p, was first discovered in *S. pombe* (Molnar *et al.*, 1995; Watanabe & Nurse, 1999), and also later identified in *S. cerevisiae* (Klein et al., 1999). *S. pombe* Rec8p was found to be essential for normal meiotic homologous recombination, being required during several stages of this complex, and poorly understood, process in *S. pombe* (Molnar *et al.*,1995). Analysis of *rec8* mutants further revealed roles for this meiotic gene in accurate meiotic chromosome segregation and spore formation (Krawchuk *et al.*, 1999; Parisi *et al.*, 1999).

Chapter 3 describes how expression of *S. pombe* cohesins, *rad21*$^+$ and *rec8*$^+$, is cell cycle-specific i.e. *rad21*$^+$ is expressed solely during the mitotic cell cycle, while expression of *rec8*$^+$ is induced only during sexual development.

In a study published by Watanabe and Nurse (1999), it was reported that by placing *rec8*$^+$ under *nmt1*$^+$ promoter control, so that it was ectopically and constitutively over-expressed during the mitotic cell cycle, the lethal effect of a *rad21*$^+$ disruption could be partially rescued, though this was accompanied by a 20% loss of viability. In contrast, *rad21*$^+$ was unable to support sexual development in the absence of *rec8*$^+$. When *rad21*$^+$ was ectopically over-expressed during the meiotic cell cycle, in cells where chromosomal *rec8*$^+$ was disrupted, meiotic defects such as abnormal spore development and precocious separation of sister chromatids during MI were reported. These findings suggested two

possibilities: that (i) the essential function of Rad21p is partially conserved in the meiotic cohesin Rec8p, and that (ii) Rec8p has functions that are specifically required for sexual development, that are not conserved in the mitotic cohesin Rad21p.

However, it is not unusual for only distantly related genes, with dissimilar function, to partially complement each other when massively over-expressed. The aim of this study was to further investigate the functional conservation between *rad21*$^+$ and *rec8*$^+$, and to look at the specific roles of these cohesins during both vegetative growth and sexual development. This was addressed in a manner similar to that employed by Watanabe and Nurse: that is by swapping cohesins so that *rad21*$^+$ was expressed during the meiotic cell cycle and *rec8*$^+$ during the mitotic cell cycle. However, the major difference in this study was that ectopic expression of cohesins was elicited both at the appropriate time at the cell cycle, and at levels comparable to those seen physiologically. This chapter investigates the effects of swapping cohesins during the mitotic cell cycle, so that *rad21*$^+$ expression is replaced with expression of *rec8*$^+$. Chapter 6 then looks at the reciprocal situation, in which the meiotic cohesin, *rec8*$^+$, is swapped for *rad21*$^+$, and the consequences upon sexual development.

Chapter 4 described how *S. pombe* strains, termed *rad21*$^+$-repressible strains, were constructed, in which expression of the mitotic cohesin *rad21*$^+$ was regulated by the thiamine-repressible *nmt1*$^+$ promoter (*nmt1*P). By placing *rad21*$^+$ under *nmt1*P control it was possible to switch off *rad21*$^+$ expression simply by supplementing the culture media with thiamine. The ability to regulate *rad21*$^+$ expression was necessary because, as it is an essential gene, irreversible inactivation of *rad21*$^+$ would lead to cell death.

DNA constructs, in which the *rec8*$^+$ gene was placed under *rad21*$^+$ promoter control (*rad21*P: *rec8*$^+$), were then introduced into *rad21*$^+$-repressible cells, both in low copy plasmid (pSP1) and in integrating plasmid form (pJK148). The meiotic cohesin *rec8*$^+$ under *rad21*$^+$ promoter control was therefore expressed during the mitotic cell cycle, both at physiological levels, and in the correct cell cycle-regulated manner. This system was used to analyse more precisely the conservation of function between the Rad21p and Rec8p cohesins. *S. pombe* strains, primers and constructs are described in Appendices I, II and III, respectively.

5.2 LETHAL *rad21*$^+$-REPRESSION IS RESCUED BY PHYSIOLOGICAL *rec8*$^+$ EXPRESSION IN MITOSIS

As described in chapter 4, the *rad21*P: *rec8*$^+$ construct was introduced into *rad21*$^+$-repressible cells (*nmt1*P::*rad21*$^+$; GG 320) either in the low copy plasmid pSP1 (creating GG 327) or integrated into the genome in single copy using pJK148 (GG 358).

To determine if *rec8*$^+$, when expressed at physiological levels, could rescue the lethal effect of *rad21*$^+$ repression in mitosis (Birkenbihl & Subramani, 1995), *rad21*$^+$-repressible cells either with or without the *rad21*P: *rec8*$^+$ construct, were first streaked to single colonies on EMM plates. Individual colonies were then streaked both onto fresh EMM plates, and to EMM supplemented with 10 μM thiamine, to repress expression of *rad21*$^+$. Plates were then incubated at 25°C until small colonies of 20-30 cells grew. Individual colonies were then picked and streaked onto fresh EMM or EMM + thiamine plates a further 2 times to allow adequate accumulation of thiamine (Figure 5.1).

When cultured on EMM (Figure 5.1A), *rad21*$^+$-repressible cells carrying the *rad21*P: *rec8*$^+$ construct (GG 327 and GG 358), as well as those with empty vector (GG 345 and GG 346) continued to grow, with colonies appearing after 2-3 days. When cultured on EMM + thiamine to repress *rad21*$^+$ expression (Figure 5.1B), GG 345 and GG 346 failed to grow. However, when *rec8*$^+$ was expressed in place of *rad21*$^+$ in these cells, both in low copy (GG 327) and in single copy (GG 358), cells continued to grow with colonies appearing after 2-3 days. These findings confirm that, in *S. pombe*, *rad21*$^+$ is essential for vegetative growth. They also demonstrate that physiological expression of *rec8*$^+$ in place of *rad21*$^+$ was able to rescue the lethal effect of *rad21*$^+$ repression and support mitotic growth.

5.3 *rec8*$^+$ INTEGRATED IN SINGLE COPY SUPPORTS MITOTIC GROWTH IN THE ABSENCE OF Rad21p

To confirm that *rec8*$^+$, when expressed at physiological levels, was able to complement the essential function of Rad21p when it was completely absent, the *rad21*P: *rec8*$^+$ construct was integrated into a *S. pombe* strain in which the *rad21*$^+$ gene was disrupted, preventing translation of functional Rad21p protein.

Because Rad21p is essential, it was not possible to work with a *rad21*$^+$-disrupted haploid strain, in which Rad21p would be completely absent. To overcome this, a diploid strain was used, in which only one copy of *rad21*$^+$ was disrupted by the *ura4*$^+$ gene (GG 417). This

strain was viable because the *rad21*$^+$ allele was able to complement the mutant *rad21*::*ura4*$^+$allele.

The pJK148/ *rad21*P: *rec8*$^+$ construct (GB 190) was integrated into GG 417 (Figure 5.2 ii; Section 2.2.4) then sporulation was induced (Figure 5.2 iii). Colonies that grew in the absence of leucine represented cells that carried the integrated pJK148/*rad21*P: *rec8*$^+$construct (Figure 5.2 iv). To confirm that sporulation had occurred and that the isolates were indeed haploids, cells were first viewed by light microscopy, and their size compared to the known haploid (GG 1) and diploid (GG 375) *S. pombe* strains. They were then replica plated onto selective media to confirm that they were *ade6*$^-$ and *leu1*$^+$. Six positive colonies were then streaked onto fresh media plates with or without uracil, to identify those carrying the mutant *rad21*::*ura4*$^+$ allele (Figure 5.3).

All six colonies grew in the presence of uracil confirming that they were *leu1*$^+$ (Figure 5.3 top). When cultured without uracil, 3 colonies failed to grow representing cells with the *rad21*$^+$ allele. The remaining 3 colonies that did grow without uracil represented cells carrying the *rad21*::*ura4*$^+$ allele (Figure 5.3 bottom). In these cells a single copy of *rec8*$^+$ integrated into the genome was able to support vegetative growth in the complete absence of Rad21p

5.4 PHENOTYPES OF *rad21*$^+$-REPRESSIBLE CELLS

So far it was established that while physiological expression of *rec8*$^+$ in place of *rad21*$^+$ was able to support mitotic growth, there was also a significant loss of viability. The next stage in the analysis was to see if this was coupled with a specific phenotype(s), and in particular any morphological defects.

5.4.1 Mutant Morphology of *rad21*-repressible *rad21*P:*rec8*$^+$ Cells

rad21$^+$-repressible cells, carrying either empty plasmid or *rad21*P:*rec8*$^+$, were cultured to mid-exponential phase (2-5x10^6 cells/ml) in liquid EMM, either with or without 10 μM thiamine. From each culture, 10 ml of cells were pelleted by centrifugation at 3000 rpm for 5 minutes, and fixed by resuspension in 100 μl of 10% formaldehyde in dH_20. Cells were then fixed to poly-L-lysine coated slides and viewed by light microscopy.

Alternatively, *rad21*$^+$-repressible cells were viewed directly on EMM plates. However, on solid media, the mutant phenotypes of *S. pombe* cells were consistently found to take considerably longer to manifest. In liquid media abnormal morphologies were seen

after only 8 hours culture (or 2 cell divisions), while on solid media, in some cases cells had undergone more than 8 cell divisions before abnormal morphologies were seen. This is a widely accepted phenomenon on *S. pombe* though the reason for it is not known To overcome this, cells were therefore streaked onto fresh media several times, to allow accumulation of thiamine and development of the resultant phenotype. Cells were streaked onto EMM plates with or without thiamine, and incubated at 25°C for 3-5 days, until small colonies of 10-20 cells had grown. Single colonies were then re-streaked onto fresh EMM plates (with or without thiamine) a further 3 times. Coverslips were placed gently over individual colonies for microscopic examination.

Photos from both slides and plates were taken using an Olympus BX60 microscope, with a 60 x lens, connected to a Princeton Instruments digital camera. Images were collected using IPLab software installed on an Apple Macintosh computer, then copied to Adobe®Photoshop® 5.0.

As a control, the parental strain (GG 95), from which all *rad21*$^+$-repressible strains were constructed, was also examined (Figure 5.4 i). These cells had a uniform rod-shaped morphology with cells usually 20-25 μm in length. The increased length of *cdc25-22* *S. pombe* cells, when compared to wild type which are 10-15μm in length, is well documented (Fantes, 1981), and is caused by a short delay in progression from G2 into M phase, at the permissive temperature of 25°C. for this conditional mutant.

The majority of cells from *rad21*$^+$-repressible strains (*nmt1*P::*rad21*$^+$) carrying empty vector, had normal phenotypes when cultured without thiamine (Figure 5.4 ii and iv top), while as mall fraction displayed abnormal morphologies. However, when cultured in thiamine, repressing *rad21*$^+$ transcription, a heterogeneous array of mutant phenotypes were seen, with many cells grossly enlarged, particularly in length. Many of these cells had hyphal phenotypes: branching, and multiple septa were observed.

When *rec8*$^+$ under *rad21*P control was expressed in place of *rad21*$^+$ in these cells, the mutant phenotype described for *rad21*$^+$-repressed cells was abolished, and the majority of cells had a normal 25°C *cdc25-22* phenotype. However, a fraction of these cells did display the mutant phenotype described for *rad21*$^+$-repressed cells. No phenotypic difference was noted between *nmt1*P::*rad21*$^+$ strains expressing *rec8*$^+$ in integrated form (GG 358) or from a plasmid (GG 327).

5.4.2 Frequency of Abnormal Morphologies

The number of cells with abnormal morphologies, compared to those of normal appearance were counted and plotted as a histogram (Figure 5.5). Abnormal cells were typically enlarged, both in length and diameter, and some branching and multiple septation was observed. For each culture, at least 300 cells were examined and the mean values calculated.

In the parental strain (GG 95) 95% of cells were normal in appearance. Only 5% of cells displayed the *cdc25-22* mutant phenotype of grossly elongated cells, normally displayed by thermal inactivation of Cdc25-22p protein at 36°C. This phenotype was caused by cells arresting at G2 phase of the cell cycle, and therefore failing to enter mitosis and divide, but continuing to grow.

However, although physiological expression of *rec8*$^+$ was able to rescue the lethal effect of *rad21*$^+$ repression, and the majority of cells had a normal phenotype, a significant percentage of cells expressing *rec8*$^+$ in place of *rad21*$^+$ displayed the mutant phenotype described for *rad21*$^+$-repressed cells carrying empty plasmid. When *rec8*$^+$ was expressed from a plasmid (GG 327) 35% of cells displayed a mutant phenotype, while 29% of cells expressing *rec8*$^+$ in integrated form were of abnormal appearance (Figure 5.5).

5.5 VIABILITY OF *rad21*$^+$-REPRESSIBLE *rad21*P: *rec8*$^+$ CELLS

The previous section showed that expressing *rec8*$^+$ in place of *rad21*$^+$ was able to rescue the lethal effect of *rad21*$^+$ repression to support vegetative growth. To investigate this further viability assays were performed (Section 2.2.7).

Wild type *S. pombe* cells (GG 95) showed 81-83% viability, with no significant difference whether in the presence or absence of thiamine. When cultured without thiamine, 58% of *rad21*$^+$-repressible cells (*nmt1*P::*rad21*$^+$) with *rad21*P: *rec8*$^+$ in pSP1 (GG 327), were viable. Similarly, when *rad21*P: *rec8*$^+$ was integrated into *nmt1*P::*rad21*$^+$ cells (GG 358), 53% were viable. Therefore, in the absence of thiamine, when *rad21*$^+$, under *nmt1*P control, was constitutively over-expressed, a significant loss in viability was seen when compared to wild type.

When *rad21*$^+$expression was repressed by thiamine, and replaced with *rec8*$^+$ under *rad21*P control, although cells continued to grow (Figure 5.1), only 28% (GG 327) and 35% (GG 358) of cells were viable (Figure 5.7). Therefore, although physiological expression of

rec8$^+$ restores the viability of *rad21*$^+$-repressed cells, it is only restored to a third of the wild type value.

5.6 DIVISION TIMES OF *rad21*$^+$-REPRESSIBLE *rad21*P: *rec8*$^+$ CELLS

S. pombe mutants can exhibit an increase, or more rarely a decrease, in the time it takes to complete the cell division cycle. This is usually due to the activation of checkpoints, mechanisms that delay cell cycle progression at specific stages, allowing the cell time to overcome any shortcomings such as incomplete DNA replication. To examine whether expressing *rec8*$^+$ in place of *rad21*$^+$ altered the time taken to complete the cell division cycle, the growth rates of *rad21*$^+$-repressible (*nmt1*P::*rad21*$^+$) cells were determined.

Cells were cultured in liquid media with and without thiamine and cell counts taken, using a Beckton Dickinson Coulter counter, at 1hour intervals throughout the period of exponential growth. Cell counts were then plotted against time to generate growth curves (Figure 5.8A).

When cultured without thiamine, and therefore *rad21*$^+$ was being expressed, the growth curves of both *rad21*$^+$-repressible strains, GG 327 and GG 358, were comparable to that of the parental strain, with cultures growing at similar rates.

When *rad21*$^+$ was repressed by culture in thiamine, and replaced with *rec8*$^+$being expressed from a plasmid (GG 327), this did not lead to any notable difference in the growth rate of cells, when compared to culture without thiamine. Similarly, in *rad21*$^+$-repressable cells expressing *rec8*$^+$ in integrated form (GG 358), the growth rates of cultures in which *rad21*$^+$ was repressed, when compared to cultures in which *rad21*$^+$ was expressed, were very similar to eachother, and also to those of GG 327 and of the *rad21*$^+$ parental strain (GG 95). Repression of *rad21*$^+$ in cells carrying empty vector (pJK148; GG 346), and therefore not expressing *rec8*$^+$, failed to grow at all, further confirming that *S. pombe rad21*$^+$ is essential for vegetative growth. These experiments were performed at least 3 times for each strain. Using the same data, the generation times of each strain were also calculated as described in section ***. This revealed more precisely any differences in cell cycle lengths (Figure 5.8B).

rad21$^+$-repressible strains carrying empty plasmid pSP1 (Figure 5.8B i; GG 345) or pJK148 integrated (Figure 5.8B iii; GG 346), had generation times of 240 minutes when cultured in EMM - thiamine at 25°C (Figure 5.8B; 'ON'), the same as that reported for wild type *S. pombe* strains cultured in minimal media. When *rad21*$^+$ transcription was repressed

in these cells (Figure 5.8B 'OFF"), and with no *rec8*$^+$ being transcribed, these strains stopped growing altogether.

When *rec8*$^+$ expressed from a plasmid and *rad21*$^+$ was being transcribed (GG 327; Figure 5.8B ii; ON), these cells had division times of 240 minutes. However, when *rad21*$^+$ was repressed (Figure 5.8B ii; 'OFF'), an increase in generation times to 258 minutes was seen, representing a lengthening of the cell division cycle by 7.5%.

When *rad21*$^+$ was being transcribed and *rec8*$^+$ expressed in integrated form (GG 358; Figure5.8B iv 'ON'), these cells had generation times of 258 minutes. These generation times were increased by 5% to 270 minutes with repression of *rad21*$^+$ (Figure 5.8B iv; 'OFF').

Therefore, in both strains expressing *rec8*$^+$ in place of *rad21*$^+$, a small but significant increase was seen in generation times when compared to the same strains expressing *rad21*$^+$.

5.7 NO S PHASE DELAY IN *rad21*-REPRESSIBLE *rad21*P: *rec8*$^+$ CELLS

Transcription of both *rad21*$^+$ and *rec8*$^+$ cohesins is induced during G1-S phase. *S. pombe rec8*$^+$ must be expressed during pre-meiotic S phase to prevent meiotic defects, and there is evidence that in *S. cerevisiae*, deleting *REC8* leads to a shortening of S phase length by ~10% (Cha *et al.*, 2000). *rad21*$^+$ has also been shown to interact genetically with *hsk1*$^+$ and *eso1*$^+$, both of which have roles in DNA replication (Takeda *et al.*, 2001; Tanaka *et al.*, 2001). Taken together, these findings indicate that Rad21p and Rec8p cohesins have S phase functions.

In an asynchronous population of wild type *S. pombe* cells, flow cytometry (FACS) analysis of the DNA distribution reveals a single peak in DNA content of 2C (Figure 5.9A, B ii). This is because following DNA replication each daughter cell has a 2C DNA content. This is halved to1C during chromosome segregation at mitosis but 1C daughter cells remain joined until S phase, when cytokinesis is completed. As a result, *S. pombe* cells at any point in the cell cycle have a 2C DNA content. However, if there is an S phase delay, cytokinesis is completed before DNA replication and an additional 1C peak in DNA content can be seen.

The previous Section demonstrated a 5-7.5% increase in cell cycle length when *rad21*$^+$ expression was replaced with *rec8*$^+$. Flow cytometry was therefore used to determine if this increase was caused by a delay in S phase progression (Section 2.4.8). Cells were first cultured to mid-exponential phase in EMM with or without thiamine. Cells from 1 ml of

culture (2-5x 10^6 cells) were pelleted and fixed in 70% ethanol then labelled with 2 μg/ml propidium iodide. The DNA distribution of these cells was then analysed by flow cytometry.

The *cdc10*$^+$ gene encodes a key component of the DSC1 transcription factor that is required for the transcription of several S phase genes. The *cdc10-129* mutant after temperature arrest at 36°C for 2 hours shows an S phase arrest in some cells, shown by FACS as a 1C peak in DNA content. A *S. pombe cdc10-129* mutant (GG 27) was therefore used as a standard for 1C and 2C DNA peaks (Figure 5.9B i). Analysis of a wild type *S. pombe* strain (GG 1) revealed a single 2C peak in DNA content.

In both *rad21*$^+$-repressible strains, when *rad21*$^+$ was expressed in addition to *rec8*$^+$ (Figure 5.9 iii, iv; green lines), a single 2C peak in DNA content was seen. Furthermore, when *rad21*$^+$ expression was repressed, replacing it with physiological expression of *rec8*$^+$, either from a plasmid (GG 327; Figure 5.9B iii) or in integrated form (GG 358; Figure 5.9B iv), did not lead to S phase delay.

5.8 DISCUSSION

Rad21p has several roles during the mitotic cell cycle. During G1-S phase Rad21p binds to chromosomes, and its association at the centromeric region is important to ensure bipolar attachment of sister chromatids, and equational chromosome segregation during mitosis. Rad21p is known to have a role in DNA damage repair and has also been implicated in DNA replication, possibly forming part of a mechanism that both monitors replication, and activates the repair of any replication defects that arise. Also, Rad21p positioned along chromosome arms is likely to function in association with Condensin, a multi-protein complex that brings about compaction of chromatin, making chromosome dynamics more manageable for the cell.

This chapter was concerned with investigating the mitotic-specific roles of Rad21p. To address this, *S. pombe* strains were created in which Rad21p was replaced with the meiotic cohesin Rec8p, and the effects on the mitotic cell cycle analysed.

5.8.1 Rec8p Partially Rescues Lethal *rad21*Δ Phenotype

5.8.1.1 CONSTITUTIVE OVER-EXPRESSION OF *rad21*$^+$

When *rad21*$^+$ was constitutively over-expressed, cells were able to grow and divide, and upon microscopic analysis were found to be wild type in appearance. This was the case both in the presence and absence of Rec8p. These findings allow two conclusions. First they

show that when *rad21*$^+$ loses its periodic expression pattern, being expressed constitutively throughout the cell cycle and at levels up to 50-fold higher than wild type, cell cycle arrest was not observed. Secondly, they show that ectopic expression of the meiotic-specific cohesin *rec8*$^+$ during the mitotic cell cycle, also did not lead to cell cycle arrest.

5.8.1.2 MORPHOLOGY OF Rad21p-DEPLETED CELLS

In the absence of Rad21p, cells stopped dividing and cell populations were made up of a heterogeneous array of mutant phenotypes. Rad21p-depleted cells were enlarged, both in diameter but particularly in length, with some cells several times longer than the *rad21*$^+$ parental strain. Some cells also had branching, while others were very small in length. This phenotype confirms that Rad21p is essential for vegetative growth.

5.8.1.3 PHENOTYPE OF *rad21*$^+$-REPRESSIBLE *rad21*P: *rec8*$^+$ CELLS

Strikingly, when Rad21p was replaced with the meiotic-specific cohesin Rec8p, expressed both at the appropriate time in the cell cycle and at physiological levels, the mutant phenotype described above was almost abolished. These cells continued to grow and divide, and the majority of cells were wild type in appearance. This was seen in *S. pombe* strains in which *rad21*$^+$ expression was repressed, as well as in cells completely lacking Rad21p due to a chromosomal *rad21*$^+$ deletion. This demonstrated that Rec8p was able to rescue the lethal phenotype of *rad21*$^+$ repression, supporting mitotic growth. These data suggest that the essential mitotic function of Rad21p is conserved in Rec8p.

However, a significant percentage of these cells displayed the morphological defects described above for Rad21p-depleted mutants. One possible explanation is that the abnormal cells represented those that had lost plasmid DNA carrying the *rad21*P: *rec8*$^+$ construct. To determine the proportion of cells that retain each plasmid, successful transformants could be re-streaked to YE then replica plated to *leu*$^-$ plates, and the percentage of colonies continuing to grow calculated. However, one of the *rad21*$^+$-repressible strains had *rad21*P: *rec8*$^+$ integrated into the genome, to prevent plasmid loss. Yet these cells had a similar percentage of cells with abnormal morphologies when compared to those carrying *rad21*P: *rec8*$^+$ on a plasmid. Furthermore, one would expect that if plasmid loss were the cause, these cells would have the distinctive small, rounded up stationary phase morphology of starved *S. pombe* cells, due to loss of the plasmid nutritional gene. Further arguing against plasmid loss being responsible for this phenotype, it was noted that on solid media, *rad21*Δ *rad21*P: *rec8*$^+$

cells had divided up to 8 times before mutant phenotypes were seen. If nutrient deficiency were the cause, cells would be expected to enter stationary phase well before 8 divisions.

5.8.1.4 EXPLAINING *rad21*Δ *rad21*P: *rec8*$^+$ PHENOTYPE

A more reasonable explanation for the high percentage of *rad21*Δ *rad21*P: *rec8*$^+$ cells exhibiting the *rad21*$^+$-repressed phenotype, is that the ability of Rec8p to rescue Rad21p depletion was only partial, and that Rad21p possesses functions that could not be complemented by the meiotic cohesin Rec8p. However, why the majority of cells are wild type in appearance while some display the phenotype of Rad21p-depleted cells is unclear.

One possibility is that Rec8p is able to carry out some mitotic functions of Rad21p but not others. As these *rad21*Δ *rad21*P: *rec8*$^+$ cells progress through the cell division cycle, minor defects that arise due to the absence of Rad21p, or that arise spontaneously and cannot be rectified without Rad21p, would be tolerated. However, these minor defects would be perpetuated by repeated rounds of cell division, and eventually trigger a checkpoint causing cell cycle arrest. Because cell cycle-arrested cells continue to grow without division, this would explain the enlarged phenotype of Rad21p-depleted cells. Because Rec8p is able to partially complement Rad21p functions, defects would arise less frequently than in Rad21p-depleted cells and more cells would have wild type morphology.

5.8.2 Expression of Rec8p in place of Rad21p leads to a Loss of Viability

5.8.2.1 *rad21*$^+$-REPRESSIBLE CELLS SHOW A LOSS OF VIABILITY WITHOUT REPRESSION OF Rad21p

All *rad21*$^+$-repressible cells showed a pronounced loss of viability. Interestingly, a ~50% loss of viability was seen even when Rad21p was present. This could be due to several reasons. Firstly, *rad21*$^+$ was regulated by the *nmt1*$^+$ promoter so that *rad21*$^+$ transcript levels were up to 50-fold higher than they would be in wild type *S. pombe* (Maundrell, 1990). Secondly, *rad21*$^+$ lost its periodicity and was expressed throughout the cell cycle. Such profound changes in the transcriptional regulation of an essential gene may have had an adverse effect on the cell. Another factor that might have affected the viability of *rad21*$^+$-repressible strains was that, in placing *rad21*$^+$ under *nmt1*$^+$ promoter control, as described in Section 4.3.1, 6 kb of foreign DNA had been inserted into the genome. This might have

caused difficulties with chromosome dynamics, which can in turn lead to chromosome loss. Alternatively, the DNA exchange event that occurred when placing *rad21*$^+$ under *nmt1*$^+$ promoter control could potentially have resulted in the loss of an important DNA fragment. All of these factors may have contributed to the loss of viability seen in these cells.

5.8.2.2 FURTHER LOSS OF VIABILITY WHEN Rad21p IS REPLACED WITH Rec8p

When Rad21p was repressed and *rec8*$^+$ expressed in its place, despite a clear rescue of the lethal *rad21*$^+$-repressed phenotype, there was a further loss of viability. Only ~30% of cells grew to a form sizeable colonies. When this is considered along with the high percentage of cells with abnormal morphology, it indicates that these cells harbour lethal defects that are caused either by a Rad21p-deficiency, or by the presence of the meiotic cohesin Rec8p.

Rec8p only partially complements the essential function of Rad21p, and the more cell division cycles that occur would see an accumulation of defects and a consequent further loss of viability. Alternatively, or in addition, the presence of Rec8p may have a harmful effect on the cell division cycle. This meiotic cohesin has roles in meiotic-specific processes such as homologous recombination, spore formation and reductional chromosome segregation. Its presence might therefore perturb the normal mitotic cell cycle, for example by causing an increase in the frequency of recombination events, or an altered programme of cytokinesis and/ or chromosome segregation.

5.8.3 Mitotic Cell Cycle Progression of Rad21p-Deficient Cells

5.8.3.1 GROWTH RATES OF *rad21Δ rad21*P: *rec8*$^+$ CELLS

When Rad21p was replaced with *rec8*$^+$ expression, the time taken to progress through the mitotic cell cycle was increased from 240 minutes to 258 minutes in the plasmid strain (GG 327), and from 258 to 270 minutes in the integrant strain. However, these times may not actually reflect increases in cell cycle length. Instead, apparent lower rates of cell cycle progression may have been caused by a percentage of these cells dying, as discussed in the previous Section. Growth rates were assessed over 8 hours (approximately two cell cycles), and only a small amount of cell death would occur over this short period. However, it might account for the subtle increases in growth rate seen in these strains.

5.8.3.2 POTENTIAL SOURCES OF MITOTIC CELL CYCLE DELAY

5.8.3.2.1 *Abnormal Kinetochore Orientation*

The increased growth rates may be due to difficulties encountered in cell cycle progression. For example, one role of Rad21p is in the bi-orientation of sister kinetochores, so that they capture microtubules emanating from opposite spindle poles, and which is important for accurate sister chromatid separation (Goshima and Yanagida, 2000; Tanaka *et al.*, 2000; He *et al.*, 2000). Rec8p instead orientates sister kinetochores so they face the same spindle pole, so that a reductional segregation takes place during the first meiotic division (Watanabe & Nurse, 1999). Replacing Rad21p with Rec8p may therefore cause defects in kinetochore orientation. This could affect kinetochore attachment to microtubules, which might in turn activate the spindle checkpoint causing cell cycle delay. Defects in kinetochore orientation may also alter the pattern of chromosome segregation, which could lead to delays in progression through M phase.

5.8.3.2.2 *Defects in DNA Damage Repair*

Rad21p is known to be required for the repair of DNA damage caused by environmental agents, defects in replication, or chromosome fragment loss due to abnormal chromosome segregation. The increased generation times for *rad21*Δ *rad21*P: *rec8*$^{+}$ cells may be due to Rec8p not being able to perform this function of Rad21p. If DNA damage checkpoints were activated, *rad21*Δ *rad21*P: *rec8*$^{+}$ cells might not be able to repair the DNA damage, and this would lead to cell cycle delay after which cells would either progress on to the next cell cycle stage and perpetuate the DNA damage, or die.

5.8.3.2.3 *Defects in DNA Replication*

Another consideration is that the absence of Rad21p could make cells more susceptible to certain types of damage. It has been suggested that DNA replication is closely linked to the establishment of cohesion. It is therefore a possibility that exchanging Rad21p for the meiotic cohesin Rec8p might have an effect on the replication process. Furthermore, one might predict that if replication defects occurred, replacing Rad21p with Rec8p could also lead to problems in their repair.

If replication defects occur this might delay S phase progression, which would give time for their repair. Yet exchanging Rad21p for Rec8p did not cause S phase delay.

However, this does exclude the possibility that replication defects were present. In fact, the major replication and DNA damage checkpoints are in late G2, and so this is when defects are more likely to be detected. And as discussed above, an extended G2 delay would account for the grossly enlarged phenotype seen in Rad21p-deficient mutants.

5.9 SUMMARY

To summarise the findings in this chapter:

1. Repression of Rad21p was lethal.
2. Ectopic expression of *rec8*$^{+}$, at the appropriate time and at physiological levels, was able to support mitotic growth in the absence of Rad21p, with the majority of these cells being wild type in appearance.
3. Replacing Rad21p with Rec8p led to a ~50% loss of viability.
4. Replacing Rad21p with Rec8p did not cause a significant increase in total cell cycle length, nor an S phase delay.

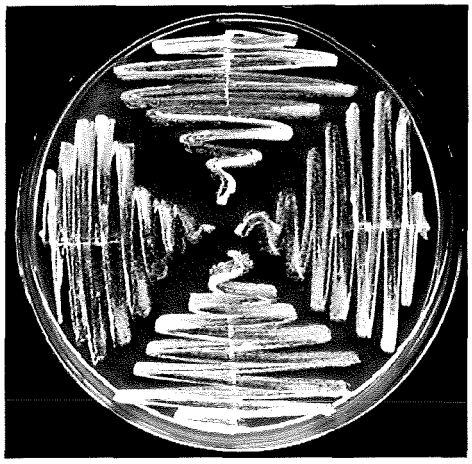
These findings suggest that the mitotic function of Rad21p is partially conserved in Rec8p. The following chapter goes on to further investigate overlapping functions between Rad21p and Rec8p, by replacing Rec8p with Rad21p during meiosis.

A

-Thiamine

pSP1
*rad21*P: *rec8*$^+$
(GG 327)

pJK148
(GG 346)



pJK148
*rad21*P: *rec8*$^+$
(GG 358)

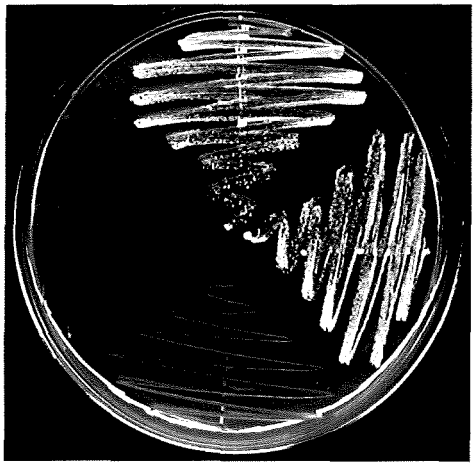
pSP1
(GG 345)

B

+ Thiamine

pSP1
*rad21*P: *rec8*$^+$
(GG 327)

pJK148
(GG 346)



pJK148
*rad21*P: *rec8*$^+$
(GG 358)

pSP1
(GG 345)

Figure 5.1 Physiological Expression of *rec8*$^+$ rescues Lethal *rad21*$^+$ Repression. *rad21*$^+$-repressible cells were grown on minimal media plates either with, or without thiamine. **A,** In the absence of thiamine (*nmt1*P::*rad21*$^+$ 'ON'), *rad21*$^+$-repressible cells, expressing *rec8*$^+$ in place of *rad21*$^+$, were able to grow. This was also seen for *rad21*$^+$-repressible strains carrying empty plasmids pSP1 and pJK148. **B,** When the essential *rad21*$^+$ gene was repressed by streaking onto plates supplemented with 10 μM thiamine (i.e. *nmt1*P:*rad21*$^+$ 'OFF'), cells carrying empty plasmids pSP1 or pJK148 were no longer able to grow. However, physiological expression of the meiotic cohesin *rec8*$^+$ in place of *rad21*$^+$ rescued the lethal effect of *rad21*$^+$ repression, and these cells continued to grow. This was seen both for the non-integrating (pSP1/ *rad21*P: *rec8*$^+$; GG 327) and integrating plasmid (pJK148/ *rad21*P: *rec8*$^+$; GG 358) strains.

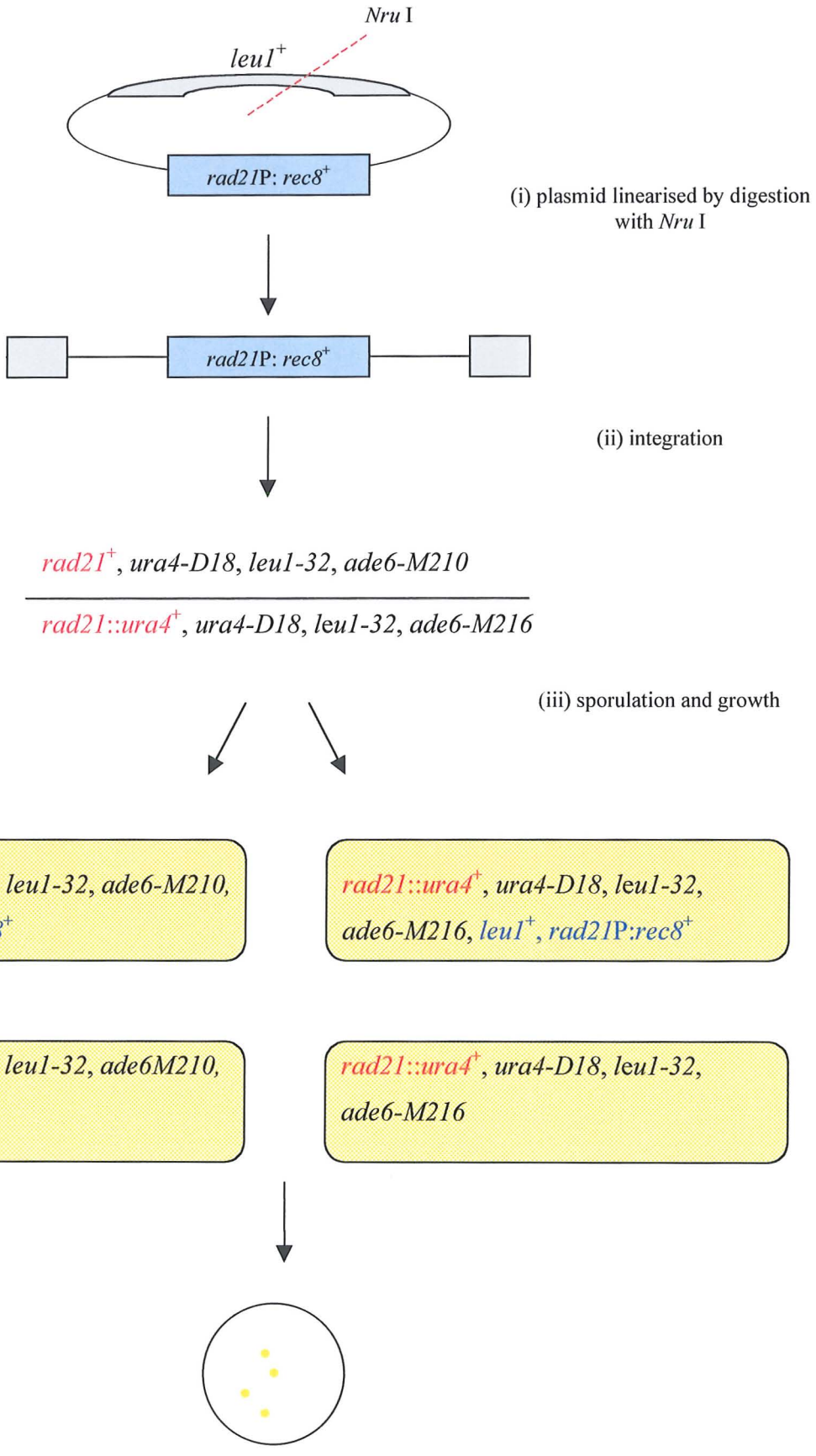
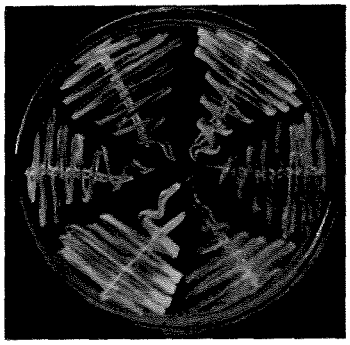

Nru I
leu1+
rad21P: rec8+
(i) plasmid linearised by digestion with Nru I
rad21P: rec8+
(ii) integration
rad21+, ura4-D18, leu1-32, ade6-M210
rad21::ura4+, ura4-D18, leu1-32, ade6-M216
(iii) sporulation and growth
rad21+, ura4-D18, leu1-32, ade6-M210, leu1+, rad21P:rec8+
rad21::ura4+, ura4-D18, leu1-32, ade6-M216, leu1+, rad21P:rec8+
rad21+, ura4-D18, leu1-32, ade6M210,
rad21::ura4+, ura4-D18, leu1-32, ade6-M216
(iv) selection of leu+ colonies

Figure 5.2 Integrating *rad21*P: *rec8*$^+$ into *rad21*Δ Cells. (i) The *rad21*P: *rec8*$^+$ construct in pJK148 (GB 190) was first linearised by digestion with *Nru* I, a unique restriction site within the *leu1*$^+$ gene. (ii) This was then integrated, by homologous recombination, into the *leu1-32* locus of a diploid fission yeast diploid strain, in which one copy of *rad21*$^+$ was disrupted by the *ura4*$^+$ gene (*rad21*::*ura4*$^+$/ *rad21*$^+$; GG 417), using the lithium acetate method of DNA uptake (materials and methods). (iii) Cells were then sporulated by culture at 25°C on minimal media (EMM + adenine + uracil), and haploids carrying the *rad21*P: *rec8*$^+$ construct identified by growth in the absence of leucine (iv). Integrated DNA is shown in blue.

- uracil



+ Uracil

Figure 5.3 *rec8*$^+$ integrated in single copy rescues lethal effect of a *rad21*$^+$ disruption. Linearised pJK148/*rad21*P:*rec8*$^+$ construct (GB 190) was integrated into a diploid fission yeast strain in which one copy of *rad21*$^+$ was disrupted by *ura4*$^+$ (*rad21*::*ura4*$^+$; GG 417) These diploids were then triggered to undergo sporulation by seeding onto minimal media. Colonies of cells that had successfully taken up the pJK148/ *rad21*P: *rec8*$^+$ construct were identified by growth in the absence of leucine. Positive isolates were also viewed by light microscopy to confirm that sporulation had occurred to form haploids. To identify any *leu1*$^+$ (and therefore pJK148/*rad21*P: *rec8*$^+$) that also carried the *rad21*::*ura4*$^+$ disruption, six haploid colonies were streaked onto minimal media either with (top) or without (bottom) uracil. *rad21*::*ura4*$^+$ colonies were identified by growth in the absence of uracil.

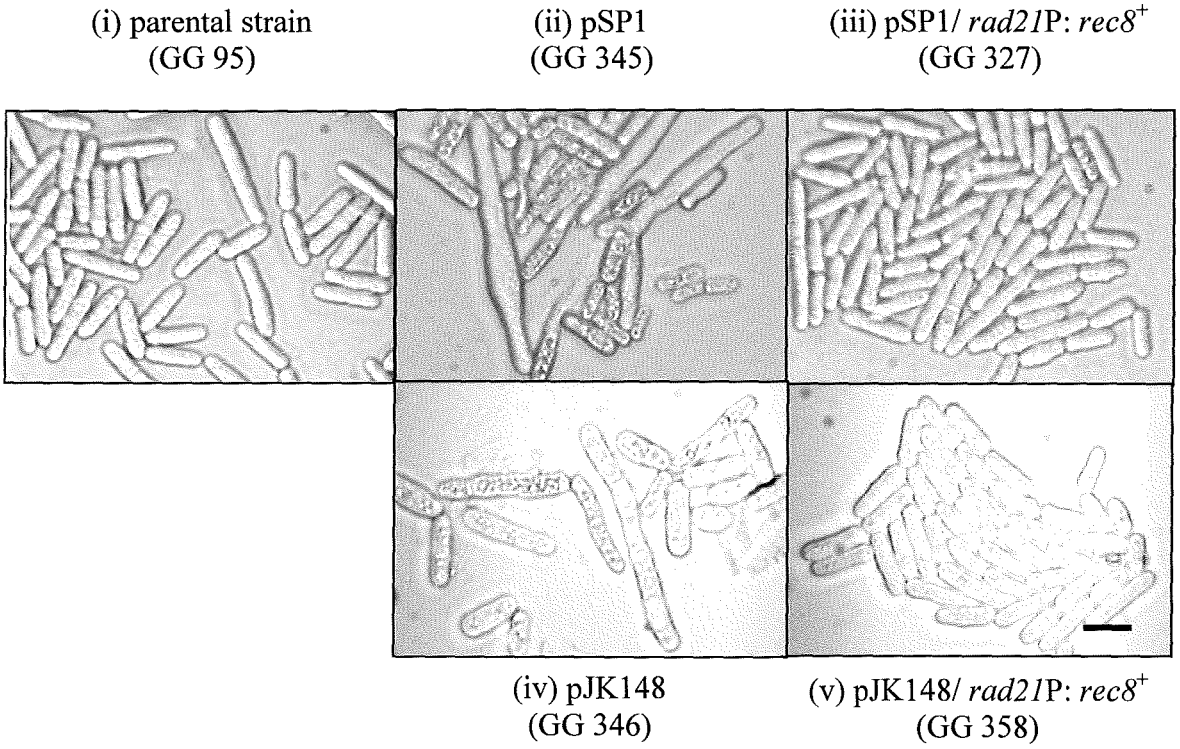


Figure 5.4 Phenotypes of *rad21*$^+$-Repressible Cells. *rad21*$^+$-repressible strains, carrying empty pSP1 (ii; GG 345), integrated pJK148 (iv; GG 346), pSP1/ *rad21*P: *rec8*$^+$ (iii; GG 327), or integrated pJK148/ *rad21*P: *rec8*$^+$ (v; GG 358), were cultured to exponential phase in liquid EMM with 10 μM thiamine. Cells were pelleted and fixed in 10% formaldehyde, then mounted onto glass slides to be viewed by phase contrast microscopy. Small colonies of 10-20 cells growing on plates were viewed directly by phase contrast microscopy. Bar = 10 μm.

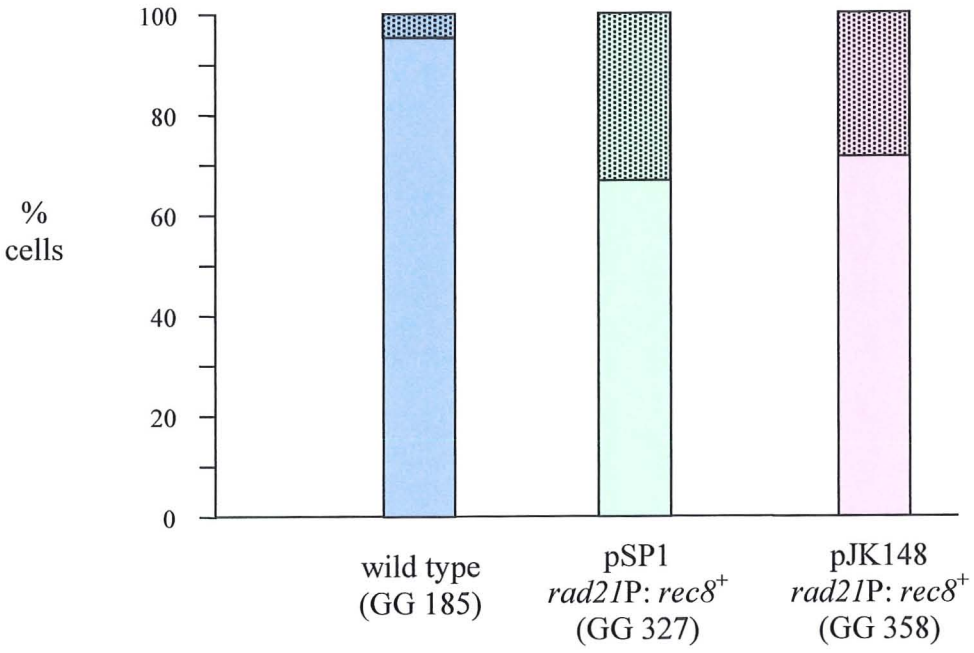


Figure 5.5 Percentage of *rad21*$^+$-Repressible Cells with Abnormal Morphology. Aliquots of *rad21*$^+$-repressible cells, from liquid cultures supplemented with 10 μM thiamine, were fixed in 10% formaldehyde and viewed by phase contrast microscopy. The ratios of cells with abnormal (shaded areas) versus normal morphology (unshaded areas) were then plotted for each strain.

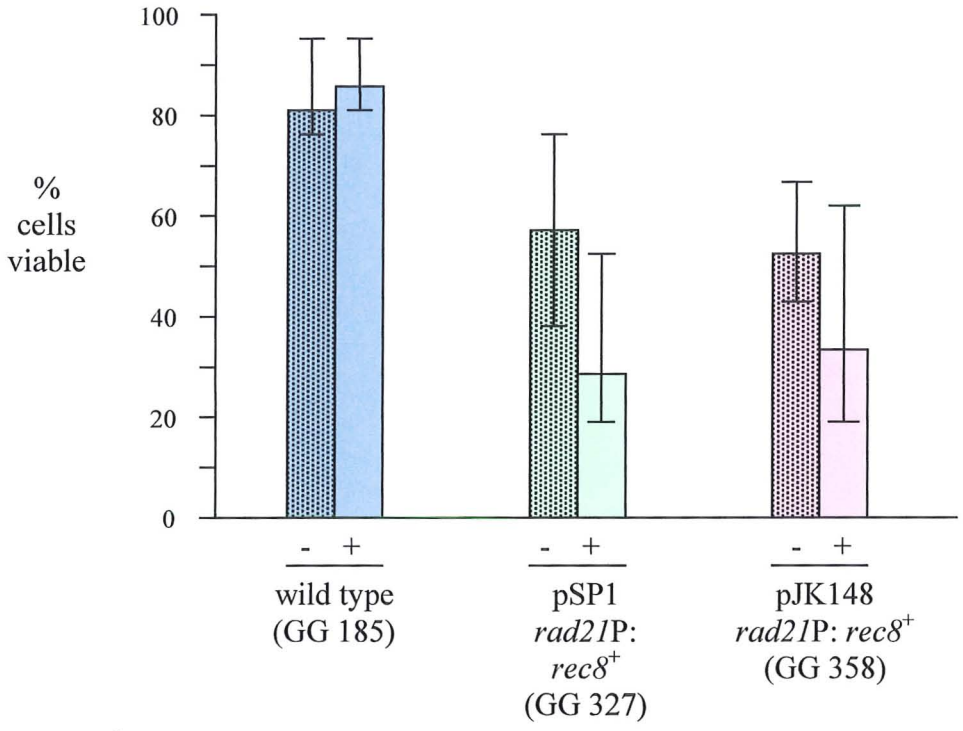
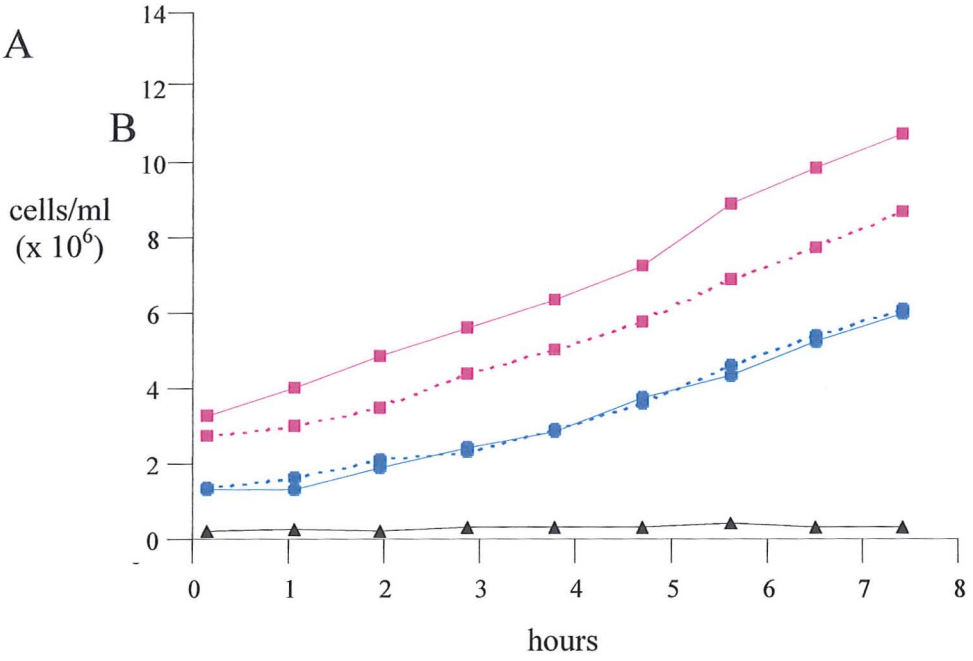


Figure 5.7 Expressing *rec8*$^+$ in place of *rad21*$^+$ Leads to a Loss of viability. Cells were grown to mid-log phase in liquid media, either with (+) or without (-) 10 μM thiamine. The exact concentration of each culture was determined using a Coulter counter. Cells from 1 ml of culture were pelleted by centrifugation then resuspended to a final concentration of 1000 cells/ml. Cells from each culture were then seeded at serial dilutions onto minimal media plates, either with or without thiamine. Plates were incubated at 25° C for 5 days, and the number of colonies, each representing growth of a single cell, was counted. Viabilities were then calculated as the percentage of colonies that grew compared to the number of colonies expected, and then plotted as a histogram. Each bar represents an experiment performed at least three times.

A

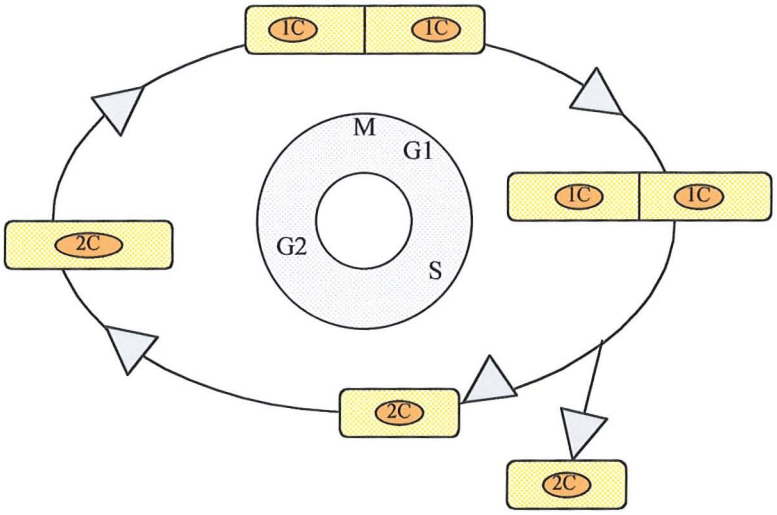


B

	*nmt1*P::*rad21*$^+$	
	ON	OFF
(i) pSP1 (GG 375)	240	no growth
(ii) pSP1 *rad21*P: *rec8*$^+$ (GG 327)	240	258
(iii) pJK148 (GG 346)	240	no growth
(iv) pJK148 *rad21*P: *rec8*$^+$ (GG 358)	258	270

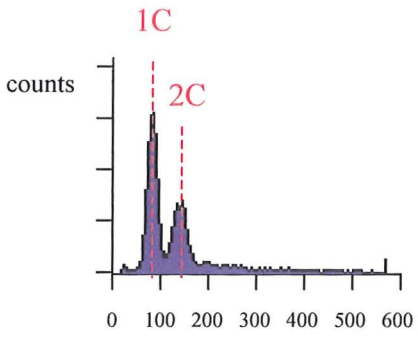
Figure 5.8 Growth Rates of *rad21*$^+$-Repressible Cells. *rad21*$^+$-repressible strains (*nmt1*P::*rad21*$^+$) were cultured in liquid minimal media, with or without 10 μM thiamine. Cell counts were then taken at 1 hour intervals throughout the period if exponential growth. **A,** Cell counts were plotted against time to compare the growth rates of each strain; black line: *nmt1*P::*rad21*$^+$ (GG 320); pink lines: *nmt1*P::*rad21*$^+$, pJK148/ *rad21*P: *rec8*$^+$ (GG 358); blue lines: *nmt1*P::*rad21*$^+$, pSP1/ *rad21*P: *rec8*$^+$ (GG 327); continuous lines: + thiamine; dashed lines: - thiamine. **B,** The generation times of *nmt1*P::*rad21*$^+$ strains were calculated using an equation as described in the text.

A

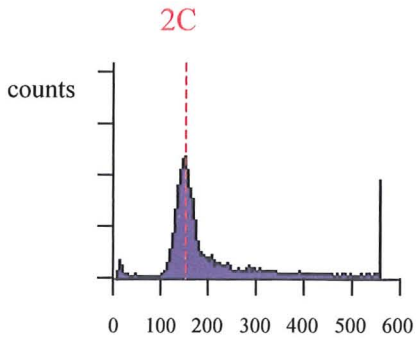


B

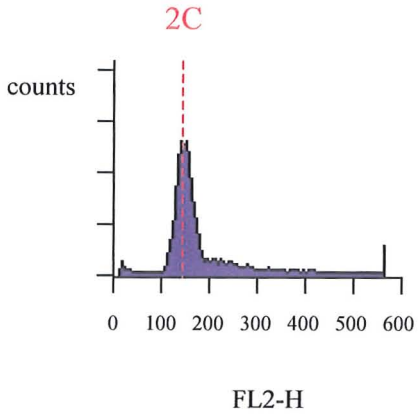
(i) 1C and 2C control
(*cdc10-129*; GG 27)



(ii) wild type
(GG 185)



(iii) *nmt1*P::*rad21*$^{+}$
pSP1/ *rad21*P: *rec8*$^{+}$
(GG 327)



(iv) *nmt1*P::*rad21*$^{+}$
pJK148/ *rad21*P: *rec8*$^{+}$
(GG 358)

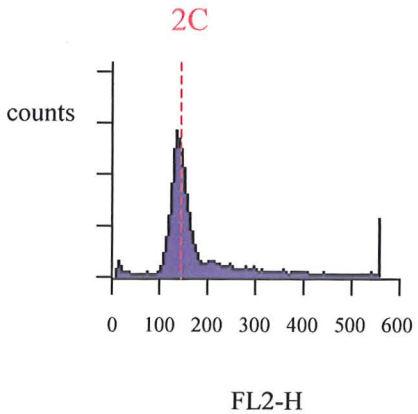


Figure 5.9 Expressing rec8$^+$ in place of *rad21*$^+$ doers not Cause S Phase Delay. A, In an asynchronous sample taken from a population of exponentially growing wild type fission yeast cells, flow cytometry reveals a single 2C peak in DNA content. This is because following DNA replication, each daughter cell has a 2C DNA content. This is halved to 1C during chromosome segregation at mitosis but 1C daughter cells remain joined until S phase, when cytokinesis is completed. As a result, cells taken at any point in the cell cycle have a 2C DNA content. However, if there is an S phase delay, cytokinesis is completed before DNA replication and an additional 1C peak in DNA content is seen. **B,** *rad21*$^+$-repressible strains were grown to exponential phase in liquid media, with (green line) and without (black line) 10 μM thiamine (iii and iv). Cells were harvested by centrifugation and fixed in 70% ethanol, rehydrated in 50 mM sodium citrate, then labelled with 2 μg/ml propidium iodide. The DNA distribution of cells from each culture was then analysed by flow cytometry (Section 2.4.8). FACS analysis of a *cdc10-129* fission yeast (GG 27) mutant gave standard 1C and 2C peaks (i).

Chapter 6:

Ectopically Expressed *rad21*$^{+}$ Partially Complements Meiotic Rec8p Function

6.1 BACKGROUND

As demonstrated in Chapter 3, the *S. pombe* cohesin, *rec8*$^+$, is expressed exclusively during sexual development. Rec8p is required for several meiotic processes including linear element formation, alignment of homologous chromosomes, and homologous recombination (DeVeaux & Smith, 1994; Molnar *et al.*, 1995). This meiotic cohesin is also essential for reductional division during the first meiotic division and normal spore development (Watanabe & Nurse, 1999; Parisi et al., 1999).

Rec8p is homologous to the mitotic cohesin, Rad21p, with the proteins sharing sequence similarity in the N and C-terminal portions. Chapter 5 demonstrated that ectopic expression of Rec8p during the mitotic cell cycle rescued the lethal effect of Rad21p deletion. This indicates functional overlap between the two cohesins, and these common functions are likely to reside in the conserved regions. The aim of this part of the study was to investigate the meiotic-specific functions of Rec8p, and to determine whether ectopic expression of *rad21*$^+$ during sexual development could rescue the defects seen in *rec8*Δ meioses.

To this end, in a *rec8*Δ *S. pombe* strain, the *rad21*$^+$ gene was placed under the control of the *rec8*$^+$ promoter, so that following meiotic induction, *rad21*$^+$ would be expressed in place of *rec8*$^+$. The effects on pre-meiotic DNA replication, meiotic chromosome segregation and ascospore development were then examined.

6.2 REPLACING THE MEIOTIC COHESIN Rec8p WITH THE MITOTIC COHESIN Rad21p DOES NOT CAUSE S PHASE DELAY

A study in *S. cerevisiae* found that deletion of *REC8* caused a delay in the timing of S phase progression of ~10% (Cha *et al.*, 2000). It has also been shown in *S. pombe* that *rec8*$^+$ must be expressed during pre-meiotic S phase, and if expression of *rec8*$^+$ is delayed until G2 phase cells undergo aberrant meiotic divisions (Watanabe & Nurse, 2001). These findings indicate that Rec8p is required during pre-meiotic S phase, and therefore might have a role in DNA replication.

To investigate the role of Rec8p during pre-meiotic S phase in *S. pombe*, diploid *pat1-114* synchronous meioses were induced and DNA replication monitored by FACS analysis. Progression of DNA replication could be seen as a doubling of DNA content from

2C to 4C. This was performed in wild type (GG 401), *rec8*Δ (GG 323), and in cells in which *rec8*$^+$ was replaced with the mitotic cohesin, *rad21*$^+$ (*rec8*Δ *rec8*P: *rad21*$^+$; GG 402).

Synchronous meiosis was induced by thermal inactivation of Pat1p, as described in section ***. Cell samples were collected at 10 minute intervals, fixed 70% ethanol and DNA labelled with 2 μg/ml propidium iodide (Section 2.4.8). To analyse the cellular DNA contents, and therefore the progression of DNA replication, each sample was then analysed by flow cytometry. Analyses were performed in isogenic diploid *S. pombe* strains, and each experiment was repeated at least five times.

In the wild type strain, the shift in DNA content from 2C to 4C, which indicates DNA replication, occurred between 110 and 170 minutes (GG 401; Figure 6.1A). In the absence of Rec8p (*rec8*Δ; GG 323; Figure 6.1B) no alteration was seen in the timing of entry into, or completion of S phase, which took place between 110 and 170 minutes as in wild type. Furthermore, replacing Rec8p with the mitotic cohesin Rad21p during meiotic induction, did not alter the timing of DNA replication (*rec8*Δ *rec8*P: *rad21*$^+$; GG 402; Figure 6.1C). As in both the wild type and *rec8*Δ strains, replication started 110 minutes after *pat1-114* inactivation and was completed by 170 minutes, as indicated by a single 4C peak in DNA content.

6.3 REPLACING Rec8p WITH Rad21p CAUSES MEIOTIC CHROMOSOME SEGREGATION ERRORS

S. pombe has a 14 Mb genome, which is distributed between three large chromosomes. As a result, when DNA is labelled with DAPI, defects in chromosome segregation can be easily visualised, and is seen in individual ascospores as unequal distributions of fluorescence.

Rec8p is required for reductional segregation during the first meiotic division (MI; Watanabe & Nurse, 1999). Deleting Rec8p causes aberrant meiotic segregation, with chromosomes separating equationally during MI. Because sister chromatids are no longer associated, this is followed by random segregation during MII, rather than equational, and results in the generation of aneuploid spores. In *S. cerevisiae*, deleting *REC8* caused random division during MI (Buonomo *et al.*, 2000). Furthermore, replacing *S. cerevisiae REC8* with the *rad21*$^+$ homologue, *MCD1*, changed the pattern of chromosome segregation during MI to equational.

To investigate how replacing Rec8p with the mitotic cohesin Rad21p affected meiotic chromosome segregation, meiosis was triggered in diploid wild type, *rec8*Δ and cells in which *rec8*$^+$ expression was replaced *rad21*$^+$ (*rec8*Δ *rec8*P: *rad21*$^+$).

Haploid strains of opposite mating type were crossed by mixing fresh cells in a drop of sterile water on ME plates, and incubating at 25°C for 2-3 days, inducing conjugation and sporulation. When many asci were visible, small aliquots were lifted from each plate and fixed by gentle re-suspension in 1 ml 70 % ethanol. To label spore DNA 1 μg/ml DAPI was then added. Ascospores were then fixed to poly-L-lysine coated glass slides, and visualised by phase contrast and fluorescent microscopy. The following meiotic crosses were performed: GG 347 x GG 435 (wild type); GG 300 x GG 301 (*rec8*Δ); GG 349 x GG 434 (*rec8*Δ *rec8*P: *rad21*$^+$). Slides were visualised with a 60 x lens on an Olympus BX60 microscope connected to a Princeton Instruments digital camera. For DAPI images an Olympus U-RFL-T fluorescent lamp was also used. Images were collected using IPLab software installed on an Apple Macintosh computer, then copied to Adobe® Photoshop® 5.0.

In the wild type cross, the majority of asci contained four clearly defined spores, each with fluorescent foci of equivalent size and intensity (Figure 6.2, left panel). This demonstrated an even distribution of DNA between the spores of individual asci, and indicated accurate meiotic chromosome segregation. Only rarely did an ascus from the wild type cross exhibit abnormal DNA distribution. In the *rec8*Δ cross, phase contrast microscopy did not reveal gross morphological defects in ascospore formation (Figure 6.2, middle panel). However, uneven distributions of DNA amongst the spores of individual asci was revealed by DAPI staining. Ascospores containing fewer or more than four centres of DAPI fluorescence were prevalent, indicating aberrant meiotic chromosome segregation. Even in those asci that contained four fluorescent foci, often they were not equivalent in size, suggesting that meiotic chromosome segregation was also defective in these asci. When Rec8p was replaced with the mitotic cohesin Rad21p, again phase contrast microscopy did not reveal any gross morphological defects in ascospore formation when compared to wild type (Figure 6.2, right panel). When viewed by fluorescence microscopy, the distribution of DNA within individual asci was often found to be unequal. The majority of asci contained four DNA foci but these often had irregular outlines, or were uneven in size. Also, a significant proportion of asci contained fewer or more than four foci indicating defects in meiotic chromosome segregation.

6.4 ANALYSING DNA DISTRIBUTION WITHIN INDIVIDUAL ASCOSPORES

To determine if replacing Rec8p with the mitotic cohesin Rad21p during meiosis altered the proportion of asci with abnormal DNA distribution, the number of DAPI foci in individual asci was scored for each cross described above: wild type (GG 347 x GG 435); *rec*8Δ (GG 300 x GG 301); *rec8*Δ *rec8*P: *rad21*$^+$ (GG 349 x GG 434). These data were then plotted on a histogram (Figure 6.3).

In the wild type meiosis, 94% of asci contained four DNA foci. When Rec8p was deleted this figure was reduced to only 51%, and most of the remaining *rec8*Δ asci contained either three or five DNA centres. However, when Rec8p was replaced with Rad21p, the number of asci containing four DNA centres was increased to 66%.

6.5 SPORE VIABILITY

If aberrant meiotic chromosome segregation takes place, the resultant spores gain or lose chromosomal DNA, and this results in a loss of spore viability. To further analyse chromosome segregation during *rec8*Δ and *rec8*Δ *rec8*P: *rad21*$^+$ meioses, crosses were set up between wild type, *rec8*Δ and *rec8*Δ *rec8*P: *rad21*$^+$ haploid *S. pombe* strains as in the experiments described above. The viability of spores generated from each cross were then analysed as described in section 2.2.2.9; Table 6.1).

In the wild type meiosis, 21 of the 27 asci dissected were tetrads i.e. contained four spores. Of the 108 spores pulled, 101 grew to form colonies giving a spore viability of 93%. In the *rec8*Δ meiosis, ascospores took longer to form when compared to wild type (3 days rather than 2), and when incubated at 36°C many ascus coats failed to dissolve at all, indicating impaired ascospore development. Of those that did dissolve, only 8 of the 30 asci dissected were tetrads, and only 8% (4 out of 50) of the spores isolated were viable. These findings indicate a crucial role for Rec8p in ascospore development. However, when Rec8p was replaced with Rad21p, spore viability was increased to 34% when compared to the *rec8*Δ meiosis. Furthermore, 19 of the 30 asci dissected contained four spores. Therefore, meiotic expression of Rad21p in place of Rec8p, was able to significantly improve both ascospore development and spore viability.

6.6 SUMMARY

6.6.1 S Phase Progression

No alteration in the timing of pre-meiotic S phase was detected in *rec8*Δ cells. That is, DNA replication both started and finished at the same time following meiotic induction as in wild type cells. This is in contrast to the findings of a study in *S. cerevisiae*, which reported that *Rec8*Δ cells spent ~10% longer in pre-meiotic S phase when compared to wild type. In the present study, the experiments were repeated several times. Furthermore, samples were taken every 10 minutes over the interval when DNA replication took place (110-170 minutes after meiotic induction), so that any subtle differences in timing would be detected. The finding that the absence of Rec8p during pre-meiotic S phase in *S. pombe* does not alter the timing of S phase progression suggests two possibilities: that Rec8p is not required during pre-meiotic S phase; or that Rec8p is required during pre-meiotic S phase, but replication defects are not detected until a later stage of meiosis.

It was also found that replacing Rec8p with the mitotic cohesin Rad21p did not alter the timing of the progression of DNA replication. Therefore, the presence of Rad21p, which is normally excluded from chromosomes during pre-meiotic S phase, did not delay pre-meiotic S phase progression. The simplest explanation for this is that neither Rad21p nor Rec8p are involved in the replication process. Deletion of Rec8p or ectopic expression of *rad21*$^{+}$ would therefore have no effect on pre-meiotic S phase. However, another possibility is that cohesins are required for DNA replication, and that replacing Rec8p with the mitotic cohesin Rad21p does cause replication defects. If so, these defects might be detected at a later stage of meiosis. Alternatively, the absence of Rec8p might prevent replication defects from being detected at all.

One way to detect replication defects would be to extend the period of meiotic induction up to 7 hours when, in *rec8*$^{+}$ meiosis, a shift in DNA content from 4C to 2C would indicate MI progression. A delay in this timing would indicate activation of the replication or DNA damage checkpoints. Other methods include determining whether DNA replication and DNA damage checkpoint kinases, Cds1p and Chk1p, become activated in these cells, eg. By enzyme assay or western blotting.

6.6.2 Meiotic Chromosome Segregation

Segregation defects were seen both in *rec8*Δ and *rec8*Δ *rec8*P: *rad21*$^{+}$ meioses. In the majority of cases these defects generated more than four DNA foci, which is indicative of precocious separation of sister chromatids (PSS) during the first meiotic division (Figure 6.4). This is consistent with previous reports that Rec8p is required for a reductional pattern of chromosome segregation during MI, with *rec8*$^{-}$ mutants displaying an array of segregation defects including MI PSS and non-disjunction of sister chromatids during MII (Krawchuk et al., 1999).

The same segregation errors were seen when Rec8p was replaced with the mitotic cohesin Rad21p. However, in the *rec8*Δ *rec8*P: *rad21*$^{+}$ meioses, the proportion of asci containing four DNA foci was increased (67%) when compared to asci from *rec8*Δ meioses (51%). This suggests that, by replacing Rec8p with Rad21p, the frequency of segregation errors that occurred was reduced when compared to *rec8*Δ meioses. It is also possible that, in those asci containing four DNA foci, segregation errors had occurred that could not be distinguished by DAPI. It is possible for aberrant meioses to produce four DNA foci that appear to contain equal amounts of DNA, when in fact the chromosomes have segregated abnormally and the DNA is distributed unequally between the four spores (Figure 6.4). If this was the case, it would indicate that expressing Rad21p in place of Rec8p during meiosis altered the type of segregation defects that were taking place.

6.6.3 Ascospore Development

Ascospore development was severely impaired in the absence of Rec8p. It took longer for ascospores to appear after *rec8*Δ meioses, and the total number of asci formed was reduced, when compared to wild type. Furthermore, ascus coats frequently failed to dissolve indicating that they were not fully developed. Of those asci that did break down, many contained less than four spores, some of which were so tiny they were unidentifiable. These findings are consistent with an important role for Rec8p in ascospore formation.

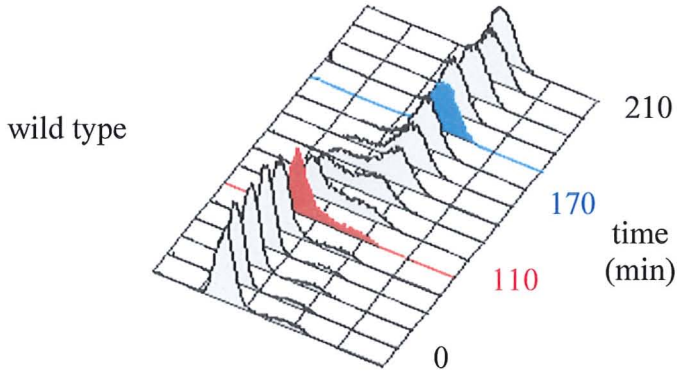
Strikingly, when Rec8p was replaced with Rad21p during meiosis, the number of ascospores formed was increased to wild type levels, and the morphology of ascospores was more similar to wild type. Ascus coats dissolved efficiently at 36°C, and released four equally sized, well-defined spores. These findings suggest that Rad21p can support

ascospore formation in the absence of Rec8p, and that this meiotic function of Rec8p is conserved in the mitotic cohesin Rad21p.

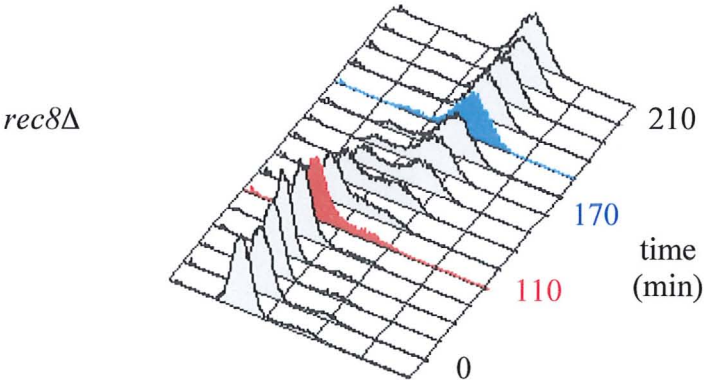
6.6.4 Spore Viability

The viability of spores generated from the wild type meiosis was 97%, demonstrating that spores had not been damaged by handling with the micro-manipulator. However, only 8% of *rec8*Δ spores were viable, which is consistent with published data (Watanabe & Nurse, 1999). Because Rec8p is not required for mitotic growth, this loss of viability must reflect defects that occurred in the preceeding meiosis. Rec8p is known to be required both for the meiotic pattern of chromosome segregation and for spore formation. Both of these processes were severely impaired in the *rec8*Δ meiosis, and the loss of viability seen in these mutants could be a consequence of either or both defects. When Rec8p was replaced with the mitotic cohesin Rad21p, spore viability was significantly increased (34%) when compared to *rec8*Δ spores. This was coupled with a small reduction in segregation defects, and greatly improved ascospore development. Therefore, although segregation defects were still seen in these cells, in the absence of Rec8p, Rad21p was able to support ascospore development. These findings indicate that the increase in spore viability when compared to *rec8*Δ, might therefore have been due to improved ascospore development.

A


wild type
210
170
time
(min)
110
0

B


rec8Δ
210
170
time
(min)
110
0

C

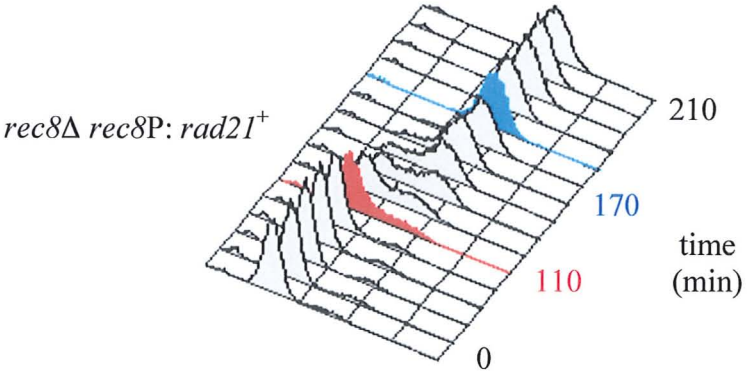

rec8Δ rec8P: rad21+
210
170
time
(min)
110
0

Figure 6.1 Replacing Rec8p with Rad21p Does Not Alter the Timing of Pre-Meiotic S Phase Progression. Synchronous meiosis was induced in fission yeast diploid strains by thermal inactivation of Pat1p. Samples (1 x 10^8 cells) were collected at 10 minute intervals, pelleted and fixed in 70% ethanol. Cells were then labelled with 2 μg/ml propidium iodide and analysed by flow cytometry to monitor the progression of DNA replication. **A,** Wild type (GG 375). **B,** *rec8*Δ (GG 323). **C,** *rec8*Δ *rec8*P: *rad21*$^+$ (GG 402).

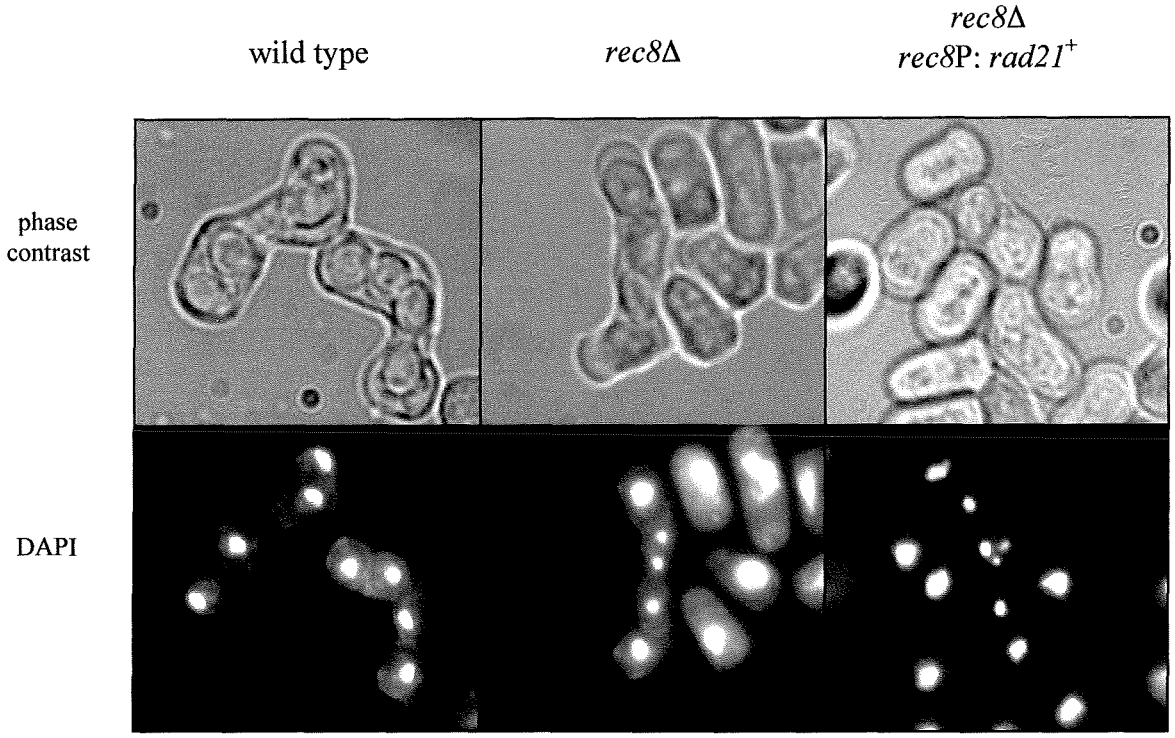


Figure 6.2 Expressing *rad21*$^+$ in place of *rec8*$^+$ Leads to Abberrant Chromosome Segregation. Mating and meiosis were induced between h^+ and h^- haploid fission yeast strains by incubating together on ME at 25°C for 2-3 days. Ascospores were gently resuspended and fixed in 70% ethanol, then labelled with 1 μg/ml DAPI. Ascospores were mounted on slides then viewed by phase contrast and fluorescence microscopy to examine the distribution of DNA within individual asci.

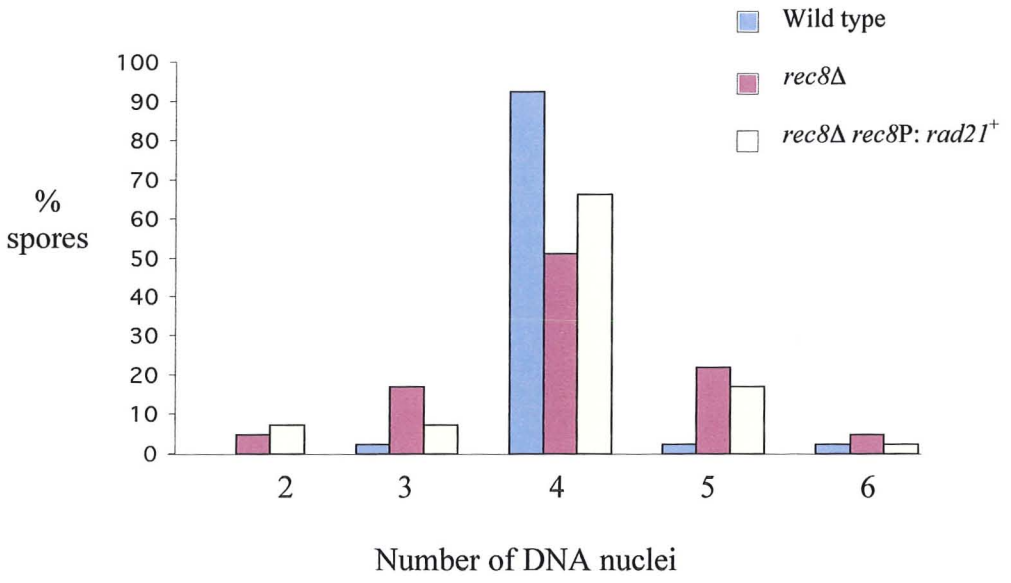


Figure 6.3 Abnormal DNA Distributions of *rec8* Mutant Meioses. Mating and meiosis were induced between h^+ and h^- haploid fission yeast strains by incubating together on ME at 25°C for 2-3 days. Ascospores were fixed in 70% ethanol, then labelled with propidium iodide. Ascospores were mounted on slides then viewed by phase contrast and fluorescence microscopy to examine the distribution of DNA within individual asci. For each cross the number of DNA nuclei per ascus was scored and plotted as a histogram. Wild type: GG 347 x GG 435; *rec8*Δ: GG 300 x GG 301; *rec8*Δ *rec8*P: *rad21*$^+$: GG 349 x GG 434.

	wild type	***rec8*Δ**	***rec8*Δ** ***rec8*P: *rad21*$^+$**
no. asci dissected	27	30	30
no. spores dissected	108	50	98
no. colonies	101	4	34
Spore viability	93%	8%	34%

Table 6.1 Reduced Spore Viability in *rec8* Mutant Meioses. Mating and sporulation was induced between h^+ and h^- fission yeast haploids on ME plates, which were incubated at 25° C for 2-3 days until lots of asci were seen. Asci were transferred to YE plates then incubated at 36° C for 2-5 hours to stimulate ascus coat breakdown. Using a micromanipulator (Singer Instruments Manual MSM), spores from individual asci were isolated and arranged in a grid pattern on the same YE plate, then incubated at 25° C for 3-7 days until colonies had grown. Spore viability was then calculated as the percentage of spores originally isolated that grew to form colonies. Wild type: GG 347 x GG 435; *rec8*Δ: GG 300 x GG 301; *rec8*Δ *rec8*P: *rad21*$^+$: GG 349 x GG 434.

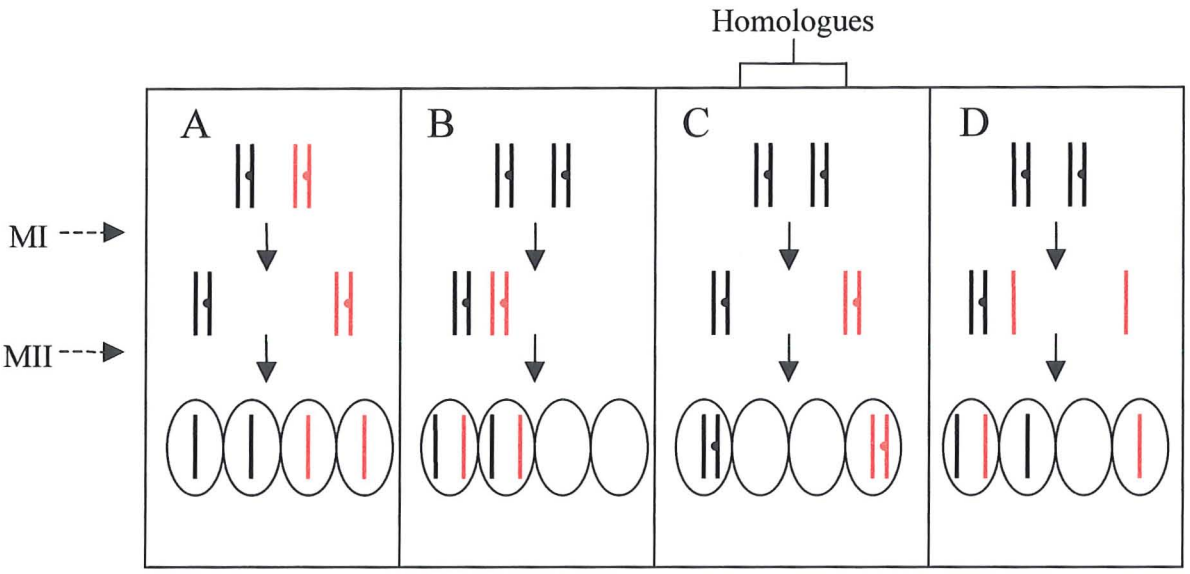


Figure 6.4 Meiotic Chromosome Segregation Errors. **A**, *Normal fission yeast meiosis*: homologous chromosomes separate during the first meiotic division (MI; reductional division) and sister chromatids remain associated. During MII, sister chromatids separate in the equational pattern of division, generating four haploid spores. **B**, *Meiosis I non-disjunction*: homologous chromosomes fail to segregate during the reductional division. **C**, *Meiosis II non-disjunction*: sister chromatids fail to segregate during the equational division. **D**, *Precocious separation of sister chromatids*: individual chromatids segregate randomly during MI and MII.

Chapter 7:

Discussion and Future Directions

7.1 INTRODUCTION

This study aimed to investigate the biological functions of two *S. pombe* cohesins, Rad21p and Rec8p. Transcription of the *rad21*$^+$ gene was found to be cell cycle-regulated and expressed exclusively during the G1/S phase of the mitotic cell division cycle. In contrast, the *rec8*$^+$ gene was expressed specifically during sexual development and, following induction of meiosis, was transcribed specifically during G1/pre-meiotic S phase.

Of particular interest was the finding that expression of the mitotic cohesin, *rad21*$^+$, was repressed during sexual development. This is an important finding because, apart from the DSC1 transcription factor co-activator Rep2p, no other known mitotic genes in *S. pombe* are repressed following meiotic induction (Mata *et al.*, 2002; Wood *et al.*, 2002). This finding suggests that the presence of Rad21p might have a harmful effect on sexual development.

A further aim of this study was to investigate the mitotic and meiotic-specific functions of Rad21p and Rec8p cohesins in *S. pombe*. To address this, the promoters of *rad21*$^+$ and *rec8*$^+$ were exchanged so that they would be ectopically expressed, both at physiological levels and at the appropriate cell cycle time. As a result, *rec8*$^+$ was transcribed during the mitotic cell cycle while *rad21*$^+$ was transcribed during meiosis. The effect of these altered cohesin expression patterns on several cell cycle processes was then analysed.

7.2 Rec8p DURING MITOSIS

7.2.1 *rad21*-repressible *rad21*P: *rec8*$^+$ Cells

Because *rad21*$^+$ is essential, the gene was placed under the control of the repressible *nmt1*$^+$ promoter, creating a '*rad21*$^+$-repressible' *S. pombe* strain. When these cells were cultured in media supplemented with thiamine, Rad21p protein levels were rapidly depleted (Figure 4.4). The *rec8*$^+$ gene was attached at its 5' end to the *rad21*$^+$ promoter region, then introduced into the *rad21*$^+$-repressible strain, either on a low copy plasmid, or integrated in single copy into the genome. Having confirmed the exchange in cohesin expression by northern blot analysis (Figures 4.5, 4.6), the *rad21*Δ *rad21*P: *rec8*$^+$ cells were then characterised.

7.2.2 Rec8p Rescues the *rad21*-repressible Lethal Phenotype

In control cells not carrying *rad21*P: *rec8*$^+$, repression of *rad21*$^+$ inhibited cell division with the cells exhibiting a heterogeneous array of abnormal phenotypes (Figures 5.1, 5.4). In the *rad21*-repressible mutant although some cells were reduced in size, most had an enlarged morphology. When cell cycle progression is delayed in *S. pombe*, the cells continue to grow but fail to divide manifesting as an elongated cell phenotype. *rad21*-repressed cells were increased in length, but also in width, suggesting that the mutant phenotype was not caused solely by a cell cycle delay. Furthermore, many of the enlarged cells had uneven outlines with bulging cell walls, as well as multiple septa, indicating that aberrant septation had taken place, or that cytokinesis was defective.

When *rec8*$^+$ was expressed in *rad21*-repressible cells under *rad21*$^+$ promoter control (*rad21*P: *rec8*$^+$), the *rad21*-repressed phenotype was largely abolished. The majority of these cells continued to grow and divide as wild type (Figures 5.1B and 5.4), suggesting that Rec8p was able to support vegetative growth, and that the essential function of Rad21p is conserved in Rec8p.

However, it should be emphasised that a small fraction of the *rad21*-repressible *rad21*P: *rec8*$^+$ cells exhibited the abnormal *rad21*-repressed phenotype. This may have been caused by cells losing the plasmid that carried the *rad21*P: *rec8*$^+$ construct. However this explanation was unlikely because the plasmid was nutritionally selected for, and the *rad21*-repressed cells did not display the typically rounded morphology of nutritionally deprived *S. pombe* cells (Figure 5.4). Another possibility was that these cells were *leu*$^+$ gene revertants, but this was unlikely to have occurred at such a high frequency. The most probable explanation was that Rec8p was unable to complement Rad21p function fully during the mitotic cell cycle, and that Rad21p has mitotic-specific functions that cannot be performed by Rec8p.

When *rad21*-repressible *rad21*P: *rec8*$^+$ cells were cultured on solid media containing thiamine, although cells were repeatedly streaked onto fresh media to allow thiamine accumulation, the *rad21*-repressed phenotype was not as prevalent as when cells were cultured in liquid media, where approximately 30% of cells looked like the *rad21*-repressed mutant (Figure 5.5). The reason for this discrepancy may be that on solid media, some of the cells in each colony would not be in direct contact with the medium. Thiamine might accumulate only in those cells in direct contact with the media, repressing *rad21*$^+$ transcription in those cells only.

7.2.3 Growth Rates of *rad21*-repressible *rad21*P: *rec8*$^+$ Cells

The division times of *rad21*$^+$-repressible strains were calculated to see if expression of *rec8*$^+$ in place of *rad21*$^+$ might cause a cell cycle delay (Figure 5.8). When cultured in media supplemented with thiamine (i.e. *nmt1*P: *rad21*$^+$ 'OFF'), the division time of the *rad21*$^+$-repressible plasmid strain was found to be unaltered when compared to the isogenic strain carrying empty plasmid. This indicates that ectopic expression of *rec8*$^+$ during the mitotic cell cycle did not increase the division time. In the *rad21*$^+$-repressible integrant strain there was a small increase in division time (7.5%) when compared to the isogenic strain with empty plasmid. In these cells, *rad21*$^+$, under *nmt1*$^+$ promoter control, was expressed constitutively and at levels approximately ten times higher than during wild type mitotic G1/S phase, and *rec8*$^+$, under *rad21*$^+$ promoter control, was ectopically expressed at physiological levels during mitotic G1/S phase. It is therefore possible that the increased cell cycle length was caused either by unnaturally high levels of Rad21p, or the presence of the meiotic cohesin Rec8p, or both. However, these factors would be expected to also affect the *rad21*$^+$-repressible plasmid strain. It is therefore more likely that the increased division time in the *rad21*$^+$-repressible integrant strain was caused by the *rad21*P: *rec8*$^+$ construct being integrated into the genome.

When cultured with thiamine (i.e. *nmt1*P::*rad21*$^+$ 'OFF'), Rad21p was depleted from the cells and *rec8*$^+$ was expressed in its place. Under these conditions the division times of both the plasmid and integrant strains were increased slightly (7.5% and 4.6%, respectively; Figure 5.8B) compared to when *rad21*$^+$ was also expressed. The increased division times were small and whether or not they were biologically significant was debatable. One could argue that the increased division times represented a cell cycle delay caused by either the absence of Rad21p, ectopic expression of *rec8*$^+$ during the mitotic cell cycle, or both. However, *rec8*$^+$ expression failed to elicit an increase in division time in the *rad21*$^+$-repressible plasmid strain, indicating that this was not responsible for the increase. Instead, the absence of Rad21p was a more probable cause. Why this would result in a greater increase in division time for the *rad21*$^+$-repressible plasmid strain when compared to the integrant strain is not clear.

7.2.4 Replacing Rad21p with Rec8p Leads to a Loss of Viability

Although physiological expression of *rec8*$^+$ during the mitotic cell cycle was able to rescue the lethal *rad21*-repressed phenotype in the majority of cells, a significant proportion exhibited the *rad21*-repressed phenotype, indicating that the complementation

of Rad21p by Rec8p was not perfect. To analyse the complementation of Rad21p by Rec8p during the mitotic cell cycle, the viability of *rad21*-repressible *rad21*P: *rec8*$^+$ cells was assessed. When cultured without thiamine with *rad21*$^+$ expressed at constitutively high levels and *rec8*$^+$ expressed at physiological levels, during mitotic G1/S phase, viability was reduced from 80% (wild type) to 58% and 56% for the plasmid and integrant *rad21*$^+$-repressible strains, respectively (Figure 5.7). This reduction may have been due to the abnormally high levels of Rad21p in these cells and its loss of cell cycle regulation. It is also possible that ectopic expression of *rec8*$^+$ caused a cell cycle defect that led to cell death.

When Rad21p was depleted so that cells expressed *rec8*$^+$ in place of *rad21*$^+$, at physiological levels and at the appropriate time in the mitotic cell cycle, viability was further decreased to 29% and 37% for the plasmid and integrant strains, respectively (Figure 5.7). Therefore, replacing Rad21p with the meiotic cohesin Rec8p, was only able to partially support vegetative growth, with the majority of cells eventually dying.

7.2.5 Replacing Rad21p with Rec8p Does Not Cause S Phase Delay

Rad21p interacts with several genes required for DNA replication (Tanaka *et al.*, 2001; Takeda *et al.*, 2001) in both *S. pombe* and budding yeast, and *rad21*$^+$ expression is induced just prior to the onset of S phase, indicating that Rad21p may be involved in the DNA replication process. The division times of *rad21*-repressible *rad21*P: *rec8*$^+$ cells were inconclusive and failed to identify a cell cycle delay. Therefore, FACS analysis was performed to see if expressing *rec8*$^+$ in place of *rad21*$^+$ led to a delay in S phase progression. In both the plasmid and integrant *rad21*-repressible *rad21*P: *rec8*$^+$ strains, no S phase delay was seen (Figure 5.9). Therefore, exchanging Rad21p for the meiotic cohesin Rec8p did not delay progression of mitotic S phase.

7.2.6 Consequences of Mitotic Rec8p Expression

7.2.6.1 CELL CYCLE DELAY

The cell division times of *rad21*-repressible *rad21*P: *rec8*$^+$ cells in liquid culture were only slightly increased when Rad21p was replaced with Rec8p, which might have been caused by a cell cycle delay (Figure 5.8). However, only 30% of *rad21*-repressible *rad21*P: *rec8*$^+$ cells were viable (Figure 5.7). An explanation for this observation is that the increased division times were not caused by cell cycle delay, but rather by a percentage of cells that died during the period of the division time assay. That only subtle

increases in division time were detected when viability was as low as 30% can be explained because the division time assay was performed over the interval of only two cell division cycles.

Expression of *rec8*$^+$ in place of *rad21*$^+$ led to a loss of viability indicating that Rec8p was only able to partially complement the mitotic functions of Rad21p. However, it is not clear why the majority of *rad21*-repressible *rad21*P: *rec8*$^+$ cells had wild type morphology, while a smaller fraction exhibited the *rad21*-repressed mutant phenotype. As discussed above, this is unlikely to have been caused by plasmid loss or gene conversions. One possible explanation is that it accrued from an accumulation of defects up to a threshold point, over which *S. pombe* cells died. These defects might have occurred spontaneously, or have been caused by an absence of Rad21p, the presence of Rec8p or by substitution of Rad21p for Rec8p. Rad21p is required for equational chromosome segregation during mitosis, so in the absence of Rad21p, chromosomes may divide randomly, as seen in the *S. pombe rad21-K1* mutant (Tatebayashi *et al.*, 1998). Conversely, Rec8p is required for reductional segregation during the first meiotic division, and the presence of Rec8p on chromatin during mitosis may shift the pattern of chromosome segregation to reductional. In either case, if aberrant segregation occurred, a significant proportion, but not all of newly formed daughter cells would be inviable.

7.2.6.2 DNA REPLICATION

The mutant phenotype of *rad21*-repressible *rad21*P: *rec8*$^+$ cells could have several causes. Because *rad21*$^+$ and *rec8*$^+$ expressions are induced during G1/S phase of the mitotic and meiotic cell cycles, respectively, it is suggested that they are required for DNA replication (Takeda *et al.*, 2001; Cha *et al.*, 2000). In support of this contention, Rad21p also interacts with several genes required for DNA replication in *S. pombe* (Tategbayashi *et al.*, 2001; Takeda *et al.*, 2001).

The finding that replacing Rad21p with Rec8p during mitotic S phase did not cause S phase delay could have several meanings. First, that Rad21p is required for DNA replication, and that Rec8p, when ectopically expressed during the mitotic cell cycle, complements this function. Second, it is possible that, if Rad21p is required for replication, exchanging Rad21p for Rec8p causes replication defects, but that these are not detected until later in the cell cycle. Indeed, if Rad21p is involved in a DNA replication checkpoint, its absence might prevent the checkpoint from being activated when replication defects occur. A third possibility is that Rad21p is not required for DNA replication per se. The passing of the replication fork over newly replicated DNA

strands may simply provide a convenient mechanism by which Rad21p can be loaded onto chromatin. Perhaps Rad21p must be loaded onto the chromosomes during G1/S phase so that the sister centromeres and their associated kinetochores are correctly orientated for accurate spindle attachment and subsequent chromosome segregation. In support of this, although DNA replication mutants often exhibit errors in sister chromatid cohesion (Tanaka et al., 2000, 2001; Kenna & Skibbens, 2003; Edwards *et al.*, 2003 2001), there are no reports of DNA replication defects occurring as a consequence of errors in cohesion.

7.2.6.3 CROSSOVER EVENTS

In eukaryotes pre-meiotic S phase is typically several times longer than mitotic S phase and this is thought to be linked to the assembly of meiotic-specific chromosomal features that prepare them for meiotic homologous recombination, which occurs approximately 100 times more frequently than mitotic crossover events. Replacing Rad21p with Rec8p during mitotic S phase might, therefore, promote the ectopic establishment of such features, resulting in an increased frequency of recombination events. If so, crossovers might occur between homologous sequences of sister chromatids, or non-homologous regions of other chromosomes. However, because none of the other genes involved in meiotic recombination are present in these cells, this could lead to failure to resolve chiasmata, and as a result, loss of chromosome fragments, and following mitotic division, the generation of inviable daughter cells.

7.2.6.4 CENTROMERE-KINETOCHORE ORIENTATION

During mitotic prophase, sister centromeres are bi-orientated so that the kinetochores that assemble upon them capture microtubules emanating from opposite spindle poles, thereby ensuring equational chromosomal segregation (Goshima & Yanagida, 2000; Tanaka *et al.*, 2000; He *et al.*, 2000). However, during pre-meiotic S phase, sister centromeres are co-orientated so that they attach to the same spindle pole, resulting in reductional chromosome segregation during the first meiotic division. Rec8p localised at the inner centromere during pre-meiotic S phase is required for this co-orientation (Watanabe *et al.*, 2001). Replacing Rad21p with the meiotic cohesin, Rec8p, might therefore shift the orientation of sister chromatids so that, during mitosis, they migrate to the same spindle pole i.e. divide reductionally, generating non-viable nullosomic aneuploid daughter cells.

7.2.6.5 CHROMOSOME COHESION

During mitosis, cohesion between sister chromatids ensures that they align correctly on the spindle apparatus. Then, as the spindle pole bodies migrate to opposite poles, this generates tension between the sister centromeres, which triggers dissolution of cohesin, and separation of the sister centromeres, which migrate to opposite poles of the dividing cell. During mitosis in *S. cerevisiae*, centromeric Mcd1p is cleaved by separin, dissolving sister chromatid cohesion so that sister chromatids are free to migrate to opposite poles of the cell (Uhlmann *et al.*, 1999; Buonomo *et al.*, 2000), and a similar mechanism is thought to operate in *S. pombe* (for review, see Yanagida, 2000). During MI, arm cohesion is dissolved but Rec8p located at the inner centromere is protected from cleavage, so that sister chromatids remain joined, resulting in reductional division. During MII, centromeric Rec8p is cleaved allowing sister chromatids, which now face opposite spindle poles, to separate (Watanabe & Nurse, 1999).

Replacing Rad21p with Rec8p during mitosis might therefore alter the pattern of sister chromatid cohesion. For example, if Rec8p was incorporated into the inner centromere, which is normally free of cohesin, in *rad21*-repressible *rad21*P: *rec8*$^+$ cells, then this might inhibit sister chromatid separation, as in meiosis I, and chromosome non-disjunction errors would occur. It would thus be interesting to perform chromatin immunoprecipitation assays to determine if ectopically expressed Rec8p was incorporated into the chromatin of *rad21*-repressible *rad21*P: *rec8*$^+$ cells during the mitotic cell cycle, and to see where it is located i.e. at the inner or outer centromere, chromosome arms.

Rad21p forms part of a multi-subunit complex called cohesin (Tomonaga *et al.*, 2000). Distinct types of cohesin, composed of different subunits, exist at the centromere and chromosome arms (Kitajima *et al.*, 2003). Rad21p has been shown to associate with specific components of cohesin depending on its chromosomal location. If Rec8p was found be incorporated into mitotic chromatin in *rad21*-repressible *rad21*P: *rec8*$^+$ cells, immunoprecipitation assays might determine if Rec8p was also able to associate with the other components of mitotic cohesin. Indeed, if Rec8p associates only weakly with mitotic cohesin, then this might explain the heterogeneous phenotype of *rad21*-repressible *rad21*P: *rec8*$^+$ cells. In these cells cohesin function would be impaired causing chromosome segregation defects and inviable daughter cells.

7.3 Rad21p DURING MEIOSIS

7.3.1 *rec8Δ rec8*P: *rad21*$^+$ Cells

Diploid strains were created in which both copies of *rec8*$^+$ were disrupted (*rec8Δ*). The *rec8*P: *rad21*$^+$ construct was then introduced into these cells, either on a low copy plasmid, or integrated into each arm of the *rec8Δ* diploid, so that upon induction of meiosis, *rad21*$^+$ would be expressed in place of *rec8*$^+$. The effects on the meiotic cell cycle were then analysed.

7.3.2 Replacing Rec8p with Rad21p Does Not Delay Pre-Meiotic S Phase Progression

In a similar study in *S. cerevisiae*, it was reported that deleting *REC8* caused a ~10% delay in pre-meiotic S phase progression, indicating that, in this yeast species, *REC8* is required for pre-meiotic DNA replication (Cha *et al.*, 2000). In *S. pombe*, *rec8*$^+$ must be expressed during G1/pre-meiotic S phase of the meiotic cell cycle for centromeric sister chromatid cohesion and for chromosomes to divide reductionally during MI, implying a link between pre-meiotic DNA replication and cohesion.

To determine if *S. pombe* Rec8p is required, pre-meiotic DNA replication was monitored in both *rec8Δ* and *rec8Δ rec8*P: *rad21*$^+$ cells by FACS analysis. In contrast to the observations in budding yeast, no alteration was seen in the timing of pre-meiotic S phase progression in cells lacking Rec8p, when compared to wild type (Figure 6.1). As described for Rad21p, this might simply be a convenient time for Rec8p to be loaded onto the newly-replicated chromosomes, with the loading of cohesin being somehow connected with the passing replication complex. Alternatively, it may be necessary for Rec8p to be incorporated at the inner centromeres as soon as sister chromatids are formed, so that sister centromeres and their associated kinetochores face the same spindle pole, and remain joined for a reductional MI division.

Another explanation for the lack of effect of *rec8Δ* on S-phase progression is that although Rec8p is required for pre-meiotic DNA replication in *S. pombe* and that the absence of Rec8p causes replication defects these might not be detected until later in the cell cycle. To investigate this it would be necessary to determine whether DNA replication checkpoints had been activated in these mutants. It is also possible that the lack of Rec8p in these cells meant that replication checkpoints were impaired, in which case it would be necessary to assess DNA replication more directly. However, to date there is no evidence that *S. pombe* Rec8p is involved directly in DNA replication.

When Rad21p was expressed in place of Rec8p during pre-meiotic S phase, no delay in S phase progression was detected (Figure 6.1). The simple explanation for this observation is that Rec8p is not required for pre-meiotic DNA replication, so replacing it with the mitotic cohesin, Rad21p, would have no effect. As already described, it would be necessary to determine whether DNA replication checkpoints were activated in these cells, or to assess replication more directly.

If Rad21p and Rec8p are required for DNA replication, one would predict that they have mitotic and mitotic-specific functions during S phase. If so, one would expect that exchanging Rec8p for Rad21p would have a deleterious effect on pre-meiotic S phase progression. However, this was found not to be the case, further implying that Rad21p and Rec8p do not function directly in DNA replication.

7.3.3 Physiological Expression of Rad21p was Able to Support Ascospore Development in the Absence of Rec8p

The role of Rec8p, and the effects of replacing Rec8p with Rad21p on ascospore development, were analysed by triggering meiosis and sporulation in *rec8*Δ and *rec8*Δ *rec8*P: *rad21*$^+$ strains. The total number of asci produced in *rec8*Δ meiosis was reduced when compared to wild type and the development of *rec8*Δ asci was found to be severely impaired. At the light microscope level, *rec8*Δ asci did not have any obvious morphological defects (Figure 6.2), but the observation that many failed to dissolve indicated that they were malformed. In addition, of those asci that did dissolve, few contained four equally-sized spores with many containing spores that were severely reduced in size, or that failed to separate from each other. These findings showed that ascus development, as well as sporulation, were defective in the *rec8*Δ mutant. These data are consistent with previous studies that have also reported a role for Rec8p in spore formation (Parisi *et al.*, 1999).

Strikingly, replacing Rec8p with the mitotic cohesin Rad21p markedly improved ascus formation when compared to *rec8*Δ. Meiosis and sporulation of *rec8*Δ *rec8*P: *rad21*$^+$ cells yielded many asci that were wild type in appearance,. Also, when dissected, *rec8*Δ *rec8*P: *rad21*$^+$ asci contained four large spores that were equal in size and separated easily. These findings suggest that Rad21p was able to support ascospore development in the absence of Rec8p. This is an interesting finding because Rad21p is mitotic –specific, while ascospores are formed only during meiosis. Indeed, there is no published evidence of a mitotic role for Rad21p in septation, which one might expect if Rad21p was able to function in ascospore development. It is not known how Rec8p is

involved in the process of ascospore development, but one would expect that this function of Rec8p is located in the conserved region of the protein, for it to be also present in Rad21p. Creating truncated Rec8p constructs might reveal the precise region involved in ascospore development, and lead to the identification of potential binding partners involved in the process.

7.3.4 Replacing Rec8p with Rad21p Leads to Meiotic Chromosome Segregation Errors

Spore DNA was labelled with DAPI to monitor chromosome segregation in *rec8*Δ *rec8*P:*rad21*$^{+}$ meioses (Figure 6.2). Because *S. pombe* contains only three chromosomes, when DNA is labelled with DAPI defects in chromosome segregation are seen in individual asci as unequal distributions of fluorescence.

Wild type asci contained four equally sized DNA nuclei that were evenly distributed in the ascus. In *rec8*Δ meioses, although many asci contained four DNA nuclei, these were often unequal in size indicating that they did not contain equal amounts of DNA. 60% of *rec8* Δ asci contained more than, or fewer than four DNA nuclei that were in some cases smaller than wild type, implying that they contained only one or two chromosomes, and in other cases larger than wild type, containing more than three chromosomes (Figure 6.2B). These phenotypes indicate that defects in meiotic chromosome segregation had occurred.

During MI, Rec8p is required for homologous recombination and for cohesion between the centromeres of sister chromatids (Molnar et al., 1995; Parisi et al., 1999; Watanabe & Nurse, 1999). During meiotic recombination, crossovers are formed between homologous chromosomes so that they become physically connected to each other. Because the homologues are also attached to microtubules emanating from opposite spindle poles, tension is generated between them which assists their accurate alignment at the spindle equator, the position at which opposing tensions become equal (Goshima and Yanagida, 2000; Tanaka *et al.*, 2000; He *et al.*, 2000). Once correctly positioned, the resolution of crossovers is triggered, arm cohesion is dissolved, and the homologues separate. Centromeric cohesion between sister chromatids persists until MII, to ensure a reductional MI division (Uhlmann *et al.*, 2000; Hauf *et al.*, 2001).

*rec8*Δ mutants undergo two types of chromosome segregation error: premature separation of sister chromatids and non-disjunction of homologous chromosomes, both occurring during MI (Krawchuk *et al.*, 1999). The frequency of crossover events is reduced in *rec8*Δ meioses (DeVeaux & Smith, 1994; Molnar *et al.*, 1995; Parisi et al.,

1999; Watanabe & Nurse, 1999), which would reduce the tension between homologous chromosomes, inhibiting their accurate alignment on the spindle, and this might cause the MI non-disjunction phenotype described for *rec8* Δ meioses. This might also account for the appearance of some larger nuclei in *rec8* Δ asci. To confirm this hypothesis it would be necessary to analyse how DNA is distributed in dyads formed after completion MI. Furthermore, the absence of centromeric Rec8p, which is required for co-orientation of sister centromeres and their association throughout MI, would cause sister chromatids to separate prematurely. Because separated sister chromatids do not attach to the spindle correctly, this leads to random segregation during MII which might account for the smaller nuclei seen in the *rec8* Δ asci in this study. But to confirm the type of segregation errors occurring, again it would be necessary to analyse the *rec8*Δ dyads.

When Rec8p was replaced with the mitotic cohesin, Rad21p, the percentage of asci containing four nuclei was increased when compared to *rec8*Δ, however, these were usually unequal in size (Figure 6.2A, B). Also, many contained more than, or fewer than, four nuclei indicating that aberrant chromosome segregation was taking place. Therefore, replacing Rec8p with the mitotic cohesin, Rad21p, did not rescue the meiotic segregation defects seen in the *rec8* Δ. The finding that the frequency of *rec8* Δ *rec8*P: *rad21*$^{+}$ asci containing four nuclei was altered when compared to *rec8* Δ, might indicate that the type of segregation error occurring had been shifted. More in depth analyses of meiotic chromosome segregation would be necessary to determine the types of segregation errors occurring in these cells.

7.3.5 Further Consequences of Meiotic Rad21p Expression

7.3.5.1 HOMOLOGOUS RECOMBINATION

As previously described, replacing Rec8p with the mitotic cohesin Rad21p, is likely to affect several meiotic processes in which Rec8p is known to be involved. For example, Rec8p is required for meiotic homologous recombination during MI (DeVeaux & Smith, 1994; Molnar et al., 1995; Parisi et al., 1999; Watanabe & Nurse, 1999). It is possible that when Rec8p was replaced with Rad21p, the frequency of meiotic recombination was reduced, and that this led to MI chromosome segregation errors as described for *S. pombe rec8* mutants. During the normal mitotic cell cycle, Rad21p is thought to promote homologous recombination between sister chromatids, to facilitate DNA double strand break repair (Tatebayashi *et al.*, 1998; Hartsuiker *et al.*, 2001). Therefore, during meiosis, Rad21p may also promote recombination between sister chromatids rather than homologous chromosomes.

7.3.5.2 CENTROMERE-KINETOCHORE ORIENTATION

Centromeric Rec8p is required for co-orientation of sister chromatids during MI (Watanabe & Nurse, 1999). Therefore, replacing Rec8p with Rad21p could prevent this co-orientation, leading to aberrant meiotic divisions. If this was found to be the case, it would be of interest to determine whether this was because ectopically expressed Rad21p had not been loaded onto the inner centromere, or if Rad21p was present at the inner centromere, it failed to co-orientate sister chromatids because this function was unique to Rec8p.

7.4 SUMMARY

7.4.1 Rec8p during Mitosis

1. *rad21*$^+$ was found to be expressed exclusively during the mitotic cell cycle and *rec8*$^+$ was found to be expressed exclusively during meiotic development.
2. Rad21p was essential for mitotic growth of *S. pombe* cells.
3. When ectopically expressed both at physiological levels, and at the appropriate time during the mitotic cell cycle, Rec8p complemented the essential function of Rad21p, although viability was reduced by 30%.
4. Replacing Rad21p with Rec8p did not cause a significant increase in cell division time, nor cause an S phase delay.

7.4.2 Rad21p during Meiosis

5. No alteration was seen in the timing of pre-meiotic S phase progression in cells lacking Rec8p.
6. Ectopic expression of *rad21*$^+$ in place of *rec8*$^+$ both at physiological levels and at the appropriate time in the meiotic cell cycle, did not alter the timing of pre-meiotic S phase progression.
7. In *rec8*Δ meioses, ascus development was severely impaired and spore viability was reduced.
8. Normal ascus development was restored and the percentage of viable spores increased, when Rec8p was replaced with Rad21p during meiosis.
9. Chromosome segregation was defective in *rec8*Δ meioses.

10. Rad21p was unable to rescue the meiotic chromosome segregation defects of *rec8*Δ meiosis.

7.5 FUTURE DIRECTIONS

Further work is required to characterise the strains described in this study to fully investigate the mitotic and meiotic-specific functions of Rad21p and Rec8p cohesins.

7.5.1 ChIP

Chromatin immuno-precipitation assays could reveal where on the chromosome the ectopically expressed cohesins had been loaded, with particular regard to the centromeric region. This might provide an insight into the link between centromeric localisation of cohesin and the regulation of chromosome segregation.

7.5.2 Effects on recombination

Recombination assays would reveal if the presence of Rec8p during mitosis increased the frequency of recombination, which could cause chromosome fragment loss. Conversely, Rad21p might reduce the frequency of meiotic recombination, which is known to lead to meiotic segregation defects. It would also be of interest to see if the type of recombination events were altered. For example, replacing Rec8p with Rad21p during meiosis might promote crossovers preferentially between sister chromatids, rather than homologues.

7.5.3 DNA double strand breaks

Rad21p is required for the repair of DNA double-strand breaks by homologous recombination between sister chromatids, during the mitotic cell cycle. To investigate this mitotic-specific function further, the repair of radiation-induced DNA double strand breaks could be analysed in cells where Rad21p was replaced with Rec8p. This could be expanded further to look at binding partners for Rad21p, and to see if these are changed in the presence of DNA damage.

7.5.4 Chromosome segregation

Labelling of centromeres with fluorescent tags would allow detailed analysis of chromosome segregation. In particular it would reveal the types of segregation errors

occurring when Rec8p was replaced with Rad21p during meiosis. A temperature-sensitive *S. pombe cdc2*$^{-}$ mutant, which arrests before the onset of MII, would allow analysis of MI and MII segregation separately. Centromere tagging would also allow analysis of mitotic chromosome segregation when Rad21p was replaced with Rec8p.

7.5.6 Ascus development

It would be of interest to investigate the ability of Rad21p to support ascus development, which might signify a mitotic role for Rad21p in septation and cytokinesis. This could be first investigated by examining septation in *rad21*$^{-}$ mutants.

7.5.7 Mutation analysis

Generating truncated and point mutants of Rad21p and Rec8p, might reveal the positions of specific cohesin functions, and whether those that overlap are located in their conserved regions. This might also provide insights into the functions of regions that are not conserved, and whether these are involved in the associations of Rad21p and Rec8p with other mitotic and meiotic-specific cohesins.

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Appendix I: *S. pombe* Strains

Strain no.	Genotype	Origin
GG 27	*h⁻ cdc10-129*	Lab stocks
GG 95	*h⁻ cdc25-22 leu1-32 ura4-D18*	Lab stocks
GG 164	*h⁻ ade6-M216 leu1-32*	Lab stocks
GG 165	*h⁺ ade6-M210 leu1-32*	Lab stocks
GG 177	*h⁻/ h⁻ pat-114 pat1-114 ade6-M210/ade6M-26 ura4-294/ura4⁺ leu⁺/leu1-32 arg1⁺/arg1-2 end1-458/end1-458*	Li & Smith (1997)
GG 190	*h⁺ pat1-114 his1-102 leu1-32 ura4-294*	Lab stocks
GG 217	*972 h⁻*	Lab stocks
GG 218	*975 h⁺*	Lab stocks
GG 278	*h⁻ rec8::ura4⁺ ura4-D18 leu1-32*	J. Kohli
GG 292	*h⁻ rec8 :: ura4⁺ pat 1-114 ura4-D18 leu 1-32*	This study
GG 297	*h⁺ rec8 :: ura4⁺ pat 1-114 ura4-D18 leu 1-32*	This study
GG 299	*h⁻ rec8::ura4⁺ pat1-114 ura4-D18 leu1-32 ade6-M216*	This study
GG 301	*h⁻ rec8::ura4⁺ pat1-114 ura4-D18 leu1-32 ade6-M210*	This study
GG 320	*h⁻ cdc25-22 ura4-D18 rad21::ura4⁺ nmt1*P::*rad21⁺*	This study
GG 323	*h⁻/ h⁻ rec8::ura4⁺/rec8::ura4⁺ pat1-114/pat1-114 ura4 -D18/ura4-D18 leu1-32/leu1-32 ade6-M216/ade6-M210*	This study
GG 327	pSP1/ *rad21*P: *rec8⁺* *h⁻ cdc25-22 leu1-32 ura4-D18 rad21::ura4⁺ nmt1*P::*rad21⁺*	This study
GG 328	pSP1/ *rec8*P: *rad21⁺* *h⁻/ h⁻ rec8::ura4⁺/rec8::ura4⁺ pat1-114/pat1-114 ura4-D18/ura4-D18 leu1-32/leu1-32 ade6-M216/ade6-M210*	This study

Strain no.	Genotype	Origin
GG 335	*h⁻ pat1-114 leu1-32 ura4-294*	This study
GG 336	*h⁺ pat1-114 leu1-32 ura4-294*	This study
GG 339	*h⁻ pat1-114 leu1-32 ade6-M210*	This study
GG 340	*h⁺ pat1-114 leu1-32 ade6 -M210*	This study
GG 341	*h⁺ pat1-114 leu1-32 ade6-M216*	This study
GG 342	*h⁻ pat1-114 leu1-32 ade6-M216*	This study
GG 345	pSP1/ *nmt1*P::*rad21*⁺ *h⁻ rad21*::*ura4*⁺ *cdc25-22 leu1-32 ura4-D18*	This study
GG 346	pJK148/ *nmt1*P::*rad21*⁺ *h⁻ rad21*::*ura4*⁺ *cdc25-22 leu1-32 ura4-D18*	This study
GG 347	pJK148 *h⁻ pat1-114 leu1-32 ade6-M216*	This study
GG 348	pJK148: *rec8* P: *rad21*⁺ *h⁻ rec8*::*ura4*⁺ *pat1-114 ura4-D18 leu1-32 ade6-M216*	This study
GG 349	pJK148: *rec8* P: *rad21*⁺ *h⁻ rec8*::*ura4*⁺ *pat1-114 ura4-D18 leu1-32 ade6-M210*	This study
GG 358	pJK148/ *rad21*P: *rec8*⁺ *h⁻ cdc25-22 leu1-32 ura4-D18 rad21*::*ura4*⁺ *nmt1*P::*rad21*⁺	This study
GG 359	pJK148 *h⁻ pat1-114 leu1-32 ade6-M210*	This study
GG 375	*h⁻/h⁻ pat1-114/pat1-114 leu1-32/leu1-32* *ade6-M216/ade6-M210*	This study
GG 401	pJK148/ pJK148 *h⁻/h⁻ pat1-114/pat1-114 leu1-32/leu1-32* *ade6-M210/ade6-M216*	This study

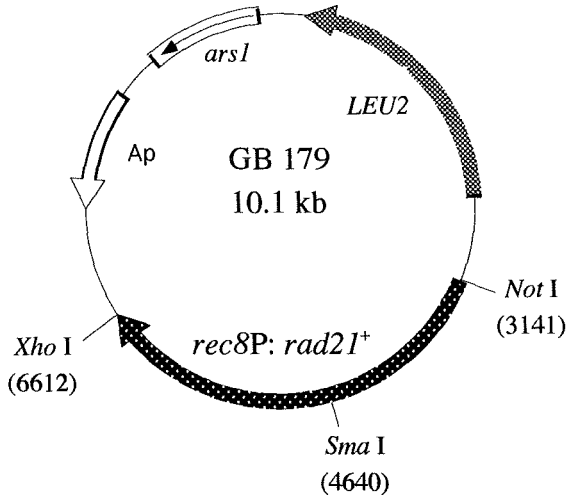
Strain no.	Genotype	Origin
GG 402	pJK148/ *rec8*P: *rad21*$^+$/ pJK148/ *rec8*P: *rad21*$^+$ *h*$^-$/*h*$^-$ *rec8*::*ura4*$^+$/*rec8*::*ura4*$^+$ *pat1-114*/*pat1-114* *ura4-D18*/*ura4-D18 leu1-32*/*leu1-32 ade6-M216*/*ade6-M210*	This study
GG 408	pSP1 *h*$^-$/*h*$^-$ *pat1-114* /*pat1-114 leu1-32* /*leu1-32* *ade6-M216* /*ade6-M210*	This study
GG 417	*h*$^+$/*h*$^-$ *rad21*::*ura4*$^+$/*rad21*$^+$ *ura4-D6*/*ura4-D6* *leu1-32*/*leu1-32 ade6-M210*/*ade6-M216*	Watanabe & Nurse (1999)
GG 434	pJK148/ *rec8*P: *rad21*$^+$ *h*$^+$ *rec8*::*ura4*$^+$ *pat1-114 ura4-D18 leu1-32 ade6-M216*	This study
GG 486	pJK148/ *rec8*P: *rad21*$^+$ *h*$^+$ *rec8*::*ura4*$^+$ *pat1-114 ura4-D18 leu1-32 ade6-M216*	This study

Appendix II: Primers

Primer no.	Gene	Sequence
GO 31	*cdc22*$^+$ forward	5'-ACA CGC GGT CCA ACC CGA-3'
GO 32	*cdc22*$^+$ reverse	5'-AGC GGA ACT TTG ATG TTC-3'
GO 118	*rad21*$^+$ forward	5'-GCG TTA GGG TCC TTT GGC CA-3'
GO 119	*rad21*$^+$ reverse	5'-CAA GCA TAA CGG TGA GGT G-3'
GO 146	*rad21*$^+$ promoter +*Xho* I	5'-GTT ACC CTC GAG TTC ATC CTT CAA TTC TCT TT-3'
GO 147	*rad21*$^+$ promoter + *Sma* I	5'-CAT TCC CCC GGG AAG AAG CTT AAT ATG ATT AG-3'
GO 148	*rad21*$^+$ ORF + *Sma* I	5'-CAT TCA CCC GGG ATG TTC TAT TCA GAG GCC AT-3'
GO 149	*rad21*$^+$ ORF + *Not* I	5'-GGT CAA GCG GCC GCT CAT AGT GAT GAA AGT AGC A-3'
GO 150	*rec8*$^+$ promoter + *Xho* I	5'-GTA CCA CTC GAG GTA GTA ATT TTG TAC TTG TT-3'
GO 151	*rec8*$^+$ promoter + *Sma* I	5'-TTA ACG CCC GGG GGT GAA AGA CAG GAA CGC GT-3'
GO 152	*rec8*$^+$ ORF + *Sma* I	5'-TCA ATT CCC GGG ATG TTT TAC AAT CAA GAT GT-3'
GO 153	*rec8*$^+$ ORF + *Not* I	5'-AAT GGA GCG GCC GCT CAA ATG GCA TCG GTG CTT T-3'
GO 167	*rec8*$^+$ forward	5'-TGA CAT GAG TTC TTT CAC TC-3'
GO 168	*rec8*$^+$ reverse	5'-CGT GGT TTG ATT GTG ATA CT-3'
GO 197	*nmt1*$^+$ promoter	5'- GAG GAA TCC TGG CAT ATC AT-3'
GO 198	*rad21*$^+$ reverse for *nmt1*P::*rad21*$^+$ construct	5'-TCA ATA ATT GCA CGT TGA CG-3'

All primer stocks were 100 pmol/ml

Appendix III: Constructs

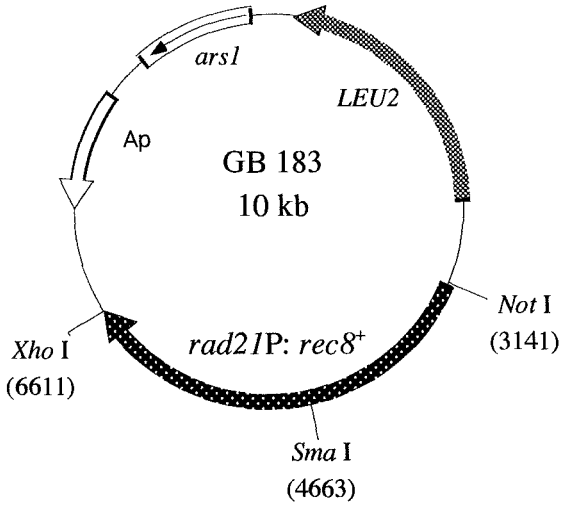


Origin: *rec8*P:*rad21*$^{+}$ inserted into pSP1

ars1: *S. pombe* autonomously replicating sequence

Ap: bacterial β-lactamase gene conferring ampicillin resistance

LEU2: *S. cerevisiae LEU2* gene

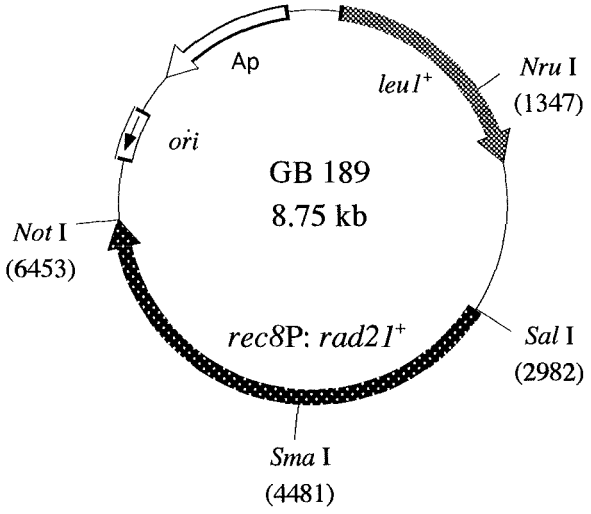


Origin: *rad21*P: *rec8*$^{+}$ inserted into pSP1

ars1: *S. pombe* autonomously replicating sequence

Ap: bacterial β-lactamase gene conferring ampicillin resistance

LEU2: *S. cerevisiae LEU2* gene

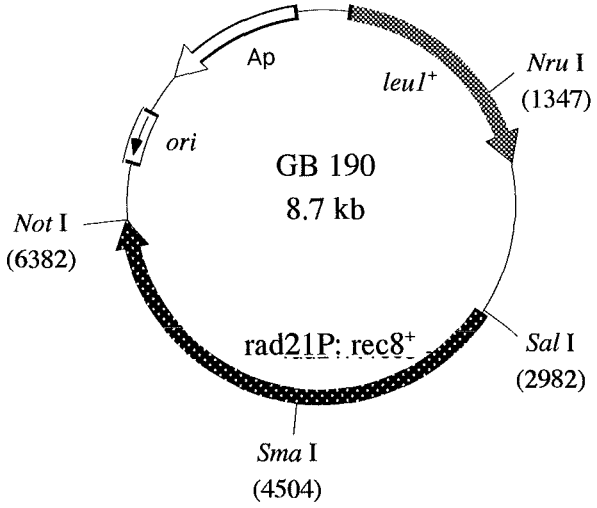


Origin: *rec8*P: *rad21*$^+$ inserted into pJK148

ori: bacterial origin of replication

Ap: bacterial β-lactamase gene conferring ampicillin resistance

leu1$^+$: *S. pombe leu1*$^+$ gene



Origin: *rad21*P: *rec8*$^+$ inserted into pJK148

ori: bacterial origin of replication

Ap: bacterial β-lactamase gene conferring ampicillin resistance

leu1$^+$: *S. pombe leu1*$^+$ gene