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**Analysis of the *S. pombe* sister  
chromatid cohesin subunit  
in response to DNA damage agents  
during mitosis**

**A thesis submitted for the degree of  
Doctor of Philosophy at the University of Glasgow**

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## Abstract

The accurate distribution of a fully replicated, intact genome perhaps defines the purpose of the cell cycle, which is a complex, organized series of events pertaining to the production of daughter cells. Every eukaryotic cell undergoes two alternative life cycles: mitosis or meiosis, the first producing identical daughter cells of a diploid genetic content, and the second for genetic diversity and cells of a haploid genetic content. Studies in various experimental systems, from yeasts to humans, have identified the conservation of many basic features, and thus produced a unified view that this process is essentially the same in all eukaryotes (Nurse, 2000). The relevance of cell cycle studies cannot be overstated and their importance in elucidating the underlying causes of many diseases has proved invaluable in modern medicine.

Chromosomes can be perceived as the packages for genetic information; they are replicated during S phase in both mitosis and meiosis to produce two identical sister chromatids which are subsequently segregated during the respective anaphases of both life cycles. Cohesin is a proteinacious structural complex holding sister chromatids together until nuclear division. Defects in this complex can cause chromosome mis-segregation inevitably causing an unbalanced genetic cell content which can either manifest as disease or give rise to inviable progeny. The subjects of this study are two homologous cohesin genes *rad21*<sup>+</sup> and *rec8*<sup>+</sup> expressed exclusively in either mitosis or meiosis respectively of the fission yeast *Schizosaccharomyces pombe*. All eukaryotes have two copies of these genes both analogously expressed and functionally similar, but undergoing life cycle specific expression.

The aim of this study was to investigate the mitotic and meiotic specific functions of *rad21*<sup>+</sup> and *rec8*<sup>+</sup>. To this end their specific pattern of expression was exchanged so that *rad21*<sup>+</sup> was expressed in the meiotic cycle and *rec8*<sup>+</sup> in the mitotic cycle. Previous work from the laboratory showed that *rec8*<sup>+</sup> when expressed in mitosis at G1-S instead of *rad21*<sup>+</sup> allows normal growth and division. Despite this initial observation, when the effects of various damaging agents applied during mitosis were examined some significant differences became apparent. In comparison to wild-type cells with functional SpRad21p, cells in which SpRec8p was expressed showed a reduction in repair efficiency which was revealed from cell survival, gene expression and chromosome integrity data.

The first results chapter of this thesis introduces the two fission yeast cohesin subunits of interest. Firstly, the similarities between SpRec8p and SpRad21p are highlighted in terms of cell cycle regulation via the control of the DSC1 complex, and their similar functions to prevent premature separation of sister chromatids during anaphase. Secondly, the main differences between the two cohesins are emphasised, in that both are solely expressed during alternative life cycles. During the course of a synchronous wild-type mitosis, northern analysis of *rad21*<sup>+</sup> mRNA levels revealed that this cohesin subunit is periodically expressed with peak transcript levels present at the G1-S boundary whilst, in contrast, no *rec8*<sup>+</sup> transcript was detected throughout mitosis. For the duration of a synchronous meiotic cycle, peak levels of *rec8*<sup>+</sup> transcript were detected, again at the G1-S boundary, whereas no *rad21*<sup>+</sup> transcript was detectable. These data are consistent with and confirm published observations (Lin *et al.*, 1992; Birkenbihl & Subramani, 1995).

Following these preliminary experiments, a more in depth analysis of the cohesin subunits was completed, utilising three strains: a wild-type control, a *rad21-45* mutant, and a repressible strain, *rad21P:rec8*<sup>+</sup> *nmt1P:rad21*<sup>+</sup>. In the latter strain cohesin expression is swapped, thus forcing expression of the meiotic cohesin *rec8*<sup>+</sup> in the mitotic cell cycle and replacing *rad21*<sup>+</sup>. The strains were treated with three damaging agents, ultraviolet-C (UV-C) radiation, methyl-methane sulfonate (MMS) and phleomycin.

Qualitative and quantitative data were collected which demonstrated that regardless of the damaging agent used, increasing concentrations resulted in decreased cell survival in the tested strains. With UV-C most cell death took place at 150 J, decrease in survival rates for MMS treated cells was more apparent at a concentration of 0.3%, and the phleomycin data showed most cell death at 20  $\mu\text{gml}^{-1}$ . Furthermore, when the survival rates of the three strains were compared between the different damaging agents the pattern of survival was consistently in the order of the wild-type control most resistant, followed by the *rad21-45* mutant, and the *rad21P:rec8*<sup>+</sup> *nmt1P:rad21*<sup>+</sup> strain most sensitive. These data allowed a visual analysis of the strains, demonstrating that the strain in which *rec8*<sup>+</sup> was expressed had the least viability irrespective of the damaging agent.

The effects of MMS were analysed further in the three strains with quantification of transcriptional profiles of the two cohesins by the northern blot technique and analysis of chromosome integrity by pulse-field gel electrophoresis (PFGE).

The northern blot experiments indicated that *rad21*<sup>+</sup> expression levels increased with higher concentrations of MMS, and cell viability decreased at concentrations above 0.1%. The induction of *rad21*<sup>+</sup> transcription after addition of MMS was also observed in cells arrested at G2-M after just 60 minutes, implying that this DNA damaging agent causes the direct induction of expression of this cohesin gene. Levels of *rec8*<sup>+</sup> transcript were also apparent in the *rad21P:rec8*<sup>+</sup> *nmt1P:rad21*<sup>+</sup> strain at the same MMS concentration, although expression was not induced. Chromosome integrity experiments were carried out using PFGE at a concentration of 0.05% MMS. Samples for PFGE and microscopic analysis were taken before, during and after MMS treatment. Through the course of the experiments samples were taken hourly to monitor growth, which demonstrated that after recovery wild-type cells resumed normal growth; similarly, the *rad21-45* mutant cells returned to an almost normal growth rate. However, the strain in which *rec8*<sup>+</sup> was expressed instead of *rad21*<sup>+</sup> did not recover a normal growth pattern. Although northern data revealed the presence of *rec8*<sup>+</sup> transcript at a concentration of 0.1%, PFGE data suggested that repair to chromosomes did not occur. Microscopic analysis revealed that only wild-type was unaffected, with the other two strains demonstrating elongated phenotypes characteristic of cell division cycle arrested cells. PFGE also revealed that after the recovery period, the only strain to recover the three chromosomes was wild-type.

Thus, data presented in this thesis suggest that the basic growth functions of *rec8*<sup>+</sup> and *rad21*<sup>+</sup> are conserved between the two genes during mitosis and meiosis, but that mitotic DNA repair is specific to *rad21*<sup>+</sup>. This research offers new insights in the function and role of these evolutionary conserved and important chromosome proteins.

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**For my dad**

چراغ بین ولد دل محمد  
بچھڑا وہ اس ادا سے کہ رت ہی بدل گئی  
اک شخص سارے شہر کو ویراں کر گیا

**Thank you for everything**

## Declaration

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

*Saeeda Bhatti*



## Abbreviations

A	Absorbance
aa	amino acid
ABC	ATP binding cassette
ACS	ARS consensus sequence
AP	apurinic/apyrimidinic
APC/C	anaphase promoting complex/cyclosome
ARS	autonomous replication sequence
Arks	Aurora related kinases
ATR	ATM and Rad3 related
ATP	adenosine triphosphate
BER	base excision repair
BSA	bovine serum albumin
<sup>60</sup> Co	cobalt-60
-CH <sub>3</sub>	methyl
ChIP	Chromatin Immunoprecipitation
CLC-CSC	complex-checkpoint sliding clamp
cnt	central
CO	cross over
CPD	cyclobutane pyrimidine dimer
<i>cut</i>	cell untimely torn
DAPI	4'-6-Diamidono-2-phenylindole
DDK	DfP1 dependent kinase
DMSO	dimethyl sulphoxide
DSC1	DNA synthesis control
DSB	double strand break
DSBR	double strand break repair
DSR	determinant of selective removal
DTT	dithiothreitol
EDTA	ethylenediaminetetraacetic acid
EMM	Edinburgh minimal media
EMM-N	nitrogen free media
FACs	fluorescent activated cell sorting
FISH	fluorescent <i>in situ</i> hybridisation
G0	stationary phase
G1	Gap 1
G2	Gap 2
GFP	green fluorescent protein
Gy min <sup>-1</sup>	Grays per minute

hr	hour
HR	homologous recombination
<i>H. sapiens</i>	<i>Homo sapiens</i>
imr	inner repeats
J m <sup>-2</sup>	joules per metre squared
kb	kilo basepair
LB	luria broth
MI	meiosis I
MII	meiosis II
MAGE	melanoma antigen encoding gene
MBF	<i>Mlu</i> I cell cycle box binding factor
MCB	<i>Mlu</i> I cell cycle box
MCM	minichromosome maintenance
ME	malt extract
min	minute
MMR	mismatch repair pathway
MMS	methyl methane sulfonate
MNE	MOPS/NaOAc/EDTA
MOPS buffer	3-(N-Morpholino)-propanesulfonic acid buffer
mRNA	messenger RNA
MTOC	microtubule organizing centre
<i>M. musculus</i>	<i>Mus Musculus</i>
M phase	Mitotic phase
N <sub>6</sub>	random hexanucleotide
NCO	no cross over
NER	nucleotide excision repair
NHEJ	non homologous end joining
NLS	nuclear localisation signal
<i>nmt</i>	no message in thiamine
<i>nmt1P</i>	<i>nmt1</i> promoter
OD	optical density
ORC	origin replication complex
ORF	open reading frame
OTR	outer repeats
PCR	polymerase chain reaction
PCNA	proliferating cell nuclear antigen
PEG	polyethylene glycol
PFGE	pulse-field gel electrophoresis
PMSF	phenylmethanesulfonyl fluoride
PMS	post meiotic segregation
post-RC	post-replicative complex

PP2A	protein phosphatase 2 A
pre-RC	pre-replicative complex
pre-IC	pre-initiation complex
R	restriction point
RFC	replication factor C
<i>rhp</i>	<i>rad</i> homologue in <i>pombe</i>
RNA	ribonucleic acid
rRNA	ribosomal RNA
RNAse	ribonuclease
S phase	synthesis phase
SARs	scaffold attachment regions
sec	second
<i>Sc</i>	<i>Saccharomyces cerevisiae</i>
SC	synaptonaemal complex
SCC	sister chromatid cohesion
<i>S. cerevisiae</i>	<i>Saccharomyces cerevisiae</i>
SIN	septation initiation network
SMC	structural maintenance chromosomes
S phase	DNA synthesis
Sp	<i>Schizosaccharomyces pombe</i>
SPAC	spindle assembly checkpoint
SPB	spindle pole body
<i>S. pombe</i>	<i>Schizosaccharomyces pombe</i>
SUMO	small ubiquitin related modifier
SWI/SNF	mating-type switching/sucrose non-fermenting
T-167	Threonine 167
TBE	Tris/Borate/EDTA buffer
TE	Tris/EDTA
TLS	translesion DNA synthesis
Tris	2-amino-2-(hydroxymethyl)-1,3-propanediol
Triton X-100	octyl phenol ethoxylate or polyoxyethylene Octyl phenyl ether
U	units
UAS	upstream activating sequence
UTR	untranslated region
UV-C	ultraviolet-C
UVDE	ultraviolet damage endonuclease
UVER	UV damage repair pathway
w/v	weight per volume
Y15	Tyrosine 15
YE	yeast extract

# Chapter 1

## 1.1 Introduction

Survival of an organism is dependant on the accurate transmission of genetic information from one cell to progeny cells. Such faithful transmission requires accuracy in the replication of DNA, precision in chromosome distribution, and the ability to survive spontaneous and induced DNA damage.

Replication of DNA takes place during S phase of the cell cycle to give two identical sister chromatids. At M phase the chromosomes align on the metaphase plate and the sister chromatids are attached via their kinetochores to microtubules radiating from the spindle poles. In mitosis one cellular division takes place at anaphase, when the sister chromatids separate with an equational pattern to be evenly distributed between two daughter cells. Meiotic replication takes place during pre-meiotic S phase and has two divisions, the first called the reductional division, when recombination events occur and homologous chromosomes separate. The second division is the separation of sister chromatids and is similar to the mitotic equational pattern; with reference to fission yeast it results in four haploid yeast spores.

Cohesin is a chromosomal protein complex comprised of four core subunits which holds replicated sister DNA strands together after their synthesis. Sister chromatids are tethered until their equational division during mitotic anaphase, or the second meiotic division. The fission yeast *Schizosaccharomyces pombe*, (*S. pombe*) has two homologous genes, *rec8*<sup>+</sup> and *rad21*<sup>+</sup>, that are specific to meiosis and mitosis respectively and encode components of the cohesin complex. Expression of both genes is regulated during their respective cell cycles, with maximum transcript and protein being present during DNA replication.

Long before the essential role of cohesin in chromosome segregation was recognized investigators found that one of the sub-units encoded by *rad21*<sup>+</sup> was also involved in DNA repair in *S. pombe*, as mutants of *rad21*<sup>+</sup> had decreased survival after exposure to ionizing radiation. An observed increase in cell survival during DNA replication was attributed to the formation of two identical sister chromatids, as the physical linkage of sister chromatids by the cohesin complex is vital for double strand break repair (DSBR) (Birkenbihl & Subramani, 1992). The protein encoded by the *rec8*<sup>+</sup> gene is required for

several meiotic processes including linear element formation, alignment of homologous chromosomes and homologous recombination. This meiotic cohesin is also essential for reductional division during the first meiotic division and normal spore development (Watanabe & Nurse, 1999).

This project will investigate whether an overlapping function exists between these two highly similar genes. The following introduction outlines the importance of regulating cell cycle events to ensure accurate chromosome segregation. The importance of model organisms will be mentioned, in particular *S. pombe*, but also contrasting it with *Saccharomyces cerevisiae* (*S. cerevisiae*). This will then lead into a discussion on mitotic and meiotic events and the mechanisms in place to ensure that progeny cells receive the full genetic complement. It then will go onto DNA replication and examine the macromolecular complex cohesin, to introduce the cohesin subunits of interest in this project, SpRad21p and SpRec8p. Finally, it will look at the importance of understanding DNA damage and why cohesin is being studied within the context of this process.

## 1.2 Genetic model organisms

Model organisms in biological research are an indispensable resource for elucidating gene function. They are studied extensively to understand biological phenomena, with the anticipation that the knowledge gained from their study can be applied to higher organisms, especially humans. This approach is feasible since evolution not only reuses fundamental biological principles but conserves metabolic and regulatory pathways. Thus, biological explanations for some complex functions in higher eukaryotes can be found in lower eukaryotes. There are many eukaryotic model organisms, such as *Drosophila* and mouse. The ideal organism should contain a small genome similar to higher eukaryotes, but additionally be easily manipulated and analysed in the laboratory.

In eukaryotes, the yeasts *Saccharomyces cerevisiae* (“baker’s” or “budding yeast”) and *Schizosaccharomyces pombe* (“fission yeast”; pombe is Swahili for beer) have been widely studied, particularly because they are easy to work with under laboratory conditions. The yeast cell cycle is very similar to the cell cycle in humans, and is regulated by homologous proteins. Both *S. cerevisiae* and *S. pombe* are at the fore front in cell cycle studies and many of the key aspects of cell cycle control were first established in these yeast models (Nurse, 1998; Forsburg, 1999).

### 1.3 A note on nomenclature

Throughout this thesis, reference will be made mostly to proteins involved in fission yeast. However, homologous proteins from budding yeast and humans will be mentioned. Therefore, for the purpose of clarity, proteins will be distinguished by the following prefixes: Sp (*S. pombe*), Sc (*S. cerevisiae*) and Hs (*Homo sapiens*). Proteins will be written with the first letter a capital, and end with the letter “p” for protein.

### 1.4 The life cycles and genetics of *S. cerevisiae* and *S. pombe*

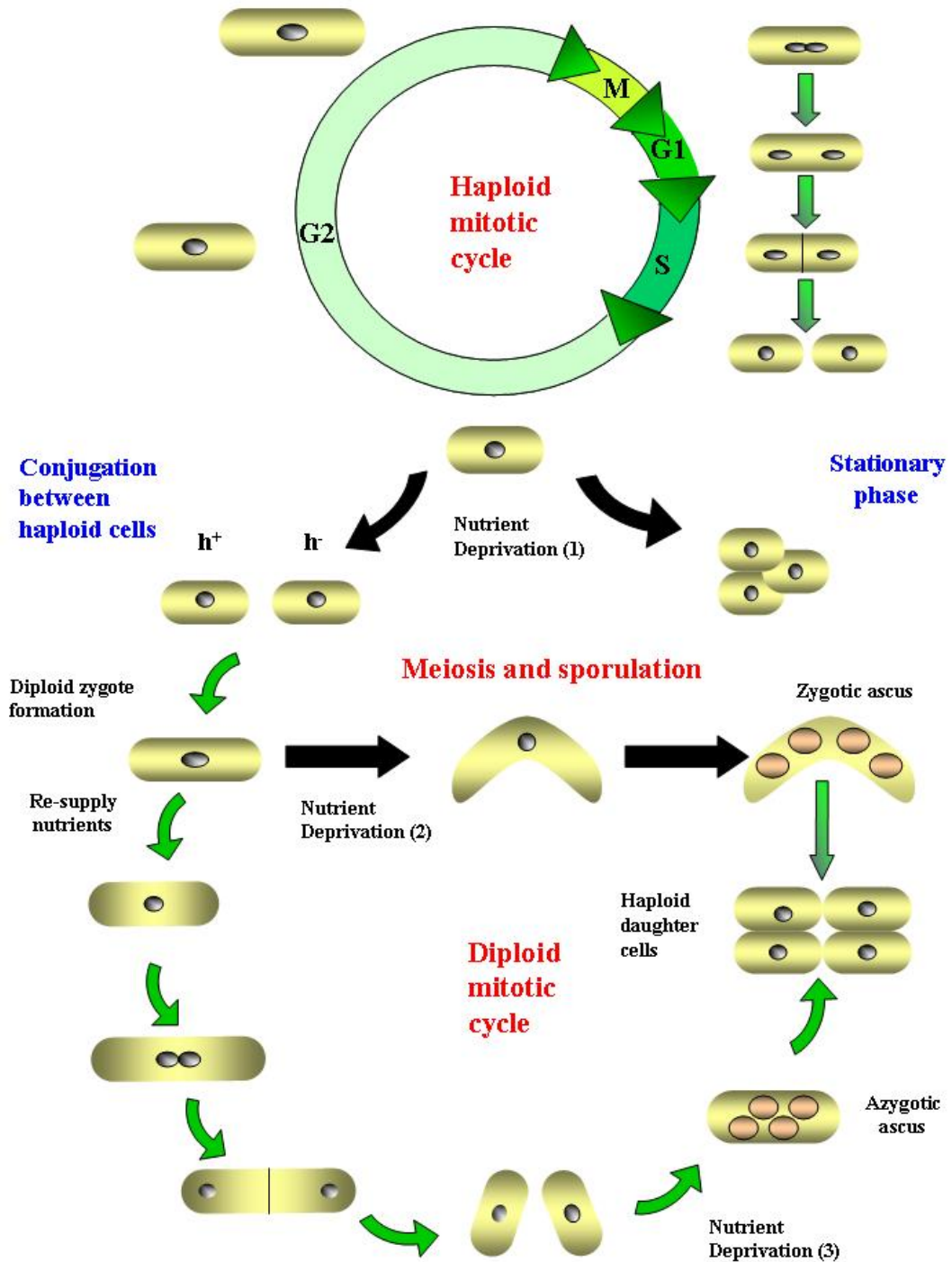
*S. cerevisiae* and *S. pombe* are both unicellular, eukaryotic ascomycete fungi which are distantly related, and are central to the study of the eukaryotic cell cycle. Despite being haploid organisms they share basic features that are common amongst higher eukaryotic systems, thus making them ideal model organisms for not only cell cycle research but also other cell biology studies (Lew *et al.*, 1997; Forsburg, 1999). The genomes of both species have been fully sequenced providing a vital tool for comparisons with other eukaryotes. *S. cerevisiae* was the first eukaryote to be sequenced and has more than 5500 genes contained in a 14 Mb genome, which is distributed amongst 16 chromosomes ranging from 230 kb to over 1 Mb in size (Goffeau *et al.*, 1996). In comparison, sequencing of the *S. pombe* genome identified nearly 5000 genes in a 14 Mb genome, which is distributed amongst 3 chromosomes 5.7, 4.7 and 3.5 Mb in size (Wood *et al.*, 2000).

#### 1.4.1 *Schizosaccharomyces pombe*

The fission yeast *S. pombe* has rod shaped cells of 7–14  $\mu\text{m}$  in length and 3–4  $\mu\text{m}$  in diameter, that grow by apical extension, and divides by septation followed by medial fission (Forsburg & Nurse, 1991; MacNeill & Nurse, 1997). Fission yeast normally exists in a haploid state and was first developed as a genetically useful organism in the 1950s by Urs Leupold, who isolated a homothallic strain called  $h^{90}$  from which he derived two heterothallic strains of opposite mating type,  $h^+$  and  $h^-$  (Leupold, 1958).

In the presence of nutrients, haploid fission yeast cells enter the mitotic cycle and multiply logarithmically. When cells are starved of nutrients several developmental fates are open to them (Figure 1.1). If cells of only a single mating type are present, fission yeast will exit

mitosis and accumulate in stationary phase. If both mating types are present,  $h^+$  and  $h^-$ , conjugation occurs, resulting in the formation of a transient diploid zygote. The diploid state is not normally stable and the diploid zygote will usually undergo meiosis and sporulation to produce an ascus containing four haploid spores. However, if diploid cells are transferred into fresh nutrient rich media before meiosis has been initiated, they can enter mitosis as diploids. Subsequent removal of nutrients then initiates entry into meiosis and sporulation, without the need for intervening conjugation (Hayles & Nurse, 1992; MacNeill & Nurse, 1997). Diploid cells are morphologically larger since DNA and RNA content, protein and cell volume are proportional to the degree of ploidy; nutritional deprivation forces diploids to enter the mitotic cycle and sporulate without the need for conjugation. However, when resupplied with nutrients they germinate to form mitotic haploids. Diploid cells undergo meiosis and sporulation and are morphologically distinct from each other. Unruptured zygotic asci are recognised as “banana shaped”, while azygotic asci appear “rod-shaped”, with rounded ends.



**Figure 1-1: The *S. pombe* life cycle.**

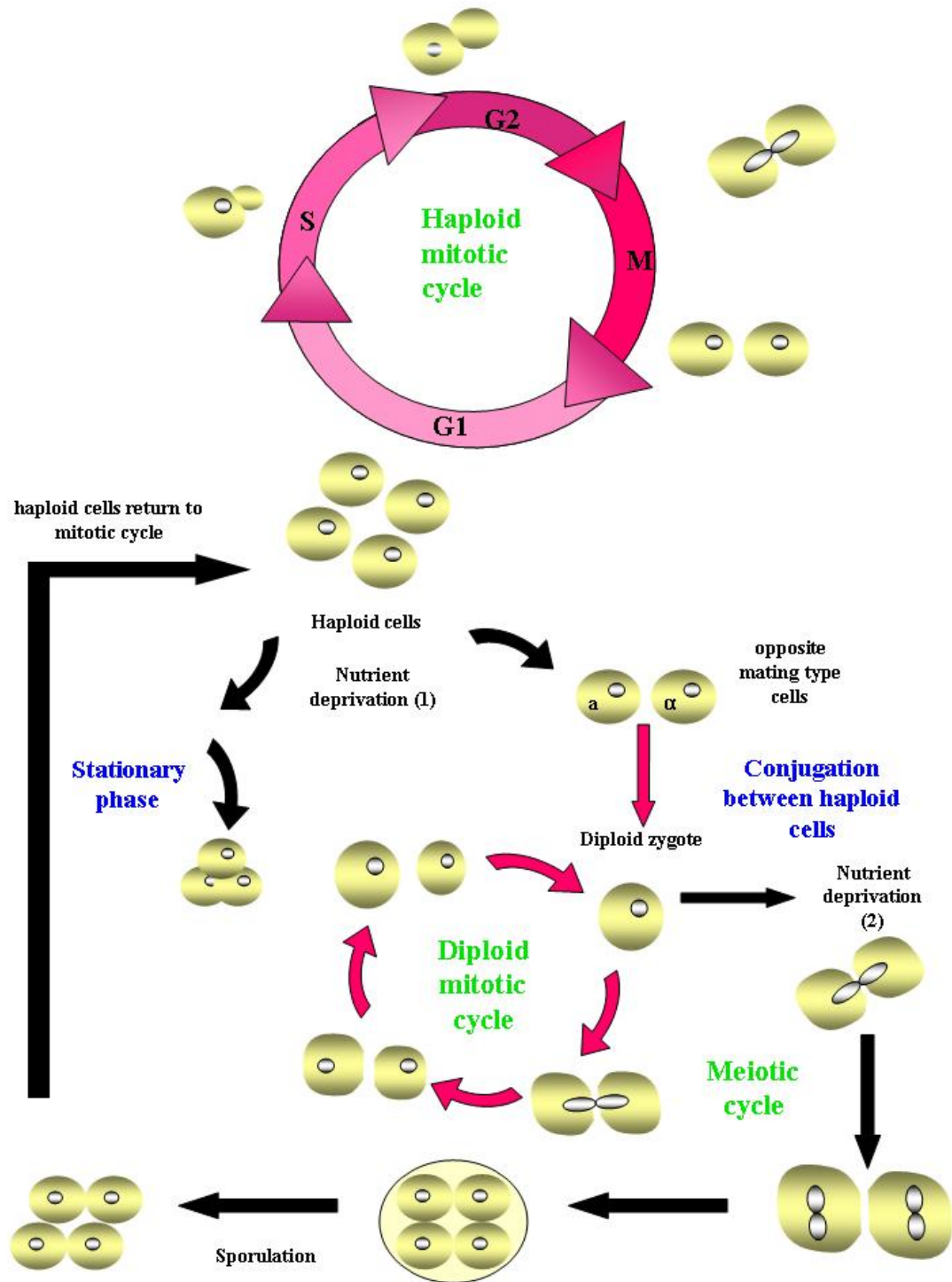
In the presence of a plentiful supply of nutrients fission yeast grows as a haploid. If cells undergo nutritional deprivation (1) they exit the mitotic cycle and can either go into stationary phase or if a cell of the opposite mating type ( $h^+$  and  $h^-$ ) is present undergo conjugation. The diploid state for *S. pombe* is unstable; if diploids undergo nutritional deprivation (2) they enter the meiotic cycle immediately following conjugation to sporulate and form four spores enclosed in an ascus, at this stage called the zygotic ascus. A temporary diploid state can be maintained by supplying fresh media, and if cells undergo nutritional starvation (3) after having entered mitosis as diploids, they can also sporulate to form four spores again enclosed in an ascus, but in this instance termed the azygotic ascus.



### 1.4.2 *Saccharomyces cerevisiae*

Similar to fission yeast, budding yeast grows in the haploid or diploid stage, although it employs a budding mode of division. In the absence of mating partners, haploid cells divide mitotically with a small bud being produced which grows throughout the cell cycle. The nucleus migrates into the neck of the bud where it then pinches off and, in comparison to the parent cell, a smaller, daughter cell is formed.

During G1 phase, budding yeast can take one of three alternative developmental fates (Figure 1.2). If nutrients are plentiful, cells can proceed through the cell cycle and divide. However, if starved, they leave the mitotic cell cycle and enter stationary phase, becoming resistant to heat and chemical treatment. Alternatively, if the cells are present as one of two opposite mating types, **a** and **α**, they can mate to form diploids. In contrast to *S. pombe*, *S. cerevisiae* diploid cells are stable. When starved for nutrients they undergo meiosis and sporulation to yield asci, enclosed by the cell wall of the original diploid cell, to contain four meiotic products. Diploid cells can choose to enter mitosis or meiosis, depending on nutrient conditions.



**Figure 1-2: The *S. cerevisiae* life cycle.**

Budding yeast grows by a process of bud formation in either the haploid or diploid state. In the presence of nutrients cells divide in the mitotic cycle as haploids. When starved, nutritional deprivation (1) they enter stationary phase, unless there is a cell of the opposite mating type (**a** and **α**) where they instead conjugate to form diploid cells. The budding yeast diploid state is stable and unless cells are nutritionally starved (2) they continue in this cycle. However, if diploid cells are starved, they enter meiosis and from sporulation four haploid spores result, which can enter the mitotic cycle when conditions become favourable again.

### **1.4.3 Phases of the yeast cell cycle**

As discussed, budding yeast grows with the formation of a small bud which grows continuously throughout the cell cycle. Bud formation and nuclear migration requires spindle pole body duplication and mitotic spindle formation relatively early in the cell cycle. This mechanism prevents a clear comparison of the budding yeast synthesis phase (S phase), Gap 1 (G1), Gap 2 (G2) and mitotic phase (M phase) with those of other eukaryotes. Budding yeast has a long G1 phase and the decision to enter a round of cell cycle occurs at the G1-S phase transition.

In contrast the cell cycle of fission yeast is very similar to that of higher eukaryotes, as it has distinct and consecutive G1, S, G2 and M phases. In rapidly growing cells the G1 and S phases are very short. This results in G1-S cells appearing as a doublet, because by the time cell separation occurs, the cells have already entered G2 phase. Compared to *S. cerevisiae*, *S. pombe* cells spend the majority of their time in G2, and cell cycle control in this yeast usually takes place by regulating the G2-M transition.

Interestingly, G1-S and G2-M control points exist and are functional in both yeasts. It is thought that the extended G2 phase in *S. pombe* makes it more resistant to DNA damage than *S. cerevisiae* since *S. pombe* haploid cells benefit by containing an extra copy of its genome which allows DNA repairs mechanisms to operate (Section 1.18).

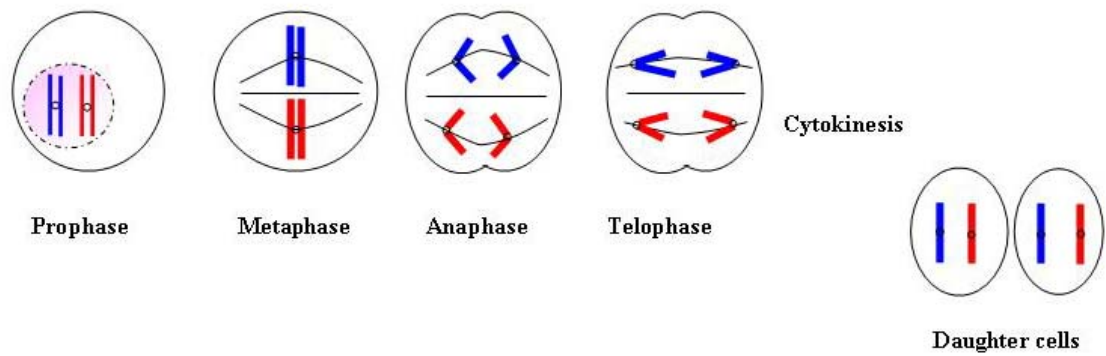
## **1.5 Overview of the eukaryote mitotic cell cycle**

The eukaryotic mitotic cell cycle is a highly regulated developmental sequence of temporal and spatial events that brings about the reproduction of the cell (Nurse, 1998). It has two main functions, firstly to allow growth of an organism, and secondly to regenerate damaged or worn out cells. The most important events of the cell cycle common to all eukaryotic cells are DNA synthesis (S phase), when chromosomes replicate, and M phase, when the replicated chromosomes segregate into the two nuclei in preparation for cell division. To ensure genomic integrity the genome must be fully and accurately replicated once per cell cycle. Cell survival is dependant on the single parent cell guaranteeing that each daughter cell receives a full complement of the hereditary material. Although an extensive study of the mitotic cell cycle in several different model organisms has lead to the view that the basic principles of the cell cycle apply to all eukaryotic cells, the details of regulation and relative amounts of time spent in the gap phases differ.

As mentioned the cellular processes characteristic of the cell cycle can be divided into four distinct phases; G1, S phase and G2 which are collectively known as interphase, and M-phase (Sullivan & Morgan, 2007). M phase itself is composed of two tightly coupled processes: mitosis, in which the cell's chromosomes are divided between two daughter cells, and cytokinesis, in which the cytoplasm physically divides.

During G1, the cell undergoes a period of rapid growth and high metabolic activity. RNA and proteins required for subsequent cell cycle progression are also synthesised. In this phase the cell also primes the DNA for replication, a process known as DNA licensing. Occurring near the end of G1 is the first checkpoint, which is known as "START" in both *S. pombe* and *S. cerevisiae*, and the "restriction point" (R) in higher eukaryotes. Also in G1 phase, the cell monitors its environment before making the decision to enter into S phase. In response to extracellular mitogens, growth factors or nutrients, cells pass through G1 and commit to mitosis and proceed with DNA synthesis. However, in unfavourable conditions, such as an inadequate supply of nutrients, cells can either proceed into the quiescence state (G0) (stationary phase in yeast) or undergo the sexual cycle. In general, the G1 phase constitutes a large proportion of the cell cycle. S phase begins when DNA replication is initiated lasting for as long as it takes for the entire DNA to be replicated. The cell then enters G2 phase where the cells continue to grow in size until the end of G2 when a second checkpoint ensures that cells have reached the minimum size required for entry into M phase and, most importantly, all chromosomes are intact and replicated completely free from damage. Thus, interphase is complete and mitosis takes place to form the daughter cells (MacNeill & Nurse, 1997).

The ensuing M phase is marked by the generation of bipolar mitotic spindles and can be further divided into four stages: prophase, metaphase, anaphase and telophase (Figure 1.3). During prophase, the replicated chromosomes begin to condense and are observable under the light microscope as two long threadlike structures called sister chromatids which are joined by a centromere. Near the end of prophase, the shortening chromosomes migrate to the cell equator. Metaphase then follows and is characterised by the alignment of the chromosomes along the equatorial plane. During anaphase the centromeres divide and the sister chromatids separate and migrate towards opposite poles of the cell. Telophase is characterised by the complete migration of the sister chromatids resulting in the equal distribution of the chromosomes between daughter nuclei. Finally, mitosis is complete when cytokinesis divides the cell to produce two identical daughter cells (Sullivan & Morgan, 2007).



**Figure 1-3: Eukaryotic mitosis.**

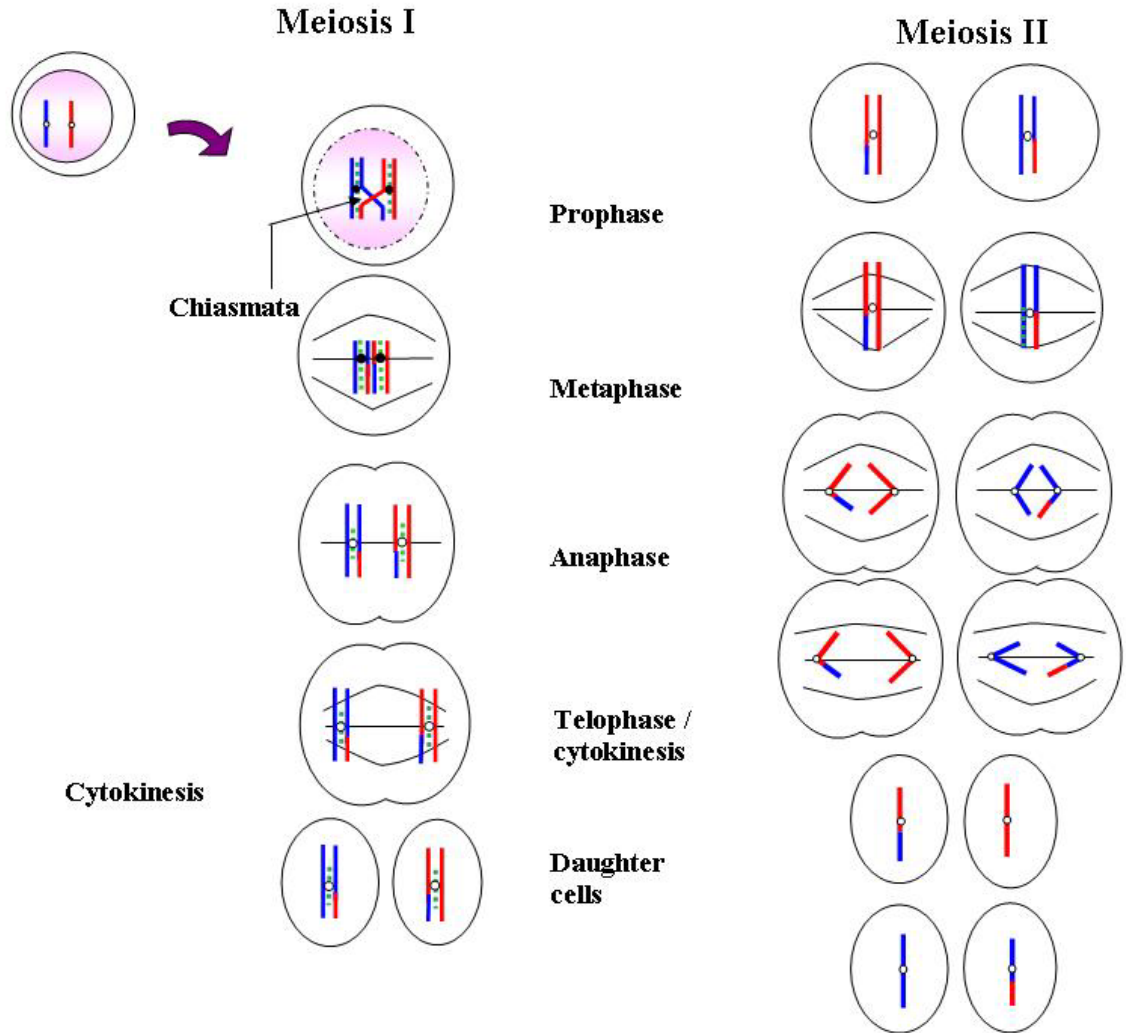
Throughout prophase chromosomes condense, becoming visible as a pair of sister chromatids and align on the mitotic spindle for metaphase. During anaphase the sister chromatids begin to separate and they reach opposite ends of the cell at telophase. Cytokinesis then occurs producing two new identical daughter cells. Yeasts do not have a nuclear membrane that is present in higher eukaryotes and shown in this diagram.

## 1.6 Overview of the eukaryote meiotic cell cycle

Meiosis occurs in the germ cells and is the means by which diploid eukaryotic organisms certify the maintenance of genetic diversity by exchanging genetic material between parental chromosomes. In comparison to mitosis there are two consecutive nuclear divisions, meiosis I (MI) and meiosis II (MII), after one round of DNA replication (Lee & Amon, 2001; Figure 1.4).

As in mitosis replication takes place during S phase or pre-meiotic S phase, to produce two identical sister chromatids. During the first meiotic division homologous chromosomes pair, recombine and segregate from each other. At prophase of MI, replicated homologous chromosomes align at the cell equator and undergo the process of recombination, where connections called chiasmata form between the homologous chromatid arms. In addition to cohesin, chiasmata also hold chromatids together and are the points where cross overs take place producing gametes genetically distinct from the parental cells (Page & Hawley, 2003; Petronczki *et al.*, 2003). At the metaphase to anaphase transition the first nuclear division takes place whereby homologous chromosomes separate; this first division, called the reductional division, is accompanied by meiotic recombination (Yamamoto, 1996) and halves the genetic content of the two resulting daughter cells. Immediately following MI, the second meiotic division occurs; MII resembles the normal mitotic division in which sister chromatids are separated. This division is called the equational division and generates four new, genetically diverse haploid cells (Roeder, 1997; Forsburg, 2002).

Aneuploidies, the most common recognised cause of mental retardation in humans, result in a gain or loss of chromosomes when compared to the normal constitution of a cell. In humans several clinically significant disorders have been defined. Approximately 35% of all conceptions have chromosomal abnormalities as a result of mis-segregation events in meiosis (Sluder & McCollum, 2000; Page & Hawley, 2003; Appendix I). Aneuploidies can involve the sex chromosomes such as monosomy of the X chromosome – Turners syndrome or disomy of the X chromosome XXY – Klinefelter syndrome; these are not as severe as autosomal aneuploidies. Autosomal aneuploidies are the most severe defects resulting in spontaneous abortions; only three autosomal aneuploidies are known to survive to a live birth, albeit the presence of severe physical and mental abnormalities. These are Down syndrome - trisomy 21, Patau syndrome or cleft palate - trisomy 13, and Edwards syndrome - trisomy 18 (Hassold *et al.*, 2007).



**Figure 1-4: Eukaryotic meiosis.**

During pre-meiotic S phase homologous chromosomes (blue, paternal; red maternal) are replicated to form sister chromatids held by cohesin (green) present along the length of chromosomes. The parental chromosomes line up and form chiasmata for cross over events to take place. At anaphase I of meiosis I, arm cohesin is lost and homologous chromosomes separate to form two daughter cells; these then undergo a second round of division which is similar to the mitotic division. In anaphase II, centromeric cohesin is lost and the two chromatids separate to four haploid meiotic products which are genetically distinct from the parental genotype.

Once a fission yeast cell commits to the meiotic cell cycle, it follows the same prototype as for all eukaryotes to result in the formation of four haploid spores (analogous to gametes) enclosed in an ascus (Figure 1.1). As mentioned previously, fission yeast is ideal for chromosome studies. It has only three chromosomes and thus even random segregation of chromosomes results in a considerable number of spores being developed which should be viable (Molnar *et al.*, 1995).

## 1.7 Cyclin dependant kinases (CDKs)

Since the majority of the experimental work described in this thesis has been carried out in mitotic cells, the following Sections will focus on the mitotic cell cycle. Mitosis has four discrete phases, G1, S, G2, M, within which there exist two checkpoints the first at the G1-S transition prior to DNA replication and the second at the G2-M transition just before chromosome segregation. These underlying mechanisms of these phases and transition points will be discussed followed by the segregation of chromosomes. However, first, cyclin dependant kinases (CDKs) will be described, since they play a pivotal role in the mitotic cell cycle.

Known as the master regulators of the cell cycle, CDKs coordinate the initiation of the two key cell cycle events, the replication of DNA followed by its equal distribution into progeny cells at mitosis (Morgan, 1995; Sullivan & Morgan, 2007). They range from 30–40 kDa in size and are all closely related with at least 40% amino acid sequence identity. CDKs are catalytically inactive in the monomeric state requiring associating with another essential molecular called a cyclin to become active. All cyclins share a region of homology known as the “cyclin box” which is responsible both for CDK association and activation (Lee & Nurse, 1987). Both the budding and fission yeasts have one CDK, encoded by the *CDC28* and *cdc2<sup>+</sup>* genes, respectively. Higher eukaryotes contain several CDKs, including CDC2/CDK1, CDK2, CDK4 and CDK6 (White, 2001).



### **1.7.1 *S. pombe* SpCdc2p is the most important mitotic cell cycle CDK**

SpCdc2p is by far the most significant CDK in the context of fission yeast cell cycle progression, required both for DNA replication and entry into mitosis (Nurse *et al.*, 1976). The *S. pombe cdc2<sup>+</sup>* gene encodes a 34 kDa serine/threonine kinase, SpCdc2p, that binds to three interphase cyclins, SpCig1p, SpCig2p and SpPuc1p, which are important in G1 (Connolly & Beach, 1994; Martin-Castellanos *et al.*, 1996), and one mitotic cyclin, SpCdc13p (Moser & Russell, 2000). Similarly, the budding yeast counterpart ScCdc28p (Reed, 1992; Nasmyth, 1993) associates with nine different cyclins, six B-type cyclins (ScClb1p-ScClb6p) required for S phase, and three G1 cyclins (ScCln1p-ScCln3p) essential for passage through START (Benito *et al.*, 1998). In contrast, in humans, HsCdc2p/HsCdk1p, which was identified on the basis of its functional homology to fission yeast SpCdc2p (Lee & Nurse, 1987), associates with relatively few cyclins. However, although HsCdc2p/HsCdk1p is the major controlling CDK in the human cell cycle, other CDKs have been identified and are present at various stages (Sherr, 1996).

Constant levels of SpCdc2p are detectable throughout the cell cycle. However the catalytic activity of SpCdc2p is determined largely by which of the four different cyclin partners it associates with at the alternating cell cycle stages, its phosphorylation and dephosphorylation status (Gould & Nurse, 1989), and the action of inhibitor proteins (Martin-Castellanos *et al.*, 1996; Benito *et al.*, 1998).

At START during G1, the major S phase cyclin, SpCig2p, associates with SpCdc2p to form an active protein kinase complex required for progression into S phase (Connolly & Beach, 1994; Martin-Castellanos *et al.*, 1996). However, the presence of SpRum1p, a negative regulator of SpCdc2p, accumulates during late M-G1, and is inhibitory to the activation of SpCig2p-SpCdc2p complex. The interaction of SpRum1p with the SpCig2p-SpCdc2p complex regulates the timing of S phase by maintaining cells in the pre-START stage until the minimum critical mass is reached; once the required size is reached cells irreversibly enter the mitotic cycle for DNA synthesis (Martin-Castellanos *et al.*, 1996).

Another substrate for SpCdc2p is SpCdc13p. SpCdc13p is the only essential cyclin in *S. pombe* functioning to prevent reinitiation of S phase and driving the events of mitosis. SpCdc13p cyclin levels rise during G2 phase and decrease slowly as cells progress through mitosis and G1. SpRum1p also blocks the activity of SpCdc13p-SpCdc2p complex,

although low levels of this complex may remain from the previous M phase. When SpRum1p binds to SpCdc13p it targets it for ubiquitin-mediated proteolysis by the 26S proteasome; if SpCdc13p-SpCdc2p was not degraded it would remain active and cause premature M phase, before chromosomes are duplicated, resulting in chromosome instabilities such as aneuploidies. Thus, the presence of SpCdc13p prevents the reinitiation of S phase and the degradation of it at the end of M phase is required to prevent premature M phase.

The phosphorylation of SpCdc2p affects its function, and the overall activity of this protein is regulated by changes in phosphorylation at two residues. Phosphorylation at the tyrosine-15 residue (Y-15) by SpWee1p and SpMik1p inhibits SpCdc2p. Whilst, in contrast, phosphorylation at threonine-167 (T-167) is essential for its activity (Gould *et al.*, 1991).

Degradation of cyclins and CDK inhibitors are important for moving from one phase of the cell cycle to the other, an activity controlled by the anaphase promoting complex/cyclosome (APC/C).

## 1.8 G1-S transition

In late G1, cells pass through START and make the commitment to enter into the mitotic cell cycle for a new round of chromosome duplication and cellular divisions. Two different mechanisms are present which mediate entry into S phase; the first controlling the transcription of genes required for DNA replication, and the second controlling SpCdc2p kinase activity.

### 1.8.1 *Transcriptional regulation*

One of the other major mechanisms of cell cycle regulation is that of transcription, or the regulation of gene expression. A large number of genes have been identified in *S. pombe* whose mRNA levels accumulate during late G1. Many of these encode functions required for the onset of and progression through S phase (McInerny, 2004; Rustici *et al.*, 2004).

Genes involved in the G1-S wave of expression contain common upstream activating sequence (UAS) in their promoter regions named the Mlu I cell cycle box (MCB) (McIntosh, 1993) as the DNA sequence, ACGCGT, is identical to the *Mlu* I restriction

enzyme recognition sequence. The DNA synthesis control 1 (DSC1), also known as the MCB binding factor (MBF), transcription factor complex binds to the MCB motif to control G1-S transcription (Lowndes *et al.*, 1992). A similar MBF-MCB system controls the expression of a group of genes at the start of S phase in *S. cerevisiae*.

During G1-S phase, DSC1 activates the expression of MCB containing genes necessary for the onset of S phase. In *S. pombe* at least eleven genes have been identified that are expressed during this transition. These include *cdc22*<sup>+</sup>, encoding the large subunit of ribonucleotide reductase, *cdc18*<sup>+</sup>, required for DNA replication initiation, *cig2*<sup>+</sup>, encoding B-type cyclin, *cdt1*<sup>+</sup>, essential for DNA synthesis and the “START” control, and *cdt2*<sup>+</sup> required for DNA replication (McInerny, 2004).

## 1.9 DNA replication – S phase

It is imperative for the genome of a proliferating eukaryotic cell to not only be duplicated accurately but exactly once during S phase of each cell division cycle. Initiation of DNA replication has to be coordinated with the ensuing chromosome segregation; no DNA segment must be left un-replicated, nor must separation take place until all DNA has been replicated. If cells enter mitosis before completion of replication or replicate their DNA a second time before division, faithful chromosome segregation is interrupted, genomic instability occurs in daughter cells, resulting in cell death or malignant cell growth.

In *S. pombe* replication of the parental genome is initiated along multiple sites strategically located along chromosome arms so that it is complete before the impending M phase. Regulation occurs both temporally and spatially so that the three *S. pombe* chromatids are replicated with high fidelity to generate the two sister chromatids which are distributed during M phase.

The complex task of one cell cycle per duplicating genome is achieved by a licensing system, where a cell carefully coordinates the events culminating in the recruitment of the components necessary to initiate DNA synthesis.

### **1.9.1 Origins of replication – autonomously replicating sequences (ARS)**

Specific sites within the DNA molecule are present along the lengths of chromosomes where DNA replication is initiated (Gomez & Antequera, 1999). Autonomously replicating sequences (ARS) elements are stretches of yeast chromosomal DNA within replication origins capable of replicating autonomously. First identified in budding yeast, these cis-acting DNA elements are defined by 100-200 bp blocks of DNA containing the ARS consensus sequences (ACS) composed of an 11 bp AT-rich consensus sequence (Nishitani & Lygerou, 2004).

In comparison to budding yeast, although an 11 bp ACS was identified in fission yeast it conferred little effect on replication activity when deleted; significantly fission yeast requires several clusters of 50-1000 bp long AT-rich stretches with one or more sequence blocks of 20-50 bp (Maundrell *et al.*, 1988; Masukata *et al.*, 2003; Nishitani & Lygerou, 2004; Dai *et al.*, 2005). Studies in eukaryotes imply that long regions of DNA containing discrete blocks of essential sequences are important for initiation of DNA replication.

Origins move from a pre-replicative state – ready to fire under favourable conditions - to a post-replicative state when they can no longer fire. The two processes are separated temporarily and are mediated by the formation of a multi-subunit complex assembling on origin DNA (Legouras *et al.*, 2006). Origins become competent for replication by the assembly of a pre-replicative complex (pre-RC) that was first identified in budding yeast (Diffley *et al.*, 1994). Although origin characteristics are not conserved the binding factors for their modifications and replication function are very similar in all organisms. (Diffley *et al.*, 1994). As cells enter S phase, CDKs activate the modified origins for initiation of DNA synthesis.

### **1.9.2 The pre-replication complex – license to replicate**

The pre-RC forms at an origin of replication conferring it the license to replicate. Created after mitosis and activated upon entry into S phase to initiate DNA synthesis, it is an ensemble of four licensing factors which are conserved from the simple eukaryotes such as yeast to the higher eukaryotes such as mammalian cells. This conservation suggests that the basic features of licensing are preserved in all eukaryotes. The four licensing factors are:

1. Origin recognition complex (ORC)
2. Cdc18p
3. Cdt1p
4. MCM complex consisting of six MCM proteins (Mcm2–7)

### **1.9.3 Origin recognition complex, cdc18<sup>+</sup> and cdt1<sup>+</sup>**

The origin recognition complex (ORC) is a hetero-hexameric complex comprised of six proteins, SpOrp1p to SpOrp6p (Moon *et al.*, 1999; Bryant *et al.*, 2001; Chuang *et al.*, 2002.). Analogous complexes also exist in both budding yeast and higher eukaryotes, also composed of six proteins (Bell & Stillman, 1992; Austin *et al.*, 1999; Kelly & Brown, 2000). During late M and G1 ORC binds to chromosomal DNA via the SpOrp4p, in fission yeast this protein contains nine copies of the AT-hook motif. This motif has a high affinity for AT rich DNA present in the ARS and is thought to mediate the binding of the *S. pombe* ORC complex. ORC serves as a platform for the recruitment of SpCdc18p and SpCdt1p, factors essential for replication (Yanow *et al.*, 2001). Both SpCdc18p and SpCdt1p have similar patterns of expression in the cell cycle, with proteins accumulating in late M phase and G1 and disappearing after the onset of S phase. This pattern of expression is the result of regulated transcription and proteolysis dependant upon CDK-dependent phosphorylation of the protein. The resulting ORC.SpCdc18p.SpCdt1p complex then serves to recruit the minichromosome maintenance (MCM) complex, completing pre-RC assembly.

### **1.9.4 Minichromosome maintenance (MCM) complex**

Recruitment of the MCM complex is restricted to a defined period of the cell cycle after exit from mitosis, but before initiation of DNA synthesis (Labib *et al.*, 2000). The MCM proteins were initially identified in budding yeast for their role in plasmid replication and cell cycle progression (Maine *et al.*, 1984; Sinha *et al.*, 1986; Kearsley *et al.*, 1996; Tye & Sawyer, 2000). At a basic level, the regulation of genome replication can be viewed simply as the regulation of the recruitment and activation of MCM complex to replication origins. Chromatin conditions alternate between two states, the pre-replication state when the MCM complex is localised at the origins, and the post-replication state when the

complex has left the origin; it is also this delocalisation which prevents re-replication (Diffley *et al.*, 1994).

The MCM genes are highly conserved in form and function, from yeasts to humans. Recent studies have shown that the MCM complex may serve a further role as the replicative helicase (Labib *et al.*, 2000; Bowers *et al.*, 2004; Forsburg, 2004). In fission yeast, the six MCM genes (*mcm2*<sup>+</sup> - *mcm7*<sup>+</sup>) encode proteins that form the pre-replication complex on DNA replication origins at the beginning of each cycle (Forsburg, 2004). Cell cycle regulated protein kinases load the MCM complex onto replication origins (Tye, 1999). This complex is the final component required for the completion of the pre-RC complex. The process of replication illustrating the pre-RC formation is shown in Figure 1.5.

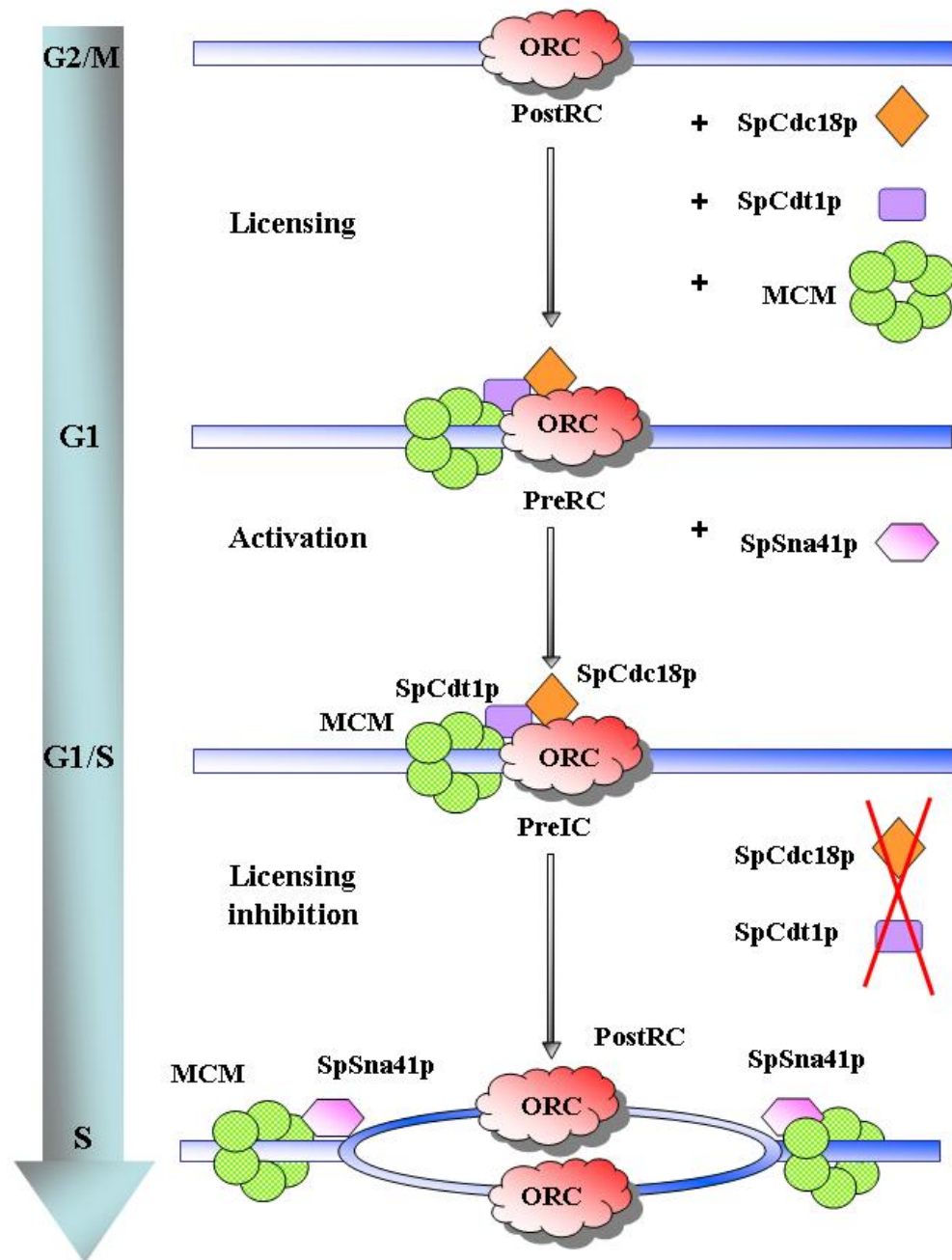
### **1.9.5 Activation of replication: CDK & DDK**

DNA replication is conserved in eukaryotes and occurs in two steps; the first is the assembly of the pre-RC and the second requires the activity of two separate kinases, SpCdc2p and SpHsk1p/ScCdc7p/HsCdc7p (Lei & Tye, 2001; Moser & Russell, 2000; Legouras *et al.*, 2006). Activities of both these kinases initiate a series of events which ultimately lead to the establishment of replication forks (Diffley & Labib, 2002).

Passage into S phase is dependant upon the association of the SpCdc2p kinase with the G1 cyclin SpCig2p. Rising levels of the resulting SpCdc2p.SpCig2p complex become active upon degradation of the SpRum1p inhibitor, the complex goes on to phosphorylate SpCdc18p dissociating it from the pre-RC and targeting it for degradation. The short life span of SpCdc18p is the major determinant for the restriction of one round of replication per cell cycle, since there is only a limited time within which it can form the pre-RC, and initiate origin firing (Yanow *et al.*, 2001). Once cells have passed START, the SpCdc2p.SpCdc13p complex is responsible for M phase entry forms; levels of this complex are kept low by phosphorylating SpCdc2p Y-15 by SpWee1p and SpMik1p (Moser & Russell, 2000; Sullivan & Morgan, 2007).

The second kinase, SpHsk1p, is essential for the initiation of DNA replication and is present at constant levels throughout the mitotic cell cycle, but only becomes active once it binds to its regulatory subunit (Masai *et al.*, 1995; Nakamura *et al.*, 2002). The regulatory

subunit of SpHsk1p is SpDfp1p and together they form the DDK, for Dfp1-dependent kinase, (Moser & Russell, 2000; Lei & Tye, 2001). DDK causes a conformational change to the MCM complex by phosphorylating SpMcm2p activating its inherent helicase quality to unwind dsDNA at replication forks and allow entry of the replication machinery (Labib *et al.*, 2001; Lee & Hurwitz, 2001). SpCdc23p/ScMcm10p is required to mediate DDK dependant phosphorylation of SpMcm2p. SpCdc23p possibly recruits SpHsk1p.SpDfp1p to the pre-RC at origins of replication (Lee *et al.*, 2003) and also the recruitment of SpSna41p/ScCdc45p/HsCdc45p (Uchiyama *et al.*, 2001; Dolan *et al.*, 2004) to initiate DNA synthesis. These factors in unison enlist the replication machinery such as the DNA polymerases for strand elongation and proteins required to fill the gaps between Okazaki fragments (Gregan *et al.*, 2003).



**Figure 1-5: DNA licensing in *S. pombe*.**

For simplicity only the main binding factors are shown. The origin recognition complex (ORC) complex, consisting of SpOrp1p to SpOrp6p, binds to multiple origin of replication sites present along the fission yeast chromosome. SpCdc18p, SpCdt1p bind to the chromatin with the minichromosome maintenance (MCM) complex, consisting of SpMcm2p to SpMcm7p, thereby forming the pre-replicative complex (pre-RC). At G1-S, additional replication factors are recruited (see text) that lead to the activation of the pre-RC to the pre-initiation complex (pre-IC), this initiates DNA replication. SpSna41p is then recruited which results in the dissociation of the MCM complex from the origins and return back to the post-replicative complex (post-RC) stage. Figure adapted from Legouras *et al.*, 2006.



### 1.9.6 The G2-M transition

At the G2-M transition, cells initiate the mitotic processes of chromosome condensation, mitotic spindle assembly, actin ring formation and cytokinesis. Before cells pass the late G2 checkpoint to enter mitosis two criteria must be met: first the cell must be a critical size, and second the chromosomes must be intact (Rhind & Russell, 2001). Entry is determined by the SpCdc2p-SpCdc13p complex, which is present after passage of START and throughout G2, but levels are controlled by its phosphorylation status. Mitotic entry takes place when, SpCdc25p a protein tyrosine phosphatase dephosphorylates SpCdc2p at Y-15, and SpWee1p is inhibited by SpCdr1p and SpCdr2p (MacNeill & Nurse, 1997; Sullivan & Morgan, 2007). The SpCdc2p-SpCdc13p complex remains active until proteolytic degradation of SpCdc13p at anaphase by the APC/C (Moser & Russell, 2000). Several other DNA integrity checkpoints are in place (Section 1.19.3).

### 1.10 Spindle dynamics

Many of the events of mitosis are dependant on the formation of the mitotic spindle, a structure composed of microtubules designed to coordinate the chromosomal movements of eukaryotic cell division. During S phase, not only is the DNA duplicated but so is the spindle pole body (SPB), also known as microtubule organizing centre (MTOC). These structures are permanently associated with the nuclear envelope and serve to nucleate the microtubules. Microtubules emanate from the MTOC in radial arrays called asters; the spindle microtubule fibres elongate from these points.

Tubulin is added to the growing end of the spindle fibre and is the major structural component of microtubules. Due to polymerisation and depolymerisation events occurring continuously and simultaneously, microtubules are highly unstable structures. Stabilisation is achieved via a capping process whereby depolymerisation is prevented by interaction with GTP- $\beta$ -tubulin (Rodionov *et al.*, 1999). For the duration of mitosis, spindle formation one end of the microtubule is stabilised by association with the SPB and the growing ends are stabilised by attaching to more specifically called the kinetochore microtubules, this period of attachment is called prophase. Spindle stabilisation is also established by cross-linking at the cells equator with microtubules from the opposite pole – polar microtubules (Hogan & Cande, 1990).

During metaphase, condensed chromosomes align on the metaphase plate, attached via their centromere to the kinetochore structure, which in turn is attached to the spindle fibres. At this point, chromosomes move back and forth on the spindle, a process thought to assist in chromosome alignment (Nabeshima *et al.*, 1998). The correct alignment of chromosomes is essential for accurate chromosome segregation. Tension is generated between sister chromatids as a result of the spindle fibre attempting to pull them apart and the cohesin which is tethering the chromatids counteracting this force (He *et al.*, 2000; Tanaka *et al.*, 2000; Yokobayashi *et al.*, 2003; Watanabe, 2006). The metaphase-anaphase transition only takes place once the appropriate tension level is reached; at this point, dissolution transpires and sisters are pulled to opposite poles pulled by the kinetochore microtubules. This event involves depolymerisation of kinetochore microtubules at their kinetochore end (anaphase A), and polar microtubules polymerisation that leads to elongation of the parent cell (anaphase B; Yamamoto *et al.*, 2001).

Completion of chromosome segregation arises during telophase when non kinetochore microtubules elongate the cell further too finally dissolve the spindle. Two daughter nuclei begin to form at the two poles of the cell where the chromosomes have gathered. The spindle dissolves followed by the rapid regeneration of cytoplasmic microtubule arrays and reorganisation of cellular structures at each end of the dividing cell. At this point, gene transcription commences and the chromosomes decondense to form the interphase cell state again. A contractile actomyosin ring appears, pinching the dividing cell into two equal halves and a septum is formed at the cells equator. Cytokinesis completes cell division by dividing the parent cell in half.

### **1.11 Anaphase promoting complex/cyclosome (APC/C)**

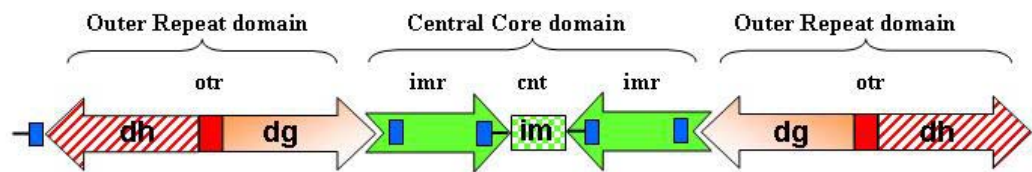
The anaphase-promoting complex/cyclosome (APC/C) is the major control at the metaphase/anaphase transition. The APC/C functions to localise substrates to the 26S proteasome for degradation by attaching multiple ubiquitin residues. This is a cell-cycle regulated ubiquitin ligase responsible for mediating the proteolysis of mitotic cyclins (Amon *et al.*, 1994; Bharadwaj & Yu, 2004). In *S. pombe* at least seven subunits have been characterised and shown to form a 20S APC/C (Harper *et al.*, 2002; Takahashi & Yanagida, 2000). APC activity rises abruptly at metaphase, resulting in the destruction of proteins that inhibit sister chromatid separation.

The APC/C is regulated at different stages of the cell cycle by the reversible binding of activator proteins. These activator proteins contain WD-repeats which can change the substrate specificity of the APC (Page & Hieter, 1997; Kim *et al.*, 1998). In *S. pombe* the essential mitotic cyclosome activator proteins are SpSlp1p and SpSte9p. SpSlp1p functions at anaphase and targets securin (SpCut2p) for ubiquitination releasing separase (SpCut1p) to cleave cohesin so that sister chromatids are no longer held together. SpSte9p is dephosphorylated and acts as an activator of the APC to promote the ubiquitination and proteolysis of SpCdc13p and SpCig1p (Blanco *et al.*, 2000).

Cyclins have sequences located near their N-termini called the destruction box which target cyclins to the ubiquitin-dependent proteolytic pathway. Degradation of the mitotic cyclins occur in an interval from anaphase until passage through START or the restriction point in late G1. This prevents the accumulation of mitotic cyclins before the formation of the G1 complexes, and thus this period of low kinase activity is thought to be important for proper assembly of pre-initiation DNA replication complexes.

## 1.12 Centromere – kinetochore complex

Centromeres are the constricted regions of a metaphase chromosome. They are structurally organized to have two separate domains: a heterochromatin domain at the outer repeats, and a central domain which anchors the kinetochore (Pidoux & Allshire, 2004). There are two types of localised centromeres, point centromeres and regional centromeres. *S. cerevisiae* has a small “point” centromere (<2.0 kb) composed of short AT-rich sequences flanked by two very short conserved regions. Regional centromeres have been identified in *S. pombe*, *Drosophila* and humans, and are composed of long sequences flanked by large quantities of repeated DNA, known as satellite DNA (Pluta *et al.*, 1995; Ekwall, 2007). *S. pombe* centromeres are 40-100 kb in length and have an asymmetric organisation. Centromeres rarely undergo meiotic recombination, and do not encode protein sequences (Lambie & Roeder, 1988; Roeder, 1997; Figure 1.6).



**Figure 1-6: Organisation of *S. pombe* centromeres.**

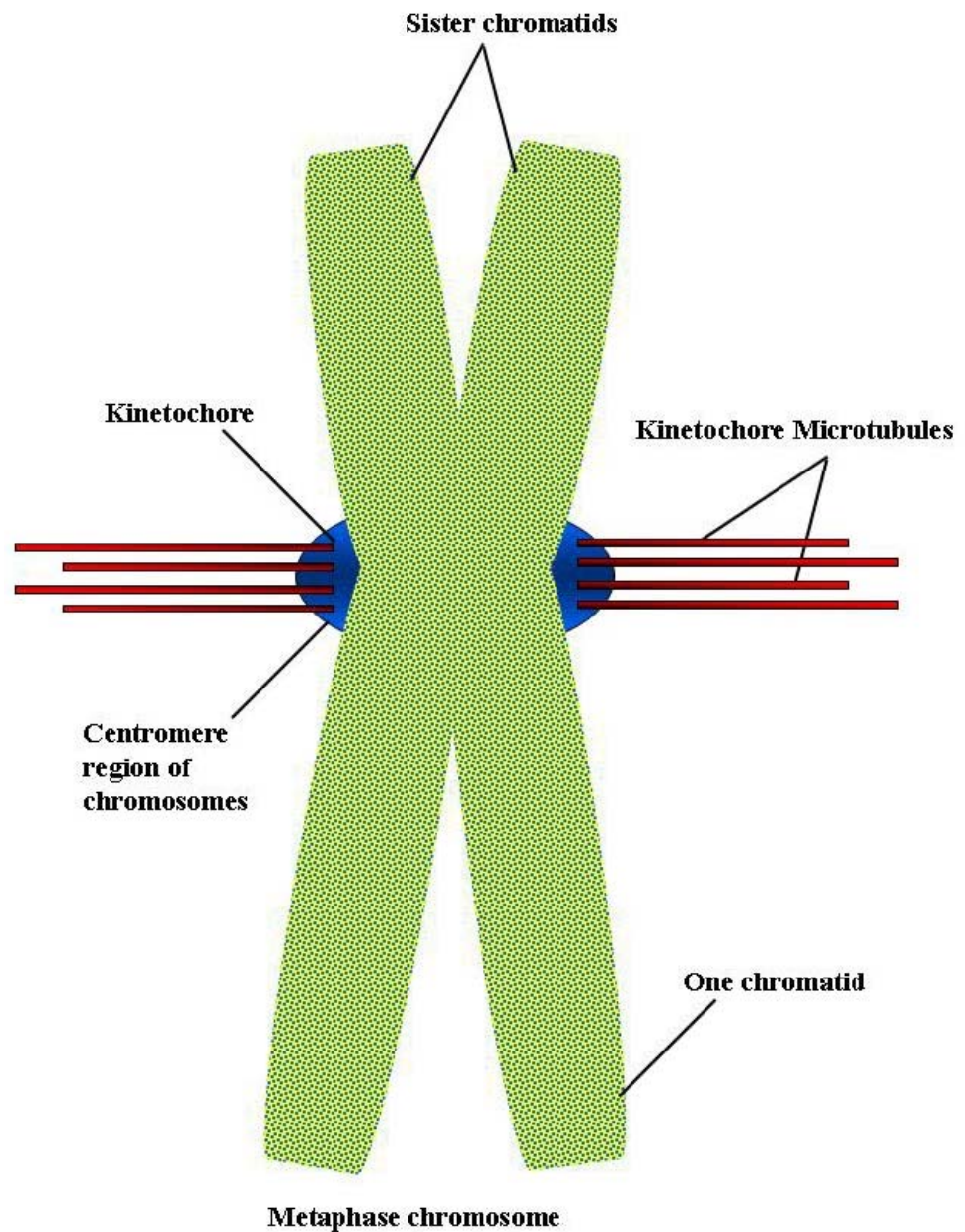
The Figure shows centromere I, however, all the three centromeres have a similar structure. A central core (cnt) of roughly 5kb is flanked by inner repeats (imr) and outer repeats (otr). The outer repeat domain incorporates the dg/dh elements and a small part of the imr element. Clusters of tRNA genes are indicated by small blue rectangles, which have been postulated to function as boundary elements between different types of chromatin-kinetochore, heterochromatin, and euchromatin. Genes are usually found 0.75-1.5kb beyond the ends of centromeres.

Histones play a central role in centromere assembly and function. The centromeres in *S. pombe*, like heterochromatic regions in vertebrates, are hypo-acetylated on histones (Ekwall *et al.*, 1997). In addition, the chromatin of the central core (cnt) contains a unique histone H3 variant, SpCnp1p, first identified in humans and encoded by CENP-A (Takahashi *et al.*, 2000). SpCn1p has several functions, namely the recruitment and assembly of kinetochore proteins, mitotic progression and chromosome segregation; it is also required for the recruitment of SpCnp3p (CENP-C). Other proteins associating with the centromere are the central domain proteins SpMis6p and SpMis12p (Saitoh *et al.*, 1997). SpMis6p is essential for sister chromatid segregation and for the localisation of SpCnp1p to the centromere. *mis6*<sup>+</sup> alleviates transcriptional silencing at the inner centromere, but not the outer repeats (Partridge *et al.*, 2000).

SpSwi6p and SpCnp1p are chromodomain proteins found at the outer repeats (Ekwall *et al.*, 1995; Partridge *et al.*, 2000), and their localisation requires the transcriptional silencer SpRik1p, and the histone methylase SpClr4p (Ekwall *et al.*, 1996; Thon & Verheinhansen, 2000). The association of SpRik1p and SpClr4p mediates the silencing of marker genes placed within these regions. Histone hypoacetylation is important for SpSwi6p binding at the outer repeats in fission yeast and binding of cohesin SpRad21p and SpPsc3p is also dependant on SpSwi6p (Bernard *et al.*, 2001; Nonaka *et al.*, 2002).

Centromeres have two main functions; after DNA replication they form at the site at which the sister chromatids are tethered together, due to a high density of cohesin present in the centromeric region (Tanaka *et al.*, 2000). Centromeric cohesion is invaluable in inhibiting premature separation of sister chromatids (Tatebayashi *et al.*, 1998; Watanabe & Nurse, 1999; Yokobayashi *et al.*, 2003; Watanabe, 2006). The second function of the centromere is to provide an anchorage point for the kinetochore. Together, the centromere and its associated proteins form the “centromere-kinetochore” complex, which alongside cohesin plays an important role in the spindle assembly checkpoint.

Kinetochores are a complex of proteins that assemble on the centromere and have a high affinity for the microtubule protein, tubulin (Pidoux & Allshire, 2000). They connect the chromosome to the mitotic spindle in both mitosis and meiosis. The kinetochore is composed of two units, an inner kinetochore which makes contact with the centromere and an outer kinetochore which interacts with the microtubules (Figure 1.7).



**Figure 1-7: Centromere kinetochore complex.**

Microtubules emanating from the spindle attach to the centromere to bi-orientate the sister chromatids at metaphase. A tension is generated as a consequence of the spindles trying to pull the chromatids apart and the cohesin joining them counteracting this pulling force. This tension contributes to the spindle assembly checkpoint which ensures that sister chromatids align correctly at metaphase. At anaphase, cohesin is cleaved and the spindle fibres attached to the kinetochores pull the two sister chromatids toward opposite poles of the spindle.

When chromosomes orientate themselves to align on the metaphase plate they become associated with kinetochores. Alternative kinetochore orientations mediate the different patterns of chromosome segregation in mitosis and meiosis (Tanaka, 2000; Pidoux & Allshire, 2003; Megee, 2006; Watanabe, 2006; Tanaka, 2007). In mitosis sister chromatids are bi-orientated so that they attach to microtubules from opposite poles; this careful alignment results in sisters being pulled to opposite poles during chromosome segregation. A different scenario is seen during meiosis when cell division occurs twice. In the first meiotic division, sister kinetochores of homologues are co-orientated so that they capture microtubules from the same spindle pole, termed monopolar attachment (Kitajima *et al.*, 2003a; Yamamoto & Hiraoka, 2003; Figure 1.10). This attachment to opposite spindle microtubules, so that sister chromatids remain joined, results in homologues being pulled to opposite poles. The second meiotic division 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 aneuploidy (Nabeshima *et al.*, 1998).

### **1.13 Spindle assembly checkpoint (SPAC)**

The spindle assembly checkpoint (SPAC) is a key control point in the metaphase to anaphase transition controlling the alignment of chromosomes. This checkpoint functions to prevent premature anaphase entry. When kinetochores attach to spindle fibres tension is generated across centromeres as a result of the fibres pulling the chromosome attached kinetochores towards themselves and the sister chromatid cohesin opposing the pulling force. SPAC functions by sensing a lack of tension resulting from unattached kinetochores, where the sensing apparatus for the spindle checkpoint appears to be assembled. Aurora related kinases (Arks) are thought to destabilise faulty attachments thereby promoting bipolar attachments (Hauf *et al.*, 2007; Kawashima *et al.*, 2007). As well as protecting centromeric cohesion, SpBub1p is a spindle checkpoint protein kinase that is necessary for delaying the activation of the APC/C until a sufficient amount of tension has been sensed from chromosomes aligned on the metaphase plate (Riedel *et al.*, 2006).

Fission yeast separase (SpCut1p) and securin (SpCut2p) are cell cycle regulatory proteins mediated by the ubiquitination system. They genetically interact to form a 30-40S large complex which is essential for the separation of sister chromatids (Funabiki *et al.*, 1996).

Securin is degraded by the proteasome to release separase, which in turn cleaves the cohesins holding sister chromatids together resulting in the onset of anaphase and their subsequent segregation. SpSlp1p targets securin for degradation so that separase is free to cleave cohesin. However, if a lack of tension is sensed, the SpMad2p (another SPAC protein) forms a complex with SpSlp1p preventing the targeting of securin. SpMad2p is only released when the chromosomes are correctly aligned and kinetochore-centromere tension is detected. In addition to the deterrence of nuclear division before anaphase, the spindle checkpoint also interferes with cytokinesis assuring that undivided nuclei are not “cut” by untimely septum formation. This signal is directed to the septation initiation network (SIN) via proteins unrelated to SPAC (Krapp *et al.*, 2004)

## 1.14 Mitosis and cytokinesis

Genetic instability and aneuploidy may lead to cell death. To ensure that the genome is stably transmitted during each cell division cycle, mitosis and cytokinesis are yet further cell cycle stages which are tightly regulated. This process is essentially similar in all observed eukaryotes (Simanis, 2003).

The primary driving force for entry into mitosis is promoted by CDK-dependant phosphorylation and de-phosphorylation of various proteins kinases. The kinase activity of the CDK, SpCdc2p, with the mitotic B cyclin, SpCdc13p, is required to reach a critical peak before mitosis can take place (MacNeill & Nurse, 1997). Association of SpCdc2p with SpCdc13p is controlled at the G2-M transition by SpCdc25p. With these initial events now in place, highly complex, sequential cellular actions are triggered to achieve chromosome condensation, segregation and finally cytokinesis. Ubiquitin-mediated proteolysis by the APC/C inactivates mitotic cyclin-CDK complexes to exit mitosis (Morgan, 1995; Harper *et al.*, 2002). The various stages of mitosis (Section 1.6) are evolutionarily conserved in all eukaryotes.

## 1.15 Chromosome compaction: from replicated DNA to metaphase chromosome

From what has been discussed so far, the cell cycle can be defined as not only the accurate replication of an organism’s genome, but also the maintenance of euploidy in both mitotic and meiotic cells to prevent chromosomal defects. Genetic material is replicated during



G1-S and segregated in G2-M. The timely and accurate distribution of chromosomes relies on chromosomal morphology and behavioural processes which must coincide with the cytoskeletal changes of the spindle apparatus taking place within a cell to participate in the processes of genome distribution at anaphase.

Many cellular mechanisms have evolved in eukaryotes to aid the distribution process. These include the presence of several evolutionarily conserved protein complexes, such as the APC/C, securin-separase complex, cohesin and condensin all playing fundamental roles in chromosome segregation.

The purpose of chromosome cohesion is to hold sister chromatids together from the point of DNA replication to chromosome separation at anaphase. The kinetochore-spindle relationship is also another important contributing factor in chromosome segregation. Sufficient tension levels must be detected by the spindle assembly checkpoint before segregation can take place. Cell microtubules attached to kinetochores generate a pulling force which is counteracted by the cohesin holding sister chromatids together. If this force is not strong enough cell cycle progression halts until events at metaphase have had a chance to be rectified. In meiosis the correct alignment of chromosomes as a consequence of spindle attachment enables a cell to distinguish between homologous chromosomes and sister chromatids, which for the purpose of meiosis is important for strand selection and meiotic cross overs.

Taking into consideration the extreme length of eukaryotic chromosomes, an efficient packaging mechanism must exist whereby the entire genome can be stored in the nucleus. Chromosomes condensation is a prerequisite for the mitotic spindle to capture chromosomes effectively and separate them without entanglement. There are several methods present which lead to the compaction or condensation of DNA such as the presence of histones and nucleosomes to form the chromatin fibres. In addition to this the protein complex condensin further compacts the dispersed interphase chromatin fibres into the mitotic chromosome visible at metaphase.

Once these conditions have been met anaphase can occur, cohesion between the sisters is finally dissolved and chromosome separation takes place, resulting in each pair of the sisters being equally distributed along the kinetochore microtubules to the opposite poles.

In the following Sections, each of the packaging mechanisms will be discussed.

### **1.15.1 The chromatin fibre – histones and nucleosomes**

Compaction of DNA fibres is essential to fit the whole genome within the limited volume of the cell nucleus. The simplest level of packing involves the winding of DNA around histones in a structure called a nucleosome, and the most highly packed state involves higher order coiling and looping of the DNA-histone complexes resulting in the metaphase chromosome.

In higher eukaryotes, metaphase chromosomes are up to 10 000 times more compacted than in interphase (Trask *et al.*, 1993). At interphase, chromatin is packaged at two levels. The first level of DNA packaging is the direct wrapping of DNA around the nucleosomes, forming chromatin fibre with the appearance of beads on a string. Histones are a family of proteins that are highly conserved in evolution; they are rich in lysine and arginine residues and thus positively-charged. For this reason histones have an intrinsic affinity to bind tightly to the negatively-charged phosphates in DNA. Nucleosomes, the basic building of chromatin, are histone octamers composed of two of each of the histones H2A, H2B, H3 and H4; these are regularly spaced along eukaryotic DNA, at intervals of 180-200 bp. The presence of additional anionic factors shields histone charges from DNA to allow chromatin assembly to take place in a regulated and ordered manner. Histone chaperones are ATP-dependant chromatin remodelling machines that cooperate to assemble DNA and histone proteins into chromatin.

The second level of DNA packaging seen in the interphase chromosome involves the histone octamer nucleosomes folding on themselves to form loop domains. In the mitotic chromosome, the looped domains undergo more coiling condensing all the chromatin further to produce the characteristic metaphase chromosome. The loops are anchored onto a framework of nuclear skeletal structures called the nuclear matrix or scaffold which is comprised of non-histone proteins. These structures are involved in the formation and maintenance of higher order chromosome structures in higher eukaryotic cells. Such nuclear structures also exist in yeasts and the nature of the DNA sequences scaffold attachment regions (SARs) that are associated with the scaffold are evolutionarily conserved (Yanagida, 1990). At the onset of mitosis, packaging increases further, in a process termed “chromosome condensation”, so that chromatin now is arranged into a tightly coiled, highly organised structure.

Therefore, in summary, at this initial compaction stage, DNA is spooled around the histones to form the nucleosomes which fold on themselves to give the chromatin fibres. After treatment with high salts and removal of histones chromosomes retain their characteristic metaphase shape, which suggests that non-histone like proteins were also involved in condensation of genetic material (Mirkovitch *et al.*, 1988; Belmont, 2002). The condensin complex is one such mechanism which enhances compaction, however before the condensin complex is discussed there will be a brief explanation on the importance of the structural maintenance of chromosomes (SMC) proteins. These proteins play an integral part in both the condensin and cohesin complex, the latter of which is the main area of interest in this thesis.

### **1.15.2 Structural maintenance of chromosomes (SMC) proteins**

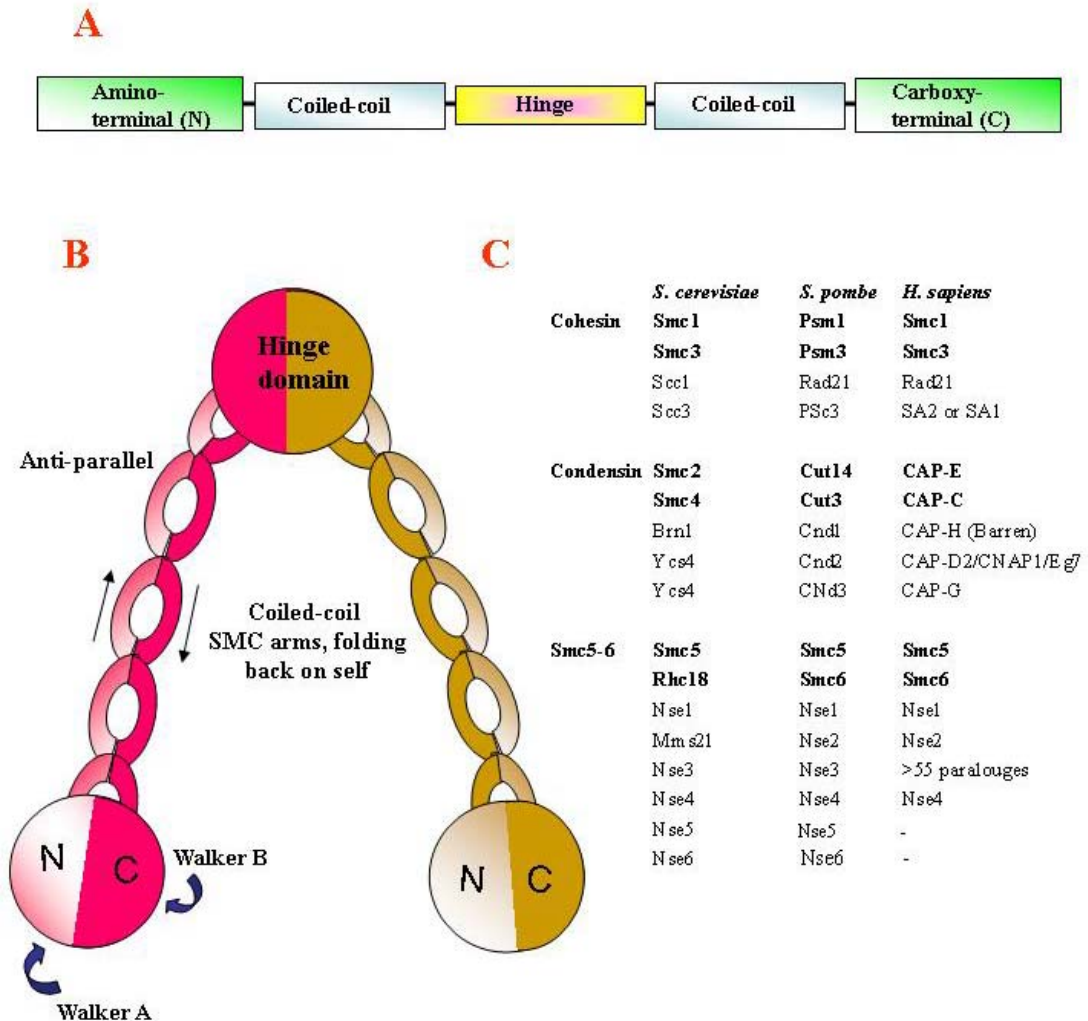
The structural maintenance of chromosome (SMC) family of proteins is highly conserved in evolution and studies have demonstrated that these proteins are key factors for a number of processes including roles in cell proliferation, chromosome segregation in mitosis and meiosis, gene regulation and recombinational repair (Hirano, 1998; Sutani *et al.*, 1999; Sakai *et al.*, 2003). An analysis of the primary sequence of members of the SMC super family indicated that they share sequence identity and a similar geometry with ScRad50p.

The SMC proteins are large polypeptides ranging between 1000 and 1500 amino acids in size. They have two nucleotide binding motifs, the Walker A and Walker B motifs, which are located within their amino- and carboxyl-termini, respectively (Hirano, 1998; Hirano & Hirano, 2002; Stray & Lindsley, 2003). The central core is composed of a conserved central hinge domain which is flanked by two long coiled-coil motifs. In eukaryotes, SMC proteins exist as heterodimers bound by additional non-SMC subunits; it is in their heterodimeric state they are thought to function. It has been postulated that, rather than the two SMC subunits associating along their lengths, the subunits fold back on themselves via their coiled-coil regions form a V-shaped heterodimer. This brings the amino- and carboxyl-terminal globular domains together to unite the ATP binding site - Walker A motif with the Walker B motif to form a potentially functional ATPase of the ATP binding cassette (ABC) (Hirano *et al.*, 1997; Sutani *et al.*, 1999; Haering *et al.*, 2002).

In eukaryotes, at least six members of the SMC protein family have been identified which form three distinct complexes with specific functions (Hirano & Hirano, 2002). SMC1 and

SMC3 form a heterodimer that functions as the core of the cohesin complex involved in sister chromatid cohesion (Losada *et al.*, 1998; Tomonaga *et al.*, 2000), and SMC2 and SMC4 form components of the condensin complex and facilitate chromosome condensation in mitosis (Sutani *et al.*, 1999; Lam *et al.*, 2006). The third dimer comprised of SMC5 and SMC6 has been implicated as having a role in DNA repair (Fousteri & Lehmann, 2000; Lehmann, 2005). Each of these SMC subunits forms heterodimers which associate with distinct non-SMC regulatory subunits to form functional complexes.

Figure 1.8 illustrates the basic structure of an SMC protein; alongside the structure, is a list of the corresponding components of each of the three SMC complexes in *S. pombe*, *S. cerevisiae* and *H. sapiens*.



**Figure 1-8: Structural maintenance of chromosomes (SMC) protein architecture.**

(A) Each SMC subunit has a hinge domain flanked by a coiled-coiled region and amino and carboxy termini which have ATPase activities and a nucleotide binding Walker A and B motifs, respectively; (B) The subunit folds back on itself by the antiparallel coiled-coiled interactions (indicated by arrows) to form the hinge domain and the ATP binding head domains. The resulting structure produces is a V-shaped molecule. (C) A list of the three SMC structures and respective subunits in *S. cerevisiae*, *S. pombe* and *H. sapiens*.

### 1.15.3 **Condensin**

In mitosis, condensation involves both the resolution of entangled interphase chromosomes and the compaction of sister chromatids into discrete segregatable units, important to maintain genome integrity by preventing strands of chromatin from breaking. Condensin is a highly conserved, multi-subunit complex that is essential for chromosome condensation during metaphase, first identified in frog (Saka *et al.*, 1994; Strunnikov *et al.*, 1995; Hirano *et al.*, 1997). The 13S condensin complex is composed of two SMC subunits SpCut14p/ScSmc2p, SpCut3p/ScSmc4p and three non-SMC subunits SpCnd1p/ScYcs4p, SpCnd2p/ScBrn1p and SpCnd3p/ScYcg1p. Although condensin was found in the cytoplasm during the oscillating stages of the cell cycle it was observed to be enriched in the nucleus during mitosis. This cell cycle dependant localization requires mitosis-specific phosphorylation of the SpCdc2p site in SpCut3p/ScPsm4p; SpCdc2p-directed phosphorylation of condensin may advance its nuclear accumulation during mitosis (Sutani *et al.*, 1999).

Each of the condensin subunits is essential for viability and mutants in any of these cause dramatic chromosome condensation and segregation defects. Apart from anomalous chromosome morphology the most pronounced phenotype seen in these condensin mutants is chromosome segregation, with masses of lagging chromatin interfering with cytokinesis to generate the so called “*cut*” (cell untimely torn) phenotype observed in fission yeast, an observation which led to its identification (Saka *et al.*, 1994; Sutani & Yanagida 1997; Gassmann *et al.*, 2004).

The molecular mechanism of condensin mediated chromosome condensation remains unclear (Haering *et al.*, 2002; Tsang *et al.*, 2007). Several models have been proposed which suggest that the intra molecular and inter molecular interactions of the SMC arms and hinge domains with DNA causes chromosome compaction (Hirano, 2006). The combined actions of protein-protein interactions and the accumulated super helical tension of DNA in the nucleoprotein structure from the presence of topoisomerases would ultimately lead to DNA condensation, and the formation of the metaphase chromosome.

## 1.16 Cohesin

After DNA replication and duplication of chromosomes the resulting sister chromatids are held along their lengths by proteins called cohesins. It is critical that the replicated products be handled as a unit until the onset of anaphase when they are divided between the two daughter cells. Cohesin proteins can be perceived as the “molecular glue” holding chromatids together until mitosis or meiosis, when nuclear and cellular division can take place. Although the complex formed is structurally similar, there are specific cohesin subunits for both mitosis and meiosis which will be discussed in the following Sections. The process of chromosome segregation has to be precise since a change in chromosome number, either by loss or gain, can cause catastrophic complications (Hassold *et al.*, 2007).

Fission yeast is the ideal model organism to enhance the understanding of the mechanisms involved in chromosome biology. With the presence of just three chromosomes and recognition of distinct cell phases, simple morphological screens affecting cell phenotype have become feasible. Several checkpoint mechanisms have been developed to coordinate chromosome states such as DNA damage, DNA replication, kinetochore-spindle interaction all coordinated to coincide with the final goal of chromosome segregation.

### 1.16.1 *The cohesin complex*

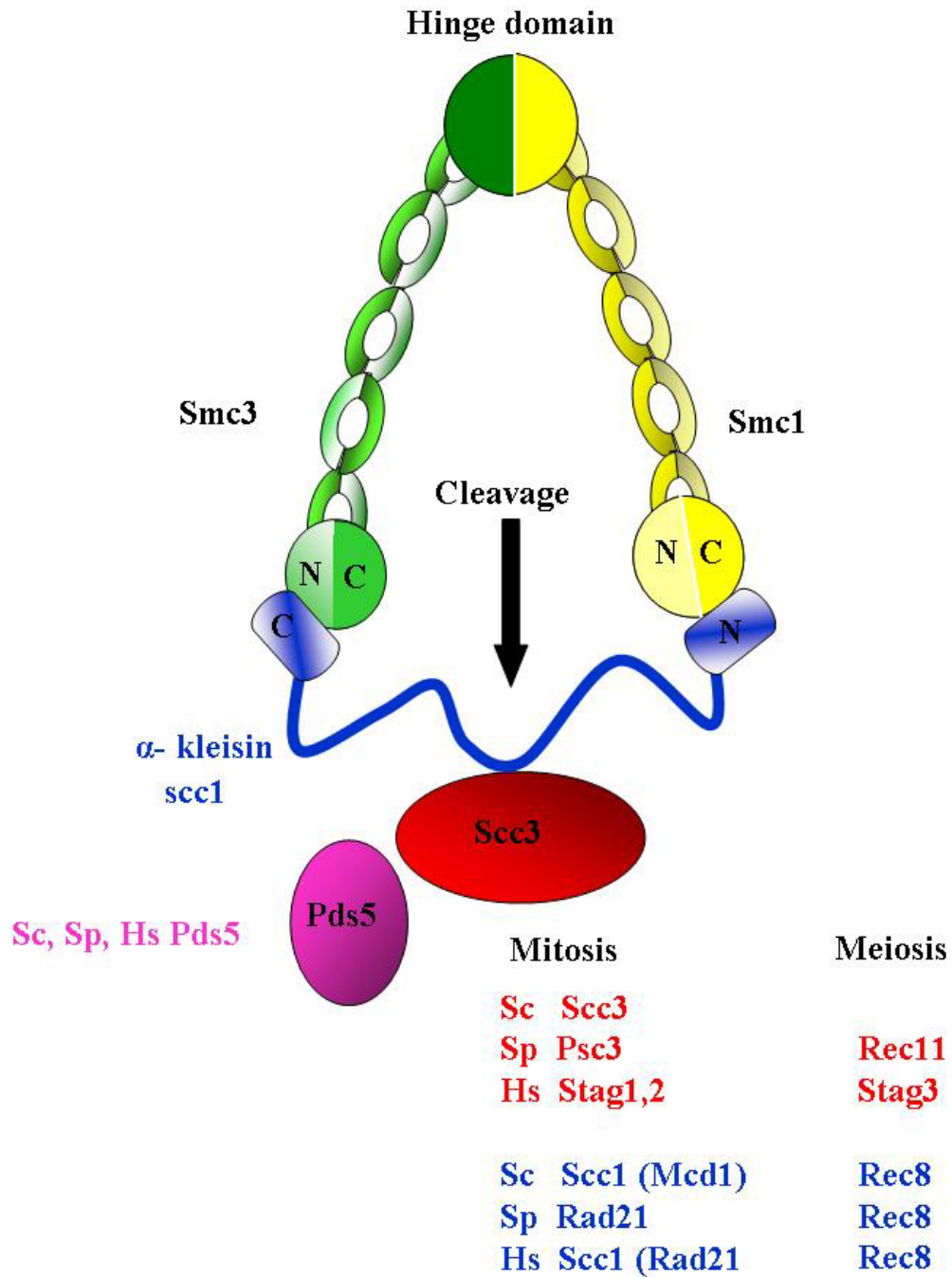
The 14S multi-subunit complex cohesin (Figure 1.9) was first characterised by genetic studies in *S. cerevisiae*. It is composed of four core subunits, two of the SMC family, ScSmc1p and ScSmc3p, and two of the sister chromatid cohesion (SCC) family, ScScc1p/ScMcd1p and ScScc3p; these subunits are evolutionarily conserved (Guacci *et al.*, 1997; Michaelis *et al.*, 1997; Toth *et al.*, 1999; Sakai *et al.*, 2003). Homologues have been found in other organisms (Losada *et al.*, 1998; Sumara *et al.*, 2000; Tomonaga *et al.*, 2000; Sonoda *et al.*, 2001). In *S. pombe* the two SMC proteins are SpPsm1p and SpPsm3p, and the two SCC proteins are SpRad21p and SpPsc3p. Similar to budding yeast, the four fission yeast proteins are essential for viability, and their mutation leads to defective sister chromatid cohesions and premature separation of sister chromatids (Tomonaga *et al.*, 2000; Uhlmann, 2001). Many organisms contain variants of the SCC subunits: for example three homologues of the Scc3 subunit have been found in human cells that have been identified as HsStag1p and HsStag2p which occupy alternative mitotic cohesin complexes, whilst HsStag3p is meiosis specific (Sumara *et al.*, 2000; Prieto *et al.*,

2001). *S. pombe* has two Scc3 homologues, SpPsc3p and SpRec11p, required for mitosis and meiosis, respectively.

The SCC1 subunit belongs to a family of proteins called kleisins and is the most extensively studied of the kleisin proteins; SCC1 is needed for sister chromatid cohesion (Michaelis *et al.*, 1997; Nasmyth & Haering, 2005). Two homologues of SCC1 are commonly found in most organisms, one mitotic specific SpRad21p/ScScc1p/ScMcd1p and the other meiotic specific SpRec8p/ScRec8p; only one is expressed at any one time (Harigaya, 2006). There are four major classes of kleisins  $\alpha$ ,  $\beta$ ,  $\gamma$ ,  $\delta$ , SpRad21p/ScScc1p/ScMcd1p fall into the  $\alpha$ -kleisin family, as do their meiotic equivalents ScRec8p/ScRec8p (Schleiffer *et al.*, 2003; Nasmyth & Haering, 2005). This particular subunit occupies a crucial position within the cohesin complex, since it is the proteolytic cleavage of Scc1 which leads to the breakdown of the complex. The mitotic *rad21*<sup>+</sup> gene and its meiotic homologue *rec8*<sup>+</sup> are the primary topic of interest in this thesis and will be discussed in more detail.

Although not part of the main cohesin complex fission yeast *pds5*<sup>+</sup>, a non essential gene, encodes SpPds5p which is co-localised to the nucleus in parallel with SpRad21p throughout the cell cycle, and is required for the maintenance of sister chromatid cohesion. It consists of large sequence motifs known as HEAT repeats which are thought to mediate protein-protein interactions (Neuwald & Hirano, 2000). SpEso1p is necessary for the establishment of cohesin and interacts physically with SpPds5p (Tanaka *et al.*, 2001). The co-localisation of budding yeast ScPds5p and ScMcd1p to chromatin are inter-dependant, but they form only a weak association indicating that ScPds5p is not an integral component of cohesin (Tanaka *et al.*, 2001; Wang *et al.*, 2002). In budding yeast, ScPds5p is essential for sister chromatid cohesion, while in fission yeast it only becomes necessary after an extended G2 period (Uhlmann, 2001).





**Figure 1-9: Schematic diagram of cohesin molecule.**

The subunits have been labelled according to their names in *S. cerevisiae*. Pds5 is found in both budding and fission yeast, however, they have different functions. There are two Scc3 homologues for *S. pombe* which are SpPsc3p for mitosis and SpRec11p for meiosis. Scc1 in *S. pombe* mitosis is SpRad21p and in meiosis it is replaced by its counterpart SpRec8p. Scc1 occupies a critical position in the cohesin complex, as the proteolytic cleavage at anaphase onset causes the complex to come apart. Subunits from three organisms listed *S. cerevisiae* (Sc), *S. pombe* (Sp) and *H. sapiens* (Hs).

### 1.16.2 **Mitotic cohesin subunit SpRad21p**

The *S. pombe rad21<sup>+</sup>* gene located on chromosome III is essential for mitotic growth and encodes a 67 kDa nuclear protein (Birkenbihl & Subramani, 1992). The gene was first identified in this organism in a screen for DNA repair mutants and was found to be involved in the DNA double strand break (DSB) repair pathway with mutants being sensitive to  $\gamma$ -irradiation (Birkenbihl & Subramani, 1992). The *rad21-45* mutant, identified for its inability to repair DSBs, is caused by a single base substitution changing wild-type isoleucine at position 67 into threonine.

An MCB element is present within the promoter region of the *rad21<sup>+</sup>* gene and *rad21<sup>+</sup>* expression is cell cycle regulated, with peak transcript and protein levels present at G1-S. Immediately following translation, SpRad21p is hypo-phosphorylated and as the cell cycle progresses it becomes phosphorylated at multiple serine and threonine residues (Birkenbihl & Subramani, 1995); thus the phosphorylation status of SpRad21p also varies throughout the cell cycle. The most hyper-phosphorylated form of SpRad21p appears at metaphase and this may be essential for APC-mediated proteolysis of SpRad21p during anaphase.

A temperature sensitive mutant of the *rad21<sup>+</sup>* gene, *rad21-K1*, shows abnormal mitosis at the non-permissive temperature. Not only is this mutant sensitive to UV and  $\gamma$ -irradiation but also to the mitotic spindle inhibitor thiabendazole, and the DNA replication inhibitor hydroxyurea, indicating additional roles for SpRad21p in microtubule and S phase function (Tatebayashi *et al.*, 1998). *rad21<sup>+</sup>* genetically interacts with the tubulin genes *nda2<sup>+</sup>* and *nda3<sup>+</sup>*, further supporting a role in microtubule function. The *rad21-K1* mutant is also defective in chromosome segregation; with cells displaying a cut-like phenotype where cell division takes place in the absence of nuclear division, leading to uneven separation of chromosomes or bisection of the nucleus with a septum.

The APC appears to have multiple roles from cyclin destruction and sister chromatid separation at anaphase, to roles in various cellular functions such as regulation of DNA replication, microtubule function and DNA repair. *rad21<sup>+</sup>* has also been implicated as having a role in the ubiquitin-mediated proteolysis pathway by either directly or indirectly regulating APC function (Tatebayashi *et al.*, 1998).

### 1.16.3 **Meiotic cohesin subunit SpRec8p**

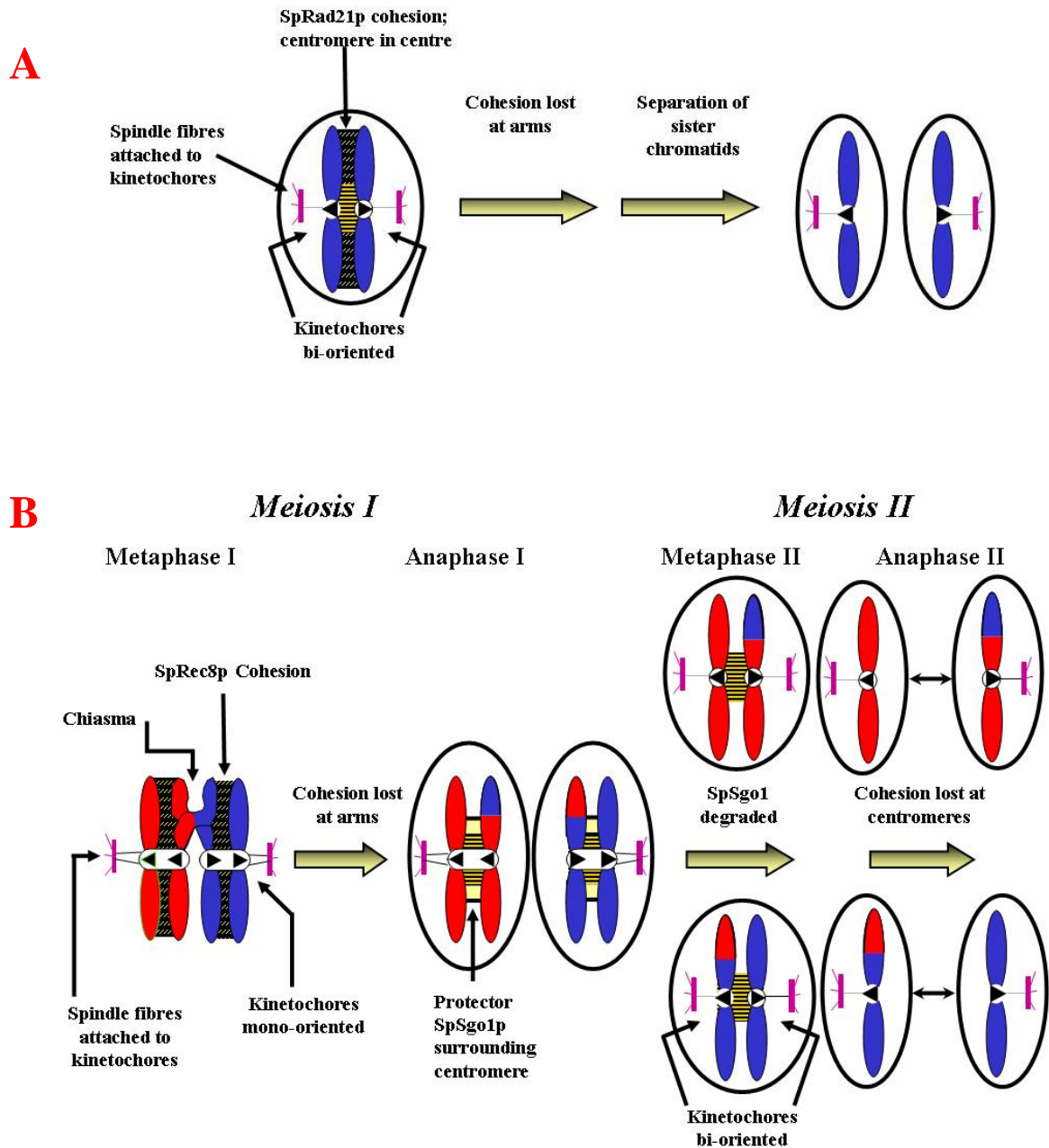
The *rad21*<sup>+</sup> homologue *rec8*<sup>+</sup> was first identified in *S. pombe* (Molnar *et al.*, 1995; Watanabe & Nurse, 1999; Molnar *et al.*, 2003). *rec8*<sup>+</sup> was identified in a screen for mutants with reduced recombination frequency at the *ade6*<sup>+</sup> locus – a hotspot for recombination (Gutz *et al.*, 1974). Mutants of the *rec8*<sup>+</sup> gene are defective in meiotic chromosome segregation, homologous recombination (HR) and the formation of linear elements which are thread like proteinacious chromosome attached structures formed during early meiotic events; thus associating this gene with several critical meiotic tasks (Molnar *et al.*, 1995; Parisi *et al.*, 1999; Watanabe & Nurse, 1999). In addition, spore formation and spore viability are reduced in *rec8* mutants. Homologues have since been identified in several species including *S. cerevesiae* and humans (Klein *et al.*, 1999; Parisi *et al.*, 1999). In humans the protein is expressed at high levels in germ line cells, further supporting a meiotic specific role for HsRec8p (Parisi *et al.*, 1999).

The *S. pombe rec8*<sup>+</sup> gene, located on chromosome II, contains two MCB elements in its promoter region (Lin *et al.*, 1992; Mata *et al.*, 2002; Cunliffe *et al.*, 2004). *rad21*<sup>+</sup> and *rec8*<sup>+</sup> share 29% and 35% sequence homology in their N- and C- termini, respectively (Watanabe & Nurse, 1999; Section 3.5). Gene expression is induced from pre-meiotic S phase through to MI. SpRec8p is a 64 kDa nuclear protein which is concentrated at the centromeric regions, becoming less abundant along the chromosome arms. The meiotic pattern of chromosome segregation is dependant on the temporal regulation of SpRec8p onto chromatin during pre-meiotic S phase (Watanabe *et al.*, 2001). Analogous to SpRad21p, SpRec8p is also a phosphoprotein; however, in comparison to its mitotic homologue, phosphorylation of SpRec8p is cell cycle regulated. The phosphorylation of SpRec8p begins in prophase I and persists beyond MI (Parisi *et al.*, 1999); this phosphorylation pattern may be relevant in the regulation of APC-mediated proteolysis of meiotic cohesin (Rogers *et al.*, 2002). Cohesin along arms is cleaved during anaphase I, allowing resolution of crossovers between homologues. Centromeric cohesin is protected from proteolysis at MI thus maintaining the sister chromatid unit (Kitajima *et al.*, 2004). At anaphase II the centromeric cohesin is lost resulting in the separation of sister chromatids in a similar manner to mitosis (Buonomo *et al.*, 2000).

#### **1.16.4 Presence of cohesin during the meiotic cycle**

The cohesin complex is described in more detail in Section 1.17.1 In brief; cohesin is composed of four core subunits conserved in all eukaryotes. In *S. pombe*, these are encoded by the *psm1*<sup>+</sup>, *psm3*<sup>+</sup>, *rad21*<sup>+</sup> and *psc3*<sup>+</sup> genes, all of which are essential for cohesion function and cell proliferation (Kitajima *et al.*, 2003a). In meiosis this complex alters slightly to that the SpRad21p subunit is replaced with a homologous protein called SpRec8p which is exclusively available in the meiotic life cycle (Parisi *et al.*, 1999; Stoop-Myer & Amon, 1999). SpRec8p binds rigidly to both outer-repeat and central-domain regions of centromeres; in the absence of SpRec8p sister chromatids separate equationally in the first meiotic division (Watanabe & Nurse, 1999).

The centromere-kinetochore complex is one mechanism to ensure accurate segregation, since the correct orientation of kinetochores with microtubules determines the separation of homologous chromosomes in MI, and then sister chromatids in MII. This is further supported by cohesin, important in retaining sister chromatids as a unit until they are ready for separation at anaphase. The pattern of meiotic cohesin differs in comparison to mitotic cohesin; in mitosis there is only one cellular division, therefore sisters need only be held together for one cellular division. In contrast, as discussed, meiosis has two cellular divisions, the first being separation of homologous chromosomes and the second the disengagement of sister chromatids at anaphase II. Thus, in the meiotic divisions cohesin is first removed from the arms by separase followed by degradation of centromeric cohesin (Brar *et al.*, 2006). As a consequence, a means must be present to comply with this mechanistic difference. Figure 1.10 shows the difference in cohesin pattern in mitosis and meiosis; as the phases have already been discussed, for simplicity only the metaphase to anaphase transitions for both types of cycles are shown.



**Figure 1-10: Mitotic and Meiotic cohesin in fission yeast.**

(A) In mitosis the SpRad21p cohesin is present along the arms of the sister chromatids. Spindle fibres are attached to the kinetochores in a bi-orientated fashion to capture microtubules from opposite spindle poles. When the arm cohesin is cleaved the two sister chromatids are pulled apart to produce two identical daughter cells. (B) In comparison, meiosis has two cellular divisions. During the first, meiosis I sister kinetochores are co-orientated so that they capture microtubules from the same spindle pole, and the kinetochores of homologues are pulled to opposite poles. The sister chromatids are held together by cohesin and chiasmata, the latter not only allows cross-over events to occur but also helps keep sisters attached. This first division is called the reductional division and results in two cells with recombinant sister chromatids. Centromeric cohesin is protected by SpSgo1p. The second meiotic division proceeds as in mitosis: sister kinetochores face opposite poles and sister chromatids are pulled away from each other. SpSgo1p is degraded and centromeric cohesin cleaved so that sister chromatids now separate, this second division is called the equational division.

### 1.16.5 **Protection of centromeric cohesin from separase in MI**

Regulation of sister chromatid cohesion during meiosis involves the protection of pericentromeric regions from separase cleavage during anaphase I. Centromeric proteins have been identified which have a role in protecting the cohesin proteins located at the centromeres; these include those encoded by the *bub1*<sup>+</sup> and *sgo1*<sup>+</sup> genes.

Aside from its function in monitoring chromosome attachment the kinase SpBub1p also functions to secure sister kinetochores as a single entity and retain centromeric cohesion. SpBub1p plays a role in maintaining SpRec8p at centromeres since, in its absence, centromeric SpRec8p is lost at anaphase I (Bernard *et al.*, 2001). Concentrated SpBub1p levels were found within the centromere regions at the time of kinetochore capture by microtubules, thus indicating that SpBub1p may have a task in monitoring that aspect of kinetochore function. Indeed, apart from observing the loss of centromeric cohesion another observed defect was the high proportion of *bub1Δ* cells in MI undergoing equational division.

In *Drosophila* Mei-S332 is a centromeric protein postulated to act as a protector for centromeric cohesion (Lee *et al.*, 2005). Another protein identified as forming a complex with the meiotic SpRec8p is SpSgo1p. SpSgo1p, “shugoshin” Japanese for “guardian spirit” protects centromeric cohesion during meiosis only (Kawashima *et al.*, 2007) so that sister chromatids remain tethered (Kitajima *et al.*, 2004); this protein was found to have similar regions to that of the *Drosophila* Mei-S332 protein. Kitajima *et al.*, (2004) also showed that in *bub1Δ* high levels of SpSgo1p were present; this suggested that SpBub1p may function in localising the protein to centromeres rather than providing a duty in stabilising the protector protein. Recent data have also shown that SpSgo1p cooperates with protein phosphatase 2A (PP2A) to protect centromeric cohesin at meiosis by dephosphorylating cohesin subunits and proteins such as separase which cleave cohesin (Yu & Koshland, 2005; Riedel *et al.*, 2006). It is the dephosphorylation event which protects cohesin from cleavage (Brar *et al.*, 2006; Kitajima *et al.*, 2006; Kawashima *et al.*, 2007).

### 1.16.6 *Meiotic recombination*

One of the main purposes of meiosis is to promote meiotic recombination for genetic diversity. Not only does the nucleus undergo morphological changes but several chromosome structures are also involved too aid recombination. Shortly after conjugation the *S. pombe* nucleus is said to take on a “horse-tail” shape as it moves backwards and forwards to enable pairing and correct chromosome alignment (Bernard *et al.*, 2001). This oscillating movement is led by the SPB (Molnar *et al.*, 2001). Chromosomes structures implicated as having a function in the recombination process include the formation of linear elements, chiasmata and cohesion.

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. Fission yeast has two ScScc3p-like proteins, SpPsc3p and SpRec11p. SpPsc3p plays an essential role in sister chromatid cohesion by forming a complex with SpRad21p in mitosis; however, SpRec11 is meiosis specific forming a complex with SpRec8p to aid recombination. Studies have shown that mutations in SpRec11p reduce recombination, thus indicating the importance of *rec11*<sup>+</sup> and *rec8*<sup>+</sup> working together (De Veaux *et al.*, 1992; Davis & Smith, 2001; Kitajima *et al.*, 2003b; Ellermeier & Smith, 2005). Chiasmata are the cytological manifestations of recombination crossover events, allowing exchange of genetic material as well as another means to keep sister chromatids together. Chiasmata are themselves stabilised by the presence of cohesin and are resolved at the metaphase-anaphase transition when cohesin is cleaved by separase.

The synaptonemal complex (SC) is a large tripartite proteinaceous structure found in *S. cerevisiae* and higher eukaryotes. The SC assists in meiotic recombination, homologue pairing during metaphase and chromosome segregation in anaphase (Bähler *et al.*, 1993). The tripartite configuration forms when the two axial elements of homologues fuse to create two lateral elements with a central protein-rich core (Molnar *et al.*, 1995; Roeder, 1995). In comparison to other eukaryotes *S. pombe* does not form tripartite synaptonemal complexes during meiotic prophase, but instead linear elements resembling axial core-like structures. As opposed to axial elements, these are discontinuous structures running along the length of a chromosome which also function in HR and chromosome segregation. Linear elements are also implicated in sister chromatid cohesion, as meiotic cohesin *rec8Δ* mutants are defective in linear element formation and meiotic chromosome segregation (Bähler *et al.*, 1993; Kohli & Bähler, 1994, Molnar *et al.*, 1995; Molnar *et al.*, 2001).

### 1.16.7 **Establishment and cleavage of cohesin**

Chromatin immunoprecipitation studies (ChIP) have shown in fission yeast that cohesin preferentially binds to intergenic regions along the length of chromosome arms in areas where there is a high A/T base composition, and is highly augmented at the centromere, telomeres and mating type locus (Nonaka *et al.*, 2002). Several factors have been implicated in the establishment of cohesin. For example, SpSwi6p is required to mediate cohesin localisation to heterochromatic regions for the loading of cohesin binding at centromeres but not along chromosome arms (Bernard *et al.*, 2006). SpSsl3/ScScs4p forms a complex with SpMis4p/ScScs2p which is necessary for the association of the cohesin complex with chromosomes. However, whilst SpMis4p co-localises with SpRad21p, the two budding yeast counterparts, although necessary for the loading of cohesin onto chromatin do not co-localise with cohesin (Furuya *et al.*, 1998; Toth *et al.*, 1999; Ciosk *et al.*, 2000). Cohesin loading is a dynamic process and its establishment during S phase remains unclear. It has been postulated that a steady state is maintained on G1 chromosomes via opposing activities. A loading activity is provided by the SpMis4/SpSsl3 complex and the removal of cohesin is provided by the *S. pombe wpl1<sup>+</sup>* gene (Bernard *et al.*, 2008).

Peak transcript levels of cohesin have been detected during G1-S period of the cell cycle, and delaying cohesin expression until G2 leads to defects in sister chromatid cohesion and chromosome segregation (Watanabe *et al.*, 2001). During the course of an unperturbed cell cycle, cohesin is loaded onto chromosomes during S phase and is possibly coupled to the replication process when cohesion can be established most efficiently. Studies with both *mis4<sup>+</sup>* and *rad21<sup>+</sup>* temperature sensitive mutant cells revealed that these cells are inviable during S phase thus implicating that cohesion between sister chromatids is likely to be established in parallel with DNA replication (Furuya *et al.*, 1998). This shows the importance of SpEso1p.

The *S. pombe* SpEso1p has two functionally distinct domains: the N-terminal is homologous to *S. cerevisiae* DNA polymerase  $\eta$  (ScPol $\eta$ p), and the C-terminal region is similar to *S. cerevisiae* ScEco1p/ScCtf7p (Tanaka *et al.*, 2000). ScEco1p is necessary for establishing cohesion during S phase, but not for its maintenance. Similarly, the ScEco1p/ScCtf7p homologous domain of SpEso1p is essential for establishing sister chromatid cohesin, whereas the Pol $\eta$  domain functions in translesion DNA synthesis during S phase to remove any chemical adducts hindering replication, this role being



important in DNA damage. *esol*<sup>+</sup> genetically interacts with the non-essential *pds5*<sup>+</sup>, homologous to the essential protein of budding yeast ScPds5p. In *S. pombe* SpPds5p is constantly associated to cohesin preventing it from forming cohesion until it is counteracted via a physical interaction from the ScEco1p/ScCtf7p domain, however once cohesion is formed SpPds5p may function to stabilise it (Tanaka *et al.*, 2001). This hindering role of *pds5*<sup>+</sup> ensures cohesion is established only during replication when sister chromatids are present and not at any other time, or on any unrelated sequence.

In both yeasts cleavage of the  $\alpha$ -kleisin SCC subunit, ScSccl1p/ScMcd1p/SpRad21p, releases cohesion between sister chromatids. Timing of cohesion loss varies: in fission yeast SpRad21p is cleaved at anaphase (Tomonaga *et al.*, 2000), whilst in budding yeast the majority of ScMcd1p is cleaved at the metaphase-anaphase transition (Uhlmann *et al.*, 2000). As already mentioned, loss of cohesin in meiosis is a step wise process first being cleaved at the arms for homologous chromosome separation followed by loss of centromeric cohesin for sister chromatid separation. This pattern is typical in all eukaryotic meiosis.

Cohesion loss is mediated by the APC/C, which has two major roles in mitosis; the first degradation of the cyclin B SpCdc13p to inactivate SpCdc2p, and the second degradation of securin (SpCut2p/ScEsp1), an inhibitor of sister chromatid separation. Securin is largely non-conserved between species apart from an N-terminal domain that targets the protein for ubiquitination by the APC (Yamashita *et al.*, 1999). Once this is degraded separase (SpCut1p/ScPds1p) is free to cleave cohesin and separate chromosomes and chromatids. Separase has sequence homology in several species, with a conserved C-terminus domain and a unique N-terminus. In *S. cerevesiae* the N-terminus of ScPds1p is thought to contain protease function, and binding of the securin ScEsp1p to this region of ScPds1p regulates its activity (Ciosk *et al.*, 1998). In *S. pombe* SpRad21p has two highly conserved separase cleavage sites in its COOH termini, also found in SpRec8p (Uhlmann *et al.*, 1999; Buonomo *et al.*, 2000). In *S. cerevesiae*, ectopically expressed ScRec8p is cleaved at a consensus separase cleavage site during mitotic metaphase-anaphase transition, indicating that the same mechanism operates in meiosis (Buonomo *et al.*, 2000; Uhlmann *et al.*, 2000; Queralt & Uhlmann, 2005). Of particular importance is that since meiosis has two cellular divisions, the centromeric cohesin in meiosis is protected by SpSgo1p to prevent precocious sister segregation (Section 1.19.5).

### 1.16.8 **Cohesin and repair via homologous recombination**

Cohesion between sister chromatids is established in S phase, has an essential function during G2 for repair, and is cleaved at anaphase to partition sister chromatids. The cohesin complex has a fundamental function in accurate chromosome segregation and efficient DNA DSB repair. The first indication of cohesin being involved in recombination repair arose when the  $\alpha$ -kleisin subunit *rad21*<sup>+</sup> gene (homologous to *S. cerevisiae* *MCD1/SCC1*) was identified as having a role in the DSB repair. The *rad21-45* mutant cells showed mild sensitivity to UV irradiation, but a more enunciated response to ionizing radiation, which was attributed to the reduced ability to repair DSBs (Birkenbihl & Subramani, 1992). SpRad50p 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. *rad21*<sup>+</sup> interacts genetically with *rad50*<sup>+</sup>, and both are thought to function in the same pathway during S phase to assist repair and possibly re-initiation of collapsed replication forks (Hartsuiker *et al.*, 2001). Studies in three organisms, chicken, budding yeast and humans have indicated the importance of cohesin in repair. The importance of SCC1 was demonstrated in the chicken DT40 cell line in which a depletion of SCC1 caused a marked increase in sensitivity towards ionizing radiation. (Sjögren & Nasmyth., 2001; Sonoda *et al.*, 2001). In human cells cohesin was found to be recruited to DSBs in S and G2 phase of the cell cycle after laser induced damage (Kim *et al.*, 2002), and that suppression of the RAD21 gene in breast cancer cell lines resulted in a notable loss of cell proliferation and cell viability (Atienza *et al.*, 2005; Yamamoto *et al.*, 2006). In budding yeast during the G2-M transition in wild-type cells SCC1/MCD1 is expressed in parallel with induction of DSBs (Ünal *et al.*, 2007).

Much of the work with cohesin has been studied in *S. cerevisiae*. As mentioned, establishment of cohesion during a normal cell cycle takes place at S phase during replication. Work in budding yeast has demonstrated that cohesin can be established also at the G2-M transition in response to DSB induction, and this also needs the presence of the cohesin loading protein ScEco1p/ScCtf7p (Ström *et al.*, 2004; Ström & Sjögren, 2005; Ünal, 2007).

Other cohesin subunits implicated as having a role in repair include ScScc1p and ScSmc1p. Cells are unable to repair damaged induced via ionizing radiation in the absence of either ScScc1p or ScSmc1p. Data from studies in mammalian cells have shown that phosphorylation of the cohesin subunit SMC1 by the protein kinase ATM is important for

DNA repair following damage caused by ionizing radiation, and that this phosphorylation is necessary to activate the DNA checkpoint pathways (Kim *et al.*, 2002; Yazdi *et al.*, 2002).

The presence of cohesin in a damage environment is supported further by studies on the securin separase complex. Under wild-type conditions, this complex promotes anaphase and loss of cohesion between sister chromatids. SpPds1p securin in budding yeast is stabilised in cells in response to checkpoint signals that have detected DNA damage thus preventing the loss of cohesin (Yamamoto *et al.*, 1996a; 1996b). Recent work in fission yeast has suggested that pre-existing cohesin has to be removed via cleavage of the SpScc1p subunit by separase before repair of DNA can occur (Nagao *et al.*, 2004; Watrin & Peters, 2006).

Thus, cohesion between sisters throughout G2 is thought to facilitate repair of double-stranded DNA breaks by HR between sister chromatids (Ström *et al.*, 2004; Watrin & Peters, 2007). Further, evidence supporting cohesins as having a role in repair have involved studying other chromosome related repair processes, such as intra-chromosomal gene conversion; this type of damage does not require a template, and thus cohesin is not detected in its repair process (Ünal *et al.*, 2004)

Eukaryotes have developed two distinct methods for repair of DSBs that are phase specific, HR in G2 which uses the sister chromatid and non-homologous end joining (NHEJ) predominantly in G1, although also used in G2 (see later).

## 1.17 DNA damage

Everything discussed so far about the cell cycle in this introduction has obviated one basic requirement, that the integrity of a cell's genome be maintained. Initiation of DNA replication must occur once per cell cycle and be complete and accurate; replicated DNA must also be compacted into manageable units to maintain euploidy when segregated, and cohesion between sister chromatids must remain intact until anaphase. However, although these mechanisms exist to ensure everything goes smoothly, damage to DNA is unavoidable.

The process of DNA repair could be considered an umbrella term encompassing a spectrum of processes whereby a cell identifies and corrects damage to DNA molecules encoding the genome. Damage to a cell can occur as a consequence of defects in DNA metabolism such as errors in replication, or environmental factors such as ionizing radiation. The vast majority of damage affecting DNA is at the structural level and thus can impede the transcription of a gene; other lesions can cause potentially harmful mutations in the genome, thus affecting the survival of progeny cells after mitosis takes place. As a result, DNA repair processes must be constantly active to repair damaged DNA immediately, and prevent genomic instability (Friedberg, 2008).

In this Section the importance of DNA repair will be discussed, the source and nature of DNA damage caused, checkpoint controls, and repair pathways. This will then lead into the discussion of DNA repair and its relevance with the concept of cohesin. However, firstly, there will be a brief introduction of the history of *S. pombe*'s involvement in the study of DNA repair, and some of the repair genes that this organism applies.

### 1.17.1 *S. pombe* and the “rad” genes

Studies using *S. pombe* for DNA repair analysis began in the early 1970s when a series of radiation-sensitive mutants were isolated, dubbed the “rad” genes (Nasim & Smith, 1975). Due to its longer G2 phase *S. pombe* is more resistant to damage from UV and ionizing radiation than *S. cerevisiae*. The DNA repair rad genes were mapped to ~35 genetic complementation groups and were grouped phenotypically as being either sensitive mainly to UV radiation, to  $\gamma$  rays, or to both. Homologues for these genes are present in budding yeast in which extensive work on DNA repair has also been completed. For this reason,

the *rad* genes in *S. pombe* also come under the synonym *rhp* for *rad* homologue in *pombe* (Phipps *et al.*, 1985; Lehmann, 1996; Ivanov & Haber, 1997). Most of the *rad* mutants are sensitive to both UV and  $\gamma$  irradiation, some are involved in checkpoint roles, whilst others are deficient in recombination repair. All the *rad* genes identified to date are listed in Tables 1a and 1b, and some of these will be discussed in the following Sections.

<i>S. pombe</i>	<i>S. cerevisiae</i>	<i>H. sapiens</i>	Function
<b>Excision repair</b>			
<i>rhp23</i> <sup>+</sup>	<i>RAD23</i>	HHR23A	ubiquitin mediated repair
<i>rad16</i> <sup>+</sup> , <i>rad1</i> <sup>+</sup>	<i>RAD1</i>	XPF	nuclease subunit
<i>rad15</i> <sup>+</sup> , <i>rhp3</i> <sup>+</sup>	<i>RAD3</i>	XPD, ERCC2	helicase, TFIIH
<i>rad8</i> <sup>+</sup>	<i>RAD5</i>	-	helicase
<i>rhp16</i> <sup>+</sup>	<i>RAD16</i>	-	helicase
<i>rhp7</i> <sup>+</sup>	<i>RAD7</i>	-	nuclease
<i>swi10</i> <sup>+</sup> , <i>rad2</i> <sup>+</sup>	<i>RAD10</i>	ERCC1	nuclease
<i>rad13</i> <sup>+</sup>	<i>RAD2</i>	XPG, ERCC5	nuclease
<i>rhp42</i> <sup>+</sup> , <i>rhp4b</i> <sup>+</sup>	<i>RAD4</i>	-	nuclease
<i>rhp14</i> <sup>+</sup>	<i>RAD14</i>	XPA	nuclease
<i>rhp26</i> <sup>+</sup>	<i>RAD26</i>	ERCC6	helicase
<b>Recombination repair</b>			
<i>rdl1</i> <sup>+</sup>	<i>PSY3</i>	-	early and late role in HR
<i>rhp54</i> <sup>+</sup> , <i>rad5</i> <sup>+</sup>	<i>RAD54</i>	HHR54	helicase
<i>rqh1</i> <sup>+</sup> , <i>hus2</i> <sup>+</sup>	<i>SGS1</i>	BLM	ReqQ-like helicase
<i>rad60</i> <sup>+</sup>	-	-	interacts with <i>smc5</i> <sup>+</sup>
<i>rad50</i> <sup>+</sup>	<i>RAD50</i>	RAD50	SMC like protein
<i>rad21</i> <sup>+</sup>	<i>SCC1, MCD1</i>	RAD21	cohesin
<i>rad32</i> <sup>+</sup> , <i>mre11</i> <sup>+</sup>	<i>MRE11</i>	MRE11	associates with Rad50
<i>nse4</i> <sup>+</sup> , <i>rad62</i> <sup>+</sup>	<i>QR12</i>	NSE4	smc5-smc6 subunit
<i>rad22</i> <sup>+</sup>	<i>RAD52</i>	HHR52	associates with Rad51
<i>rti1</i> <sup>+</sup>	<i>RAD52</i>	HHR52	<i>rti1</i> <sup>+</sup> is a homologue of <i>rad22</i> <sup>+</sup>
<i>rad18</i> <sup>+</sup> , <i>smc6</i> <sup>+</sup>	<i>RHC18</i>	SMC6	smc subunit
<i>rhp51</i> <sup>+</sup> , <i>rad5</i> <sup>+</sup>	<i>RAD51</i>	RAD51	strand exchange
<i>rhp55</i> <sup>+</sup>	<i>RAD55</i>	RAD55	meiotic viability
<i>rhp57</i> <sup>+</sup>	<i>RAD57</i>	XRCC3	strand exchange, meiosis

**Table 1a: *S. pombe rad* and *rhp* named genes.**

Table compiled using *S. pombe* database; Edwards & Carr, 1995; Lehmann, 1996 and Humphrey, 2000.

<i>S. pombe</i>	<i>S. cerevisiae</i>	<i>H. sapiens</i>	Function
<b>DNA damage checkpoints</b>			
<i>rad17</i> <sup>+</sup>	<i>RAD24</i>	-	RFC like
<i>rad1</i> <sup>+</sup>	<i>RAD17</i>	-	nuclease
<i>rad24</i> <sup>+</sup> , <i>rad25</i> <sup>+</sup>	<i>BMH1, BMH2</i>	14 3 3	conserved checkpoint
<i>rad3</i> <sup>+</sup>	<i>MEC1</i>	ATR and ATM	kinase
<i>rad9</i> <sup>+</sup>	<i>DDC1</i>	RAD9	clamp component
<i>chk1</i> <sup>+</sup> , <i>rad27</i> <sup>+</sup>	<i>CHK1</i>	CHK1	kinase
<i>crb2</i> <sup>+</sup> , <i>rhp9</i> <sup>+</sup>	<i>RAD9</i>	CRB2	replication checkpoint
<i>mek1</i> <sup>+</sup> , <i>cds1</i> <sup>+</sup> , <i>chk2</i> <sup>+</sup>	<i>MEK1, CHK2</i>	CHK2	kinase
<i>rad4</i> <sup>+</sup> , <i>cut5</i> <sup>+</sup> , <i>dre3</i> <sup>+</sup>	<i>DPB11</i>	-	replication
<i>rad26</i> <sup>+</sup>	<i>DDC2, LCD1</i>	ERCC6	kinase
<b>Miscellaneous proteins</b>			
<i>rad2</i> <sup>+</sup>	<i>RAD27</i>	DnaseIV, MF1, FEN1	replication nuclease
<i>rhp6</i> <sup>+</sup> , <i>ubc2</i> <sup>+</sup>	<i>RAD6</i>	HHR6A, HHR6B	ubiquitin conjugation
<i>rhp18</i> <sup>+</sup>	<i>RAD18</i>	RAD18	ubiquitin conjugation
<i>ssb1</i> <sup>+</sup> , <i>rpa1</i> <sup>+</sup> , <i>rad11</i> <sup>+</sup>	<i>RFA1</i>	-	binds to SSB
<i>rad31</i> <sup>+</sup> , <i>uba4</i> <sup>+</sup>	<i>AOS1</i>	-	sumo activator
SPBC365.08C	YDR411.C, <i>DER1</i>	-	ER degradation
SPAC26H5.04	YIL017C	-	negative regulation of gluconeogenesis
SPAC1687.17c	-	-	ER degradation
<i>dfp1</i> <sup>+</sup> , <i>rad35</i> <sup>+</sup> , <i>him</i> <sup>+</sup>	<i>DBF4</i>	DBF4	SpHsk1p kinase subunit
SPBC18H10.19	-	UVRAG	radiation resistance

**Table 1b: *S. pombe* rad and rhp named genes - continued.**

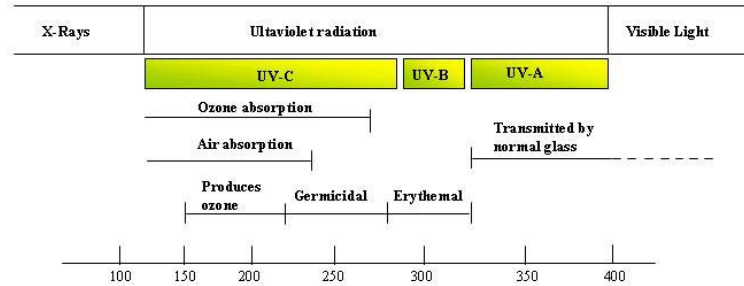
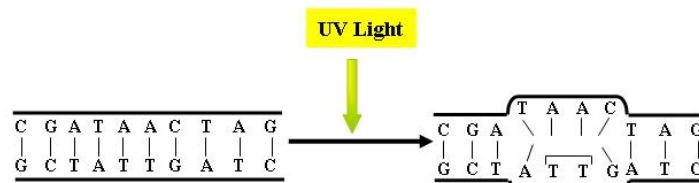
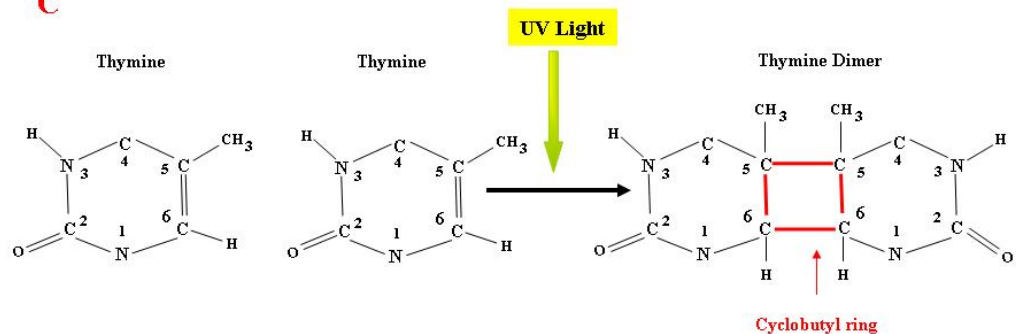
Table compiled using *S. pombe* database; Edwards & Carr, 1995; Lehmann, 1996 and Humphrey, 2000.

### 1.17.2 Sources and types of damage

DNA damage can arise in many ways. On an exogenous level through environmental agents such as UV-C and ionizing radiation, or endogenously by reaction with genotoxic chemicals that are by-products of normal cellular metabolism. DNA is also susceptible to damage caused from chemicals such as polycyclic hydrocarbons in smoke. Chemical damage can cause a multitude of DNA adducts, oxidised bases, alkylated phosphotriesters and DNA cross-linking. The sources used in this thesis to induce damage are ultraviolet-C (UV-C) radiation, methyl-methane sulfonate (MMS), phleomycin and ionizing radiation; each of these will be explained.

#### 1.17.2.1 Ultraviolet (UV)

Exposure to UV light results in the crosslinking of adjacent pyrimidines cytosine and thymine to create the pyrimidine dimer which can alter the helical structure of DNA. There are three types of UV light, the wavelength used for the experiments in this thesis is 250 nm i.e. UV-C (Figure 1.11). The most common UV products arising as a result of irradiation is the cyclobutane pyrimidine dimer (CPD). This is a bulky helix-distorting lesion that results in the arrest of replication and transcription, and is the major causes of cell killing by UV. Cyclobutane pyrimidine dimers occur when adjacent pyrimidines become covalently linked by the formation of a four-member ring structure resulting from saturation of their respective 5, 6 double bonds. The most common CPDs are thymine dimers that lead to single strand lesions in DNA. CPDs are repaired within seconds by the nucleotide excision repair machinery (NER), however, if not repaired they result in GC-AT transition mutations (purine – purine, pyrimidine – pyrimidine) and can cause skin cancer. In contrast to *S. cerevisiae* and other lower eukaryotes, fission yeast is extremely resistant to the detrimental affects of UV and ionising radiation (McCready *et al.*, 2000). This is because it spends more than 70% of its time in G2, thus having an extra copy of its genome for longer which proves to be useful in recombinational repair. Not only is the G2 time period prolonged in fission yeast, but it has two repair pathways to remove the bulky photoproducts caused by UV-C damage. The first is NER and the second pathway utilises the ultraviolet damage endonuclease (UVDE), to remove cyclobutane photodimers by incising 5' to the photoproducts (Bowman, *et al.*, 1994; Yonemasu *et al.*, 1997; Yasuhira & Yasui, 2000).

**A****B****C****Figure 1-11: UV irradiation.**

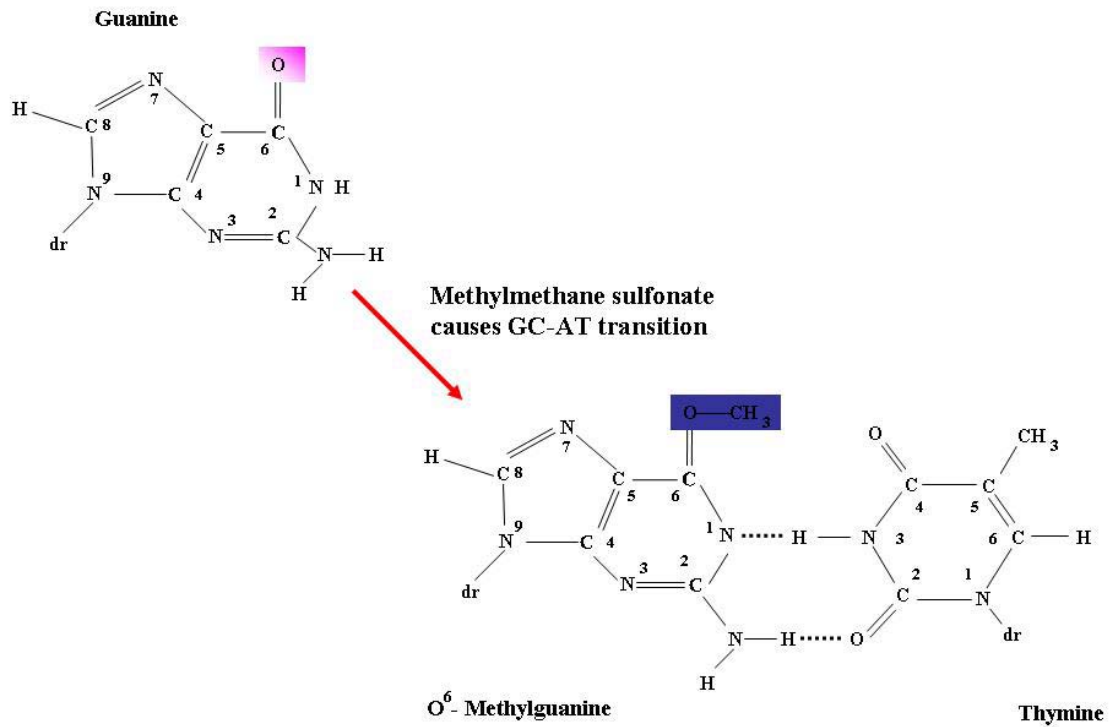
(A) UV radiation spectrum. (B) UV irradiation causes thymine dimers which causes distortion of the DNA double helix. (C) Production of thymine dimers by UV light irradiation. The most common type of mutation formed by UV irradiation is thymine dimers. Caused by two adjacent pyrimidines forming a four-membered cyclo butyl ring (red area) linking them together.



### 1.17.2.2 Methyl-methane sulfonate (MMS)

By introducing a methyl group ( $-\text{CH}_3$ ) onto DNA at a number of places, MMS has the ability to modify a single base causing a chemical change, in turn altering its base-pairing properties so that it pairs with the wrong base. After treatment with MMS, some guanines and some thymines in the DNA become methylated. A methylated guanine will pair with thymine rather than cytosine, giving GC to AT transitions and a methylated thymine will pair with guanine rather than adenine, giving TA to CG transitions. Methylation is more common on a guanine base (Figure 1.12).

Damage induced by DNA alkylation in living cells is repaired by two processes; direct transfer of the modifying alkyl group to the repair protein, or the removal of the modified base by a glycosylase, leaving an apurinic or apyrimidinic site which is then repaired by an excision-repair process. Nucleotide excision repair, normally involved in repairing adducts that distort the DNA helix, has recently been shown to play a supporting role in repairing lesions normally repaired by alkyl transferases or glycosylases (Beranek, 1990). The alkyl-transfer enzymes repair DNA lesions by an error-free mechanism since only the modified alkyl group is removed from the DNA. In the case of glycosylase repair, the entire modified nucleotide is removed, and the apurinic and apyrimidinic site is excised and repaired using the opposite strand as a template. Since this type of repair depends on the replication of the excised strand, errors may arise from mispairing and nucleotides to the template or by misincorporation of nucleotides during synthesis (Beranek, 1990).



**Figure 1-12: Methyl-methane sulfonate (MMS).**

MMS adds a methyl group onto 6-oxygen of guanines to produce O<sup>6</sup>-methylguanine. The methylated guanine will pair with thymine rather than cytosine, giving GC-to-AT transitions and problems in replication.

### 1.17.2.3 Phleomycin

Phleomycin is a bleomycin-like compound isolated from a mutant strain of *Streptomyces verticillus* (Wheatley *et al.*, 1974). Drugs of this family bind to DNA and block S phase entry during the cell cycle, causing loss in DNA bases and both single and DSBs (Steighner & Povrik, 1990).

The action of phleomycin is similar to bleomycin, although phleomycin is more cytotoxic, hence it has not been used for cancer therapy. Bleomycin has been used as an anti-cancer drug and induces damage by causing DSBs. *In vitro* studies have indicated that bleomycin requires oxygen and a metal ion, and functions by chelating primarily iron ions to produce a pseudo-enzyme that reacts with oxygen to produce superoxide and hydroxide free radicals which subsequently cleave DNA. Both produce inter-nucleosomal cleavage and chromosomal degradation (Moore, 1988). At higher doses, unrepaired breaks are substantially more abundant with phleomycin than with bleomycin, and thus contribute to more lethal effects. Such DSBs can be repaired by HR, or by NHEJ. In the experiments presented in this thesis phleomycin has been used to mimic the type of damage caused by ionizing radiation.

### 1.17.2.4 Ionizing radiation

Ionizing radiation is present in the surrounding environment and can affect genomic integrity with severe implications; exposure to this damaging source causes DSBs, one of the most fatal types of damage seen in a cell. The range of radiation includes both x-rays and gamma rays. In the laboratory a commonly used source for radiation purposes is cobalt-60 ( $^{60}\text{Co}$ ). Repair occurs using either HR or NHEJ (see later).

### 1.17.3 DNA checkpoints

DNA checkpoints can be perceived as the brakes in the cell cycle machinery. They function to halt cell cycle progression, thus safe-guarding completion of one phase before another can begin. There are four DNA integrity checkpoints which respond directly to changes in DNA structure; G1, S<sub>dNTP</sub>-M, intra-S and the G2-M checkpoint.

The checkpoint attributes usually affiliated with G1 phase in the cell cycle include cells growing to reach an appropriate cell size and the synthesis of proteins for the next phase. However, due to the short duration of G1 in *S. pombe* this phase is difficult to study in this organism. The S<sub>dNTP</sub>-M checkpoint was first identified in *S. pombe* and its biochemical basis involves phosphorylating SpCdc2p at the tyrosine 15 (Y15) residue by the kinases SpWee1p and SpMik1p. This negatively regulates SpCdc2p by inhibiting its activity and preventing initiation of mitosis. Hydroxyurea (an inhibitor of ribonucleotide reductase) causes depletion of the nucleotides required for DNA synthesis. Thus, when replication is blocked in S phase by hydroxyurea mitosis will not take place. The block is removed only when replication is complete and the mitotic cycle is re-entered. The intra-S checkpoint functions to ensure that S phase events are complete before entry into mitosis. Mitosis can only occur if all chromosomal DNA has been replicated, with the simultaneous establishment of sister chromatid-cohesion and centrosome duplication. If un-replicated DNA and stalled replication forks are detected, the SpCdc2p-SpCdc13p complex is inhibited and mitotic entry blocked. The G2-M checkpoint (Section 1.9.6) is another period during which the cell undergoes growth and makes sure that not only is the DNA completely and accurately replicated, but also any damage that might have taken place is repaired (Rhind & Russell, 2000).

### 1.17.4 Checkpoint proteins

At one point checkpoint proteins were understood simply as causing a delay in the cell, however, studies have demonstrated that they may play a role in coordinating and possibly even participating in DNA repair (Caspari & Carr, 2002). When there is damaged DNA in S phase, or when S phase proceeds in a disorderly manner due to dNTP depletion, both checkpoint and recombination proteins are required to prevent cell death. Ordinarily, both classes of proteins work independently of each other; the presence of recombination proteins is necessary only in a perturbed cell cycle.

### 1.17.5 Checkpoint loading complex-checkpoint sliding clamp

Checkpoint proteins are evolutionarily conserved and have been classified into several categories, the HsATRp-HsATRIPp kinase complex (SpRad3p-SpRad26p), the replication factor C (RFC) and proliferating cell nuclear antigen (PCNA) like complexes (SpRad1p, SpRad9p, SpHus1p and SpRad17p), the mediator proteins (SpCrb2p and SpMrc1p) and the effector kinases (SpChk1p and SpCds1p).

SpRad3p, a 2386 amino acid protein with one clear functional domain (a PI3-kinase domain), is the predominant protein underlying the DNA integrity checkpoints in *S. pombe*. It is homologous to ATM and Rad3 related (ATR) and shares a significant homology with HsATMp itself, the protein encoded by the gene that is mutated in ataxia telangiectasia patients. SpRad3p/ScMec1p/HsATRp associates with SpRad26p/ScDDcp/HsATRIPp to form a kinase complex (Al-Khodairy *et al.*, 1994; Bentley *et al.*, 1996; Edwards *et al.*, 1999).

It is thought that complexes form at the site of damage which recognises damage to DNA. Four proteins have been implicated as forming the checkpoint loading complex-checkpoint sliding clamp (CLC-CSC) complex. These are SpRad17p/ScRad24p, SpRad1p, SpRad9p and SpHus1p. The RFC complex, a recognition complex which is required for DNA replication, loads the PCNA heterotrimer onto the DNA. Three PCNA like proteins SpRad9p, SpRad1p and SpHus1p, dubbed the “9.1.1”, interact with the RFC like protein SpRad17p/ScRad24p which forms an RFC like complex with the four subunits of RFC and loads the 9.1.1 complex onto damaged chromatin (Caspari *et al.*, 2000; Venclovas & Thelen, 2000; Caspari & Carr, 2002). The activation of the DNA-damage checkpoint depends on the effector kinase SpChk1p, which is phosphorylated by SpRad3p in response to DNA damage (Martinho *et al.*, 1998).

Other proteins essential in the damage response include those encoded by the *rad4<sup>+</sup>/cut5<sup>+</sup>*, *crb2<sup>+</sup>*, *mrc1<sup>+</sup>*, and *cds1<sup>+</sup>* genes. SpRad4p/SpCut5p is an essential protein required for DNA replication, as well as for all the DNA integrity checkpoints. The checkpoint function of SpRad4p/SpCut5p is known to be required for S phase, the S-M checkpoint and for the G2-M DNA damage checkpoint (McFarlane *et al.*, 1997). By analogy from its homologue ScDpb11p this protein is also assumed to associate with replication origins and coordinate the process of DNA replication. Different complexes function in S phase and G2 phase (Furuya & Carr, 2003).

In the G2 phase, the effector kinase SpChk1p is phosphorylated in response to DNA damage by SpRad3p, and transfers the checkpoint signal to the cell cycle apparatus. This phosphorylation requires SpRad3p-SpRad26p, the four CLC-CSC proteins, SpRad4p/SpCut5p and SpCrb2p. SpCrb2 (identical to SpRhp9p) interacts with SpRad4p/SpCut5p. SpCrb2p has two C-terminal BRCT domains as does its homologue ScRad9p, and has been shown to interact with SpChk1p by mediating checkpoint signalling (Saka *et al.*, 1997).

In S phase, the effector kinase SpCds1p (homologous to ScRad53p) is phosphorylated and activated to arrest replication as an affect of DNA damage or reaction to hydroxyurea. Similar to activation of the effector kinase SpCkh1p in G2, SpCds1 also requires SpRad3p/SpRad26p and the CLC-CSC proteins.

As mentioned, SpRad4p/SpCut5p is not only required for the G2 phase but also for the S<sub>dNTP</sub>-M checkpoint. However, SpMrc1p is thought to play the equivalent role for SpCds1p in S phase that SpCrb2p plays for SpChk1p in G2 (Alcasabas *et al.*, 2001; Nitani *et al.*, 2006). The distinct responses of SpCds1p and SpCkh1p in S phase and G2 respectively is determined by the association of either SpMrc1p (in S phase) or SpCrb2p (in G2) with SpRad4/SpCut5p, and that this allows the core checkpoint complexes (SpRad3/SpRad26 and CLC-CSC) to connect with the appropriate kinase (either SpCds1p or SpCkh1p, respectively).

### **1.17.6 Repair pathways**

DNA repair systems are highly conserved throughout all organisms from both prokaryotic cell systems to eukaryotes. Many common repair pathways are present within cells such as the mismatch repair pathway (MMR), nucleotide excision repair (NER), base excision repair (BER); these pathways will be discussed here briefly. Within the context of this thesis and relative to the damaging agents used for experimental work, the pathways for the repair of DSBs; HR, NHEJ will be discussed in more detail.

#### **1.17.6.1 Mismatch repair (MMR) pathway**

Mismatches in DNA arise by spontaneous or induced base modifications, by replication errors, or by heteroduplex formation during recombination. MMR systems have to complete at least three processes which include recognising the mismatched base pairs,

determining which base in the mismatch is the incorrect one and, finally, excising the incorrect base and carry out repair synthesis.

#### **1.17.6.2 Nucleotide excision repair (NER) and transcription coupled repair**

This repair pathway is highly conserved between eukaryotes and is involved in the removal of chemical adducts such as UV damage, and also the repair of alkylation damage (Yonemasu, 1997; Lehmann, 2000; Memisoglu & Samson, 2000). Several NER genes have been identified in *S. pombe* (Table 1a and 1b). The involved proteins “slide” along the double stranded DNA surface looking for irregularities. Once identified, the proteins recruit the transcription factor TFIIH, whose helicase subunits, using ATP as an energy source, partially unwind the double helix to form a bubble. This involvement of the RNA polymerase II component is called transcription coupled repair. An endonuclease cuts the damaged bases, releasing them, and the gap is filled by DNA polymerase activity, and DNA ligase seals the nick.

#### **1.17.6.3 Base excision repair (BER)**

As well as using NER, alkylation damage can also be removed via this pathway. BER initiation occurs by the action of DNA glycosylases that recognise abnormal DNA bases and cleave the glycosidic bond linking the base to the sugar phosphate backbone. Following cleavage, the base is released to leave an apurinic/aprimidinic (AP) site. An AP endonuclease Flap endonuclease 1 (SpFen1p/SpRad2p) nicks the damaged DNA on the 5' side of the AP site to create a free 3'-OH. This allows DNA polymerase to synthesis a new DNA strand to fill the gap which is then sealed with DNA ligase (Memisoglu & Samson, 2000).

#### **1.17.6.4 Translesion DNA synthesis (TLS)**

The methods described for repair have all involved in some way where the damage bases have been excised. Cells can also repair their damaged during the replication process. DNA polymerases possess an intrinsic exonuclease activity and can “proof read” their DNA as synthesis proceeds. When a polymerase encounters a replication blockage it pauses excises the misincorporated base and then continues to synthesis. In addition to regular DNA polymerases cells have specialised DNA polymerases which evolved to

bypass blockages. This process is called translesion DNA synthesis (TLS) and allows strand extensions across template lesions (Friedberg *et al.*, 2002; Friedberg *et al.*, 2005; Lehmann, 2005)

### **1.17.7 *SpSmc5p-SpSmc6p complex in DNA repair***

The SpSmc5p-SpSmc6p forms the third complex in the SMC family; the other two being condensin and cohesin. This complex has been implicated as having a role in sister chromatid repair (Lindroos *et al.*, 2006; Ström *et al.*, 2007). As with all the SMC proteins, the SpSmc5p-SpSmc6p arms make up the core of the complex. In this complex there are six non-SMC subunits, SpNse1p-SpNse6p and, with the exception of SpNse5p and SpNse6p, all the subunits are essential for viability (Sergeant *et al.*, 2005; Palecek *et al.*, 2006).

The SpSmc5p-SpSmc6p complex was identified by the sensitivity of *smc6* mutants to DNA damage; these were found to be deficient in repair of radiation-induced DSBs, and thus thought to function in the recombination repair pathway. More recently in human cells HsSmc6p has been indicated as regulating the localisation of cohesin to DNA breaks in human cells (Potts *et al.*, 2006). SpSmc6p is also required for the DNA integrity checkpoint (Verkade *et al.*, 1999). Epistasis analysis of the gene indicated that it had a role in repair of UV damage although not in the NER but in the UVDE pathway (Fousteri & Lehmann, 2000). *smc6*<sup>+</sup> was previously given the name *rad18*<sup>+</sup>; structural analysis of the encoded protein indicated many similarities to the SMC family (Lehmann *et al.*, 1995). *nse1*<sup>+</sup> encodes a protein with E3 ubiquitin ligase activity, and *nse2*<sup>+</sup> a protein with sumo ligase activity. Absence of sumoylation of SpSmc6p sensitizes cells to DNA damaging agents and studies have shown that cells defective in SUMO (small ubiquitin related modifier) conjugation are sensitive to DNA damaging agents such as UV and ionizing radiation (Andrews *et al.*, 2005). *nse3*<sup>+</sup> encodes a protein related to the MAGE protein family (melanoma antigen-encoding gene), and no sequence motifs have been identified yet for *nse4*<sup>+</sup>, *nse5*<sup>+</sup> or *nse6*<sup>+</sup>.

How the components interconnect has only recently been revealed (Sergeant *et al.*, 2005; Palecek *et al.*, 2006). SpNse4p is a member of the  $\delta$ -kleisin sub-family and is structurally related to the SCC subunits; thus SpNse4p bridges the head regions of SpSmc5 and SpSmc6p. Although the relative locations are firmly established, the SpNse1p-SpNse3p-SpNse4p and SpNse5p-SpNse6p sub complexes bind to SpSmc5p-SpSmc6p head domains



but at different sites. Since SpNse4p binds the two SMC domains, a ring structure similar to that formed by the cohesin complex is predicted; however, studies have not demonstrated any cleavage events occurring. This might be due to the presence of the sub complexes also which are located quite close to the head domains (Palecek *et al.*, 2006).

### **1.17.8 Homologous recombination (HR) for repair of double strand breaks (DSBs)**

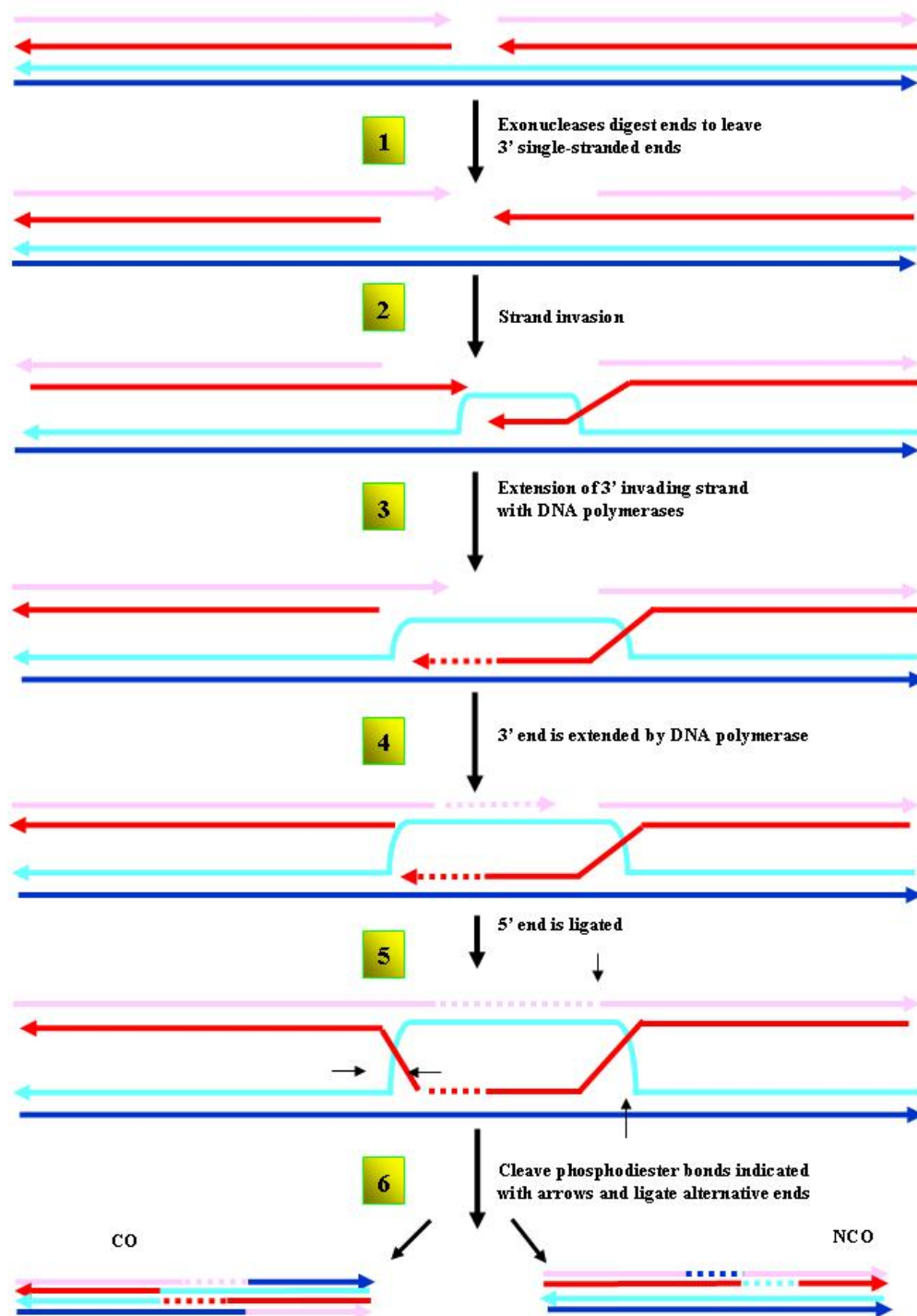
The best template for repair is one which is as identical to the original chromosome as possible; this is the basis for homologous recombination. DSBs are the most detrimental lesions that a cell's DNA can endure. Left un-repaired severe chromosomal defects can predispose cells to cancer, such as translocation events giving rise to the Philadelphia chromosome t(9;22)(q34;q11) seen in chronic myeloid leukaemia (Bonthron *et al.*, 1998), or mutations in the BRCA1/BRCA2 breast cancer genes which are involved in the repair of DSBs via HR (Liu & West, 2002).

Ionizing radiations such as X rays and  $\gamma$  – radiation cause DSBs in DNA. Cells have evolved two systems to repair these types of lesions, NHEJ and HJ. The first involves joining the non homologous ends of two DNA molecules. The process is error prone even if the two fragments were to come from the same chromosome. Since the two ends are simply ligated together several base pairs are lost at the joining point.

The second mechanism is HR and occurs when the damaged chromosome can “copy” the information from its identical partner to repair itself. As mentioned, this is the reason why *S. pombe* cells are more resistant to damage than *S. cerevisiae* when exposed to agents which cause DSBs since they spend the majority of their time in G2, when diploid genome content is available.

The process of HR is illustrated in Figure 1.13. After a DSB occurs in the DNA, an exonuclease cleaves the ends to leave 5' single-stranded ends (step 1). A single strand binding protein in *S. pombe*, SpRad51p catalyzes the invasion of one strand from one homologue into the other. The 3' end of the invading strand is extended by a DNA polymerase which causes the parental strand to form a D-loop (light blue) (step 2). Once the strand is long enough, base-pairing occurs between the complementary bases of the broken non-invading strand (pink) and the D-loop, and DNA synthesis happens (step 4). The two newly synthesised 3' ends are ligated to the 5' ends generated in step 1. This

generates two Holliday structures in the paired molecules (step 5). Repair is finalised when the strands are cleaved at the positions shown by the arrows, and depending on how cleavage takes place either recombinant chromosomes with cross over events (CO) arise or there are no cross over (NCO) events. Ligation of the alternative 5' and 3' ends at each cleaved Holliday structure generates two recombinant chromosomes that each contain the DNA of one parental DNA molecule on one side of the break point (pink and red) and the DNA of the other parental molecular (light blue and dark blue). Each chromosome also contains a heteroduplex located near the initial breakpoint; here one strand from one parent is base-paired to the complementary strand of the other parent (pink or red base-paired to dark or light blue).



**Figure 1-13: Double strand break repair (DSBR) by homologous recombination.**

The double helix of each homologous chromosome is represented as two parallel lines with sister chromatids coloured accordingly. Polarity of the strands is indicated by arrowheads at their 3' ends. The upper molecular has a DSB, which is digested to generate 3' single stranded ends (1). A single strand binding protein mediates strand invasion (2) which is then extended by DNA polymerases until the displaced single-stranded DNA (light blue) base pairs with the other (pink) (3), this 3' end is then extended with the DNA polymerase (4), the ends are ligated (5) and then depending on how the phosphodiester bond is cleaved either cross over (CO) events take place or no cross overs take place (NCO). Figure adapted from Heyer, 2004.

## 1.18 Project Aims

The aim of this study is to determine why fission yeast has two closely related genes *rad21*<sup>+</sup> and *rec8*<sup>+</sup> which, despite having related roles and being regulated similarly, are controlled in such a way that they are expressed in one life cycle and not the other. Previous work on these genes, where the cohesins were expressed in the alternative life cycles, indicated that at physiological levels in the mitotic cycle *rec8*<sup>+</sup> expression was able to complement the loss of *rad21*<sup>+</sup>, and *rad21*<sup>+</sup> was able to partially complement the absence of SpRec8p. These initial data suggested that the two cohesins, at least in these conditions, have similar and overlapping functions.

Here, we analyse the more specific role of SpRad21p, which was identified in a screen for DNA damage repair mutants. To this end, three strains were utilised: wild-type with functional SpRad21p, a *rad21* mutant strain, and a strain in which the meiotic cohesin *rec8*<sup>+</sup> was expressed in the mitotic cycle, replacing SpRad21p. Expression of *rec8*<sup>+</sup> in this manner serves as a useful tool for investigating the meiotic specific role of SpRec8p and allows us to find out if an overlap in cohesin function exists.

Various sources to induce damage are used; exposure to UV-C, treatment with MMS and phleomycin. These experiments revealed mitotic and meiotic specific roles for the two cohesins. When SpRec8p was expressed in the mitotic cycle replacing the native SpRad21p cell survival decreased, thus indicating that SpRec8p does not have a role in DNA repair.

## Chapter 2

### 2.1 Fission Yeast Materials

#### 2.1.1 Fission yeast media

Media used for the propagation of fission yeast were as described by Moreno *et al.*, (1991).

#### 2.1.2 Fission yeast strains and plasmid vectors

All fission yeast strains used in this thesis are described in Appendix II. The annotation GG refers to the Glasgow lab fission yeast collection number.

#### 2.1.3 Fission yeast chemicals

Chemicals used specifically for fission yeast in this thesis such as phloxin B, poly-L-lysine, phleomycin, methyl methane sulfonate (MMS), glucilase were purchased from Sigma.

### 2.2 Fission yeast methods

Standard genetic procedures of Gutz *et al.*, 1974 were followed.

#### 2.2.1 Culturing of fission yeast strains

Yeast strains were maintained as glycerol stocks (25% glycerol and 75% yeast extract YE) at -70°C. The required strain was streaked onto the appropriate solid medium either YE (complete medium) or EMM (Edinburgh minimal media) with or without supplements or in the presence or absence of thiamine (Moreno *et al.*, 1991). Unless indicated all cells were routinely grown at 25°C, on plates for 2 nights, followed by a 10 ml overnight pre-culture and finally culturing of the cells in the required volume and type of medium. At each stage, cells were checked microscopically to ensure no contamination had occurred, and that the cells were growing healthily.

To repress *nmt1P:rad21<sup>+</sup>*, cells were grown in the presence of 5  $\mu\text{g ml}^{-1}$  thiamine (Maundrell, 1993). Synchronous mitotic cultures using the elutriation method, and synchronous meiotic cultures using *pat1-114* were created as described (Cervantes *et al.*, 2000; White *et al.*, 2001). Cell numbers were counted using a Z series Coulter Counter.

## **2.2.2 Yeast mating, random spore analysis and tetrad analysis**

### **2.2.2.1 Yeast mating**

For mating reactions, freshly growing cells were mixed together, using a sterile toothpick, in 100  $\mu\text{l}$  of sterile  $\text{dH}_2\text{O}$  on malt extract (ME) 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. 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.

### **2.2.2.2 Random spore analysis**

Small aliquots of the resulting mating mixture was suspended in solution containing 20% helicase (Sigma) overnight at 37°C. This is a crude snail gut enzyme preparation that breaks down the ascus wall, releasing the spores unaffected and killing vegetative cells by partially digesting the cell wall.

Spores were then washed in sterile  $\text{dH}_2\text{O}$  and diluted before plating on solid YE medium and incubation at permissive temperature, typically for 3-4 days. Correct isolates were then identified by appropriate genotyping using EMM plates and supplements.

### **2.2.2.3 Tetrad analysis**

After mating, small aliquots of asci were lifted and spread very thinly onto YE plates. Individual asci were isolated and, using the Singer micro-manipulator, moved to new positions on the YE plate so that they were aligned in a column on the grid system. Asci were incubated at 36°C for 2-5 hr to promote breakdown of the ascus coat. Using the Singer 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 incubated at 25°C until colonies formed.

### **2.2.3 Identifying the *pat1-114* allele**

Cells carrying the temperature-sensitive *pat1-114* mutation were identified by comparing YE with phloxin plates 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 SpPat1p (Bähler *et al.*, 1991), an inhibitor of meiosis is inactivated at 36°C and cells undergo meiosis. However, 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 (dead cells) when compared to light pink cells on YE with phloxin at 25°C (viable cells).

### **2.2.4 Construction and identification of strains with the *LacI-GFP* construct and *LacO* repeats**

The three experimental strains, *rec8*<sup>+</sup>, *rec8Δ*, *rec8*<sup>+</sup>*P:rad21*<sup>+</sup> were required to have the *LacI-GFP* construct and the *LacO* repeats. These were constructed via, a series of mating reactions, followed by random spore experiments. Master plates were made from the spores resulting from each mating reaction. The master plate was then replica plated onto selective EMM to determine the required genotype. These cells with the correct genotype were then streaked onto fresh YE plates to obtain singles and checked against the parental strain.

### **2.2.5 Adenine mutants**

To determine which adenine allele a strain was mutant for, strains were grown on low adenine medium (10 mg l<sup>-1</sup>, or 0.5 ml of a 7.5 mg ml<sup>-1</sup> stock solution per 400 ml minimal medium or YE media without ade). Due to the accumulation of a metabolic precursor the adenine mutants vary in colour, *ade6-M210* is dark red, *ade6-M216* is light red/pink.

### **2.2.6 Mating type determination**

Freshly growing cells from the *S. pombe* strain of unknown mating type were patched twice onto ME plate. Freshly growing cells from wild-type strains of known mating type (*h*<sup>+</sup> and *h*<sup>-</sup>) were then patched alongside, and the cells mixed together in 100 µl of sterile dH<sub>2</sub>O.

The plate was left to air-dry, then incubated at 25°C for 2-3 days until asci had formed. Iodine crystals were dropped into the base of the ME plate and left for 15 mins for the vapour to stain the spores. The iodine crystals were then discarded and the ME plate left open for 5-15 mins for the excess vapour to dissipate. The mature spore wall is rich in amylase-like materials and is stained brown by iodine vapour. Patches that had stained with iodine represented those that had undergone conjugation and meiosis, and therefore contained cells of opposite mating type.

### **2.2.7 Preparation of competent yeast cells**

100 ml of the appropriate strain of *S. pombe* was grown to exponential phase ( $2-5 \times 10^6$  cells ml<sup>-1</sup>) and pelleted at 3000 rpm for 5 mins. The cells were washed in 5 ml of LiAc:TE (100 mM LiAc, 1 x TE) pelleted at 3000 rpm for 5 mins and re-suspended in 1 ml LiAc:TE plus 1 ml 2 M sorbitol. Cells were aliquoted into 100 µl and stored at -70°C.

### **2.2.8 Lithium acetate transformation**

The lithium acetate method (Schiestl & Geitz 1989, Geitz *et al.*, 1992) was carried out as follows: 1 µg of DNA and 50 µl of carrier salmon sperm DNA (5 µl of 10 mg/ml stock) was added to 100 µl of competent yeast cells in a microfuge tube. 700 µl of PEG mix (40% PEG 3350, 100 mM LiAc, 1 x TE) was added and the mixture was vortexed and incubated at 30°C for 30 mins. 43 µl of dimethyl sulphoxide (DMSO) was added before the cells were heat shocked at 42°C for 15 sec and then pelleted for a very light spin. Cells were re-suspended in 200 µl sterile dH<sub>2</sub>O and spread onto selective plates and left to grow at 30°C for 2-5 days.

### **2.2.9 Meiosis induction**

500 ml cultures of the appropriate *pat1-114* fission yeast strain were grown at 25°C to a cell density of approximately  $2-4 \times 10^6$  cells ml<sup>-1</sup>, in EMM containing the appropriate supplements at a concentration of 100 µg l<sup>-1</sup>. The cells were harvested (2000 rpm 2 mins) and washed twice in nitrogen free EMM before being re-suspended in nitrogen free EMM plus supplements at a concentration of 50 µg l<sup>-1</sup>. The culture was incubated at 25°C overnight to arrest the cells in the G1 phase of the cell cycle. The next day, to give a more synchronous meiosis, ammonium chloride was added back to the cultures to a concentration of 0.5 g l<sup>-1</sup> and a further 50 µg l<sup>-1</sup> of the appropriate supplements added. To



raise the temperature as quickly and as evenly as possible, the flasks were shaken in a 50°C water bath until the contents reached 35°C and immediately transferred to a 35°C shaking water bath. 35 ml samples were then taken at time 0 and every 15 mins for 3 hr. The cells were harvested (2000 rpm 2 mins) and the pellet washed in 500 µl RNA-ase free water. The cells were then removed to a microfuge and spun (12 000 rpm, 30 s) to remove the supernatant and the pellets stored at -70°C until required.

### **2.2.10 Mitotic synchronisation**

1 litre of culture was grown in YE at 25°C to a density of  $2 \times 10^6$  cells ml<sup>-1</sup>. The cells were then subjected to centrifugal elutriation using a Beckman JM/E with JE-6B rotor. When the centrifugal chamber was filled with cells, small cells in early G2 were selected by a gradual reduction in centrifugal pump speed. The isolated cells were then allowed to grow at an appropriate temperature for subsequent manipulations. In parallel, a small volume of cells was collected to monitor the success of cell synchrony.

### **2.2.11 Spore viability**

Cells were prepared for tetrad analysis (Section 2.2.2.3). The separated spores were left to grow at 25°C and spore viability was then calculated as the percentage of spores originally isolated that grew to form colonies.

### **2.2.12 Microscopy**

Glass slides and coverslips were rinsed in 100% ethanol and air-dried. Slides were coated in poly-L-lysine (0.1% w/v, in water); before use this was diluted by a factor of 10 in dH<sub>2</sub>O. Excess poly-L-lysine was removed from the slides by rinsing with dH<sub>2</sub>O and the slides then dried at 80°C. Cells were either viewed live or fixed using a 1:10 dilution of formaldehyde and stored in PBS for viewing later. Cells were pipetted onto a poly-L-lysine coated slide covered with a cover slip and left for 30 mins to allow cells to fix. For Nomarski imaging, cells were visualised using a Zeiss Axiostar microscope with images captured using a Sony DS-75 digital camera.

To check the fluorescence of each LacO LacI strain vegetative cells were analysed. Cells were grown exponentially in EMM. An aliquot taken, centrifuged and the supernatant discarded, the resulting pellet is re-suspended in water, 10 µl of cells were then put onto a

pre-prepared slide a cover slip placed and sealed around the edges with nail varnish. Fluorescent microscopy work was carried out using a Zeiss Axiovert microscope.

### **2.2.13 Measurement of survival rates after DNA damage treatments**

#### **2.2.13.1 DNA Damaging agents**

An Amersham UV- C 500 cross linker emitting primarily 254 nm light was used for ultra violet-C (UV-C) irradiation, UV light causes cross linking between adjacent cytosine and thymine bases creating pyrimidine dimers. Ionizing radiation causes breaks in DNA strands and was administered using a  $^{60}\text{Co}$  source. Methyl methane-sulfonate (MMS) was also used which adds a methyl ( $-\text{CH}_3$ ) group to the DNA base altering its binding properties. Phleomycin is a glycopeptides antibiotic of the bleomycin family, and inhibits DNA synthesis by reacting with DNA causing strand scission.

#### **2.2.13.2 Qualitative analysis**

For qualitative analyses, cells were routinely grown to a concentration of  $5 \times 10^6 \text{ ml}^{-1}$ , with 200  $\mu\text{l}$  of culture serially diluted six times (1:5 dilution). 5  $\mu\text{l}$  of each dilution was spotted on solid EMM with thiamine and irradiated with  $0\text{-}200 \text{ J m}^{-2}$ , or spotted on solid EMM with thiamine containing varying concentrations of MMS or phleomycin. After five days of growth at  $25^\circ\text{C}$ , sensitivity was determined by direct comparison with wild-type and *rad21-45* cells. All experiments were completed in duplicate. Where MMS was used, qualitative analyses, were also carried out using a 1:10 dilution of growing culture (v/v). The concentrations optimized from this set were used for further study to analyse effects on transcript levels and chromosome integrity. Ionizing radiation was administered using a  $^{60}\text{Co}$  source on neat and diluted cells (1:10 dilution).

#### **2.2.13.3 Quantitative analysis**

For quantitative analyses, cells were grown to a concentration of  $5 \times 10^6 \text{ ml}^{-1}$  and a 1:1000 dilution plated on solid EMM with thiamine and irradiated with increasing doses of UV light, or plated on EMM with thiamine with either MMS or phleomycin, at varying concentrations. These were left to grow for 5 days at  $25^\circ\text{C}$  and colonies counted. All counts were completed in triplicates of five.

## **2.3 Bacterial Materials**

### **2.3.1 Bacterial chemicals**

All chemicals used were of the highest grade available commercially and the distilled water was of Millipore-Q quality. Ampicillin, was from Sigma and the QIAprep<sup>®</sup> Spin Miniprep Kit was supplied by QIAGEN.

### **2.3.2 Bacterial media**

All strains of *E.coli* were grown in Luria Broth (LB; 10 g Bacto tryptone, 5 g Bacto yeast extract and 10 g NaCl per litre pH 7.5). LB plates were made by adding 7.5 g bacto agar to 500 ml LB. The media was autoclaved before use and supplemented with ampicillin ( $50 \mu\text{g ml}^{-1}$ ).

### **2.3.3 Bacterial vector**

The bacterial vector used in this thesis is listed in Appendix III. The annotation GB refers to the Glasgow lab bacteria collection number.

## **2.4 Bacterial methods**

The bacterial methods used were based on those devised by Sambrook & Russell, (2001).

### **2.4.1 Plasmid miniprep**

10 ml of LB plus antibiotic was inoculated and grown from a single colony. Plasmid DNA was purified from the culture using the QIAprep<sup>®</sup> Spin Miniprep Kit using the protocol in the manufacturer's manual. After no more than 16 hr incubation at 37°C cells were pelleted at 13000 rpm for 1 min and then re-suspended in 250  $\mu\text{l}$  buffer P1 (50 mM Tris/HCl pH 8.0, 10 mM EDTA, RNase A 100  $\mu\text{g ml}^{-1}$ ). Cells were then lysed by addition of 250  $\mu\text{l}$  buffer P2 (0.2 M NaOH, 1% SDS) and incubated for 5 mins. 350  $\mu\text{l}$  of buffer P3 (2.55 M KOAc pH 4.8) was added and the mixture was then centrifuged at 13000 rpm for 10 min to pellet cell debris. 2 x 220  $\mu\text{l}$  of supernatant was put through a QIAprep column to allow DNA to bind to the column. The column was washed with 750  $\mu\text{l}$  buffer PE and spun twice

to remove all trace of buffer. Finally, to elute the DNA, 50  $\mu$ l of nuclease-free water was added. Agarose gel electrophoresis was used to determine DNA quality and yield. The DNA was then used for restriction digest.

### **2.4.2 Restriction digest**

DNA fragments were routinely digested in 1.5 ml microfuge tubes. A typical 20  $\mu$ l reaction consists of ~200 ng of DNA, a final 1 x concentration of the appropriate 10x buffer, 1 U of enzyme, and water to 20  $\mu$ l. Depending on the restriction enzyme, reactions were incubated at the appropriate temperature and time period, after which the reaction mixture was loaded on an agarose gel (Section 2.4.3).

## **2.5 DNA Materials**

Specialised chemicals such as Sephadex G-50, [ $\alpha$ -P<sup>32</sup>] dCTP (3,000 Ci/mmol) were obtained from Amersham Pharmacia Biotech. N<sub>6</sub> (random hexanucleotide mixture), Klenow buffer and DNA molecular weight marker X (0.07-12.2 Kb) were obtained from Roche and glass beads from Sigma.

### **2.5.1 DNA Enzymes and kits**

All restriction enzymes, T4 DNA ligase, calf intestinal alkaline phosphatase, dNTPs, *Taq* DNA polymerase were obtained from Promega. QIAquick<sup>®</sup> Gel Extraction Kit was supplied by QIAGEN. Ribonuclease A was obtained from Sigma. Coated slides for the comet assay were obtained from Trevigen.

### **2.5.2 Oligonucleotides**

Oligonucleotides required were synthesised by MWG-AG Biotech. All oligonucleotides used in this thesis are listed in Appendix III. The annotation GO refers to the Glasgow lab oligonucleotide collection number.

## 2.6 DNA Methods

### 2.6.1 *Flow cytometry*

Flow cytometry was performed to measure the DNA content of cells during cell cycle experiments. This provided a method for monitoring progression of the cells through S phase. 0.5–1 ml cell samples were taken at hourly time points during a cell cycle experiment. The cells were spun down (30 sec 13000 rpm) and washed in 1 ml ice-cold dH<sub>2</sub>O. The cells were then spun down again before being gradually re-suspended in 1 ml of ice-cold 70% ethanol whilst vortexing. These fixed cells were then stored at 48°C until required.

For analysis, 300 µl of the fixed cell sample was spun down (30 sec 13000 rpm) and rehydrated by washing in 1 ml 50 mM sodium citrate. The cells were then re-suspended in 1 ml of 50 mM sodium citrate containing 0.5 µg RNase A and 0.4 µg ml<sup>-1</sup> propidium iodide and left to stain at 37°C for 2–4 hr. A Becton Dickinson FACScan machine and CellQuest software were used.

### 2.6.2 *Gel purification of DNA*

DNA of 70 bp to 10 kb was extracted and purified from agarose gels using the QIAquick Gel Extraction Kit (Qiagen 28704). All solutions were provided with the kit, the required 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 Buffer QG added (e.g. 900 µl of Buffer QG was added to 300 mg of gel). The gel slice was incubated at 50°C for 10 mins, vortexing every 2–3 mins 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 13000 rpm for 1 min and the flow through discarded. To wash the column, 0.5 ml of Buffer QG was added, centrifuged at 13000 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 mins, then centrifuged at 13000 rpm for 1 min. The flow through was discarded and the column given a repeat spin to remove any remaining Buffer PE. The column was then placed into a clean microfuge tube. To elute the DNA from the column, 50 µl of dH<sub>2</sub>O was added directly to the column

membrane, left to stand for 1 minute, and centrifuged for 1 minute at 13000 rpm. The purified DNA was then stored at -20°C.

### **2.6.3 Agarose gel electrophoresis**

For the required percentage gel, routinely 1%, the appropriate amount (1 g 100 ml<sup>-1</sup>) of agarose was dissolved in 1 x TBE (45 mM tris-borate, 1 mM EDTA) then 2-3 µl ethidium bromide was added to give a slab of agarose gel. Samples for analysis were diluted 5 fold by the addition of 6 x loading buffer (0.25% (w/v) bromophenol blue, 0.25% (w/v) xylene cyanol FF, 0.15% (w/v) Ficoll) before being loaded on the agarose gel. Gels were run at 100 V in 1 x TBE buffer for between 40 min and 1 hr depending on product size or until dye was about 1 cm from bottom of gel. Gels were viewed using a UV transilluminator and photographed using E.A.S.Y imaging software.

### **2.6.4 Polymerase chain reaction (PCR)**

Standard PCR reactions were carried out in an MWG-Biotech Primus Thermal Cycler in 0.5 ml thin walled PCR tubes with Taq DNA polymerase. A typical 100 µl reaction volume contained 10 x Taq buffer at a final concentration of 1 x, 200 µM each of dNTPs, 100 ng each of the appropriate primers, 50 ng of DNA template and 2U of Taq DNA polymerase. The annealing temperatures and extension times (\*) varied depending on the primers used and the length of the expected product. Primer annealing temperatures (T<sub>m</sub>) were calculated using the equation:  $T_m = 4 \times (G+C) + 2 \times (A+T)$ . The annealing temperature used was that of the primer with the lowest T<sub>m</sub>.

Typical cycle parameters were as follows:

	<b>Denaturation</b>	94 °C 5 mins
<b>Loop 30 cycles</b> {	<b>Denaturation</b>	94 °C 30 sec
	<b>Annealing</b>	55 °C 1 min*
	<b>Extension</b>	68 °C 1 min *
	<b>Final extension</b>	68 °C 4 mins
	<b>Incubation</b>	4 °C Indefinite

### **2.6.5 Preparation of genomic DNA**

10 ml fission yeast cultures were grown to saturation (2–3 days) in 50 ml centrifuge tubes. Cells were collected by spinning for 5 mins, at 3000 rpm, in a chilled desktop centrifuge. The supernatant was removed and the cells re-suspended in 0.5 ml dH<sub>2</sub>O. The re-suspended cells were then transferred to screw top tubes and spun for 30 sec at 13000 rpm in a microfuge tube to pellet the cells. The supernatant was decanted and the tubes vortexed to re-suspend the cells in the residual liquid. 0.2 ml solution A (2% Triton X-100, 1% SDS, 100 mM NaCl, 100 mM Tris pH 8, 1 mM EDTA), 0.2 ml 1:1 phenol/chloroform and 0.3 g acid washed glass beads were added to each tube. The tubes were then agitated in a ribolyser (Hybaid) for 5 x 40 sec at setting 4.0.

0.4 ml TE was added and the tubes spun for 5 mins at 13000 rpm in a microfuge tube. The aqueous layer was removed to a fresh microfuge tube leaving behind the liquid directly at the protein interface. 1 ml 100% ethanol was added and the tubes spun again for 5 mins in a microfuge tube at 12000 rpm to precipitate the nucleic acid. All the ethanol was carefully removed and the pellet re-suspended in 0.4 ml TE and 0.3 µg RNase A. This mixture was left at 37°C for 5 mins to remove the RNA from the pellet. 8 µl 5M ammonium acetate and 1 ml 100% ethanol were then added to each tube and the contents thoroughly mixed before freezing at -70°C for 30 mins. The tubes were spun for 5 mins at 13000 rpm in a microfuge tube and the supernatant was removed. The DNA pellets were air dried and left to re-suspend in 100 µl dH<sub>2</sub>O with gentle mixing to avoid shearing of the DNA.

### **2.6.6 Pulsed field gel electrophoresis (PFGE)**

#### **2.6.6.1 Preparation of DNA plugs**

Exponentially growing *S. pombe* cultures were grown with the appropriate concentration of MMS with the required volume centrifuged to give a final concentration of  $1 \times 10^9$  cells ml<sup>-1</sup>. The cells were washed in 1 ml SP1 buffer (50mM citrate/phosphate (pH 5.6), 40 mM EDTA, 1.2 M sorbitol), excess buffer was carefully removed and the cells either stored in -70°C or treated with Zymolyase-100T (Seigaku Corp).

Cells were treated with 1 mg ml<sup>-1</sup> Zymolyase-100T in SP1 buffer for 10-30 mins at 37°C to spheroplast. Spheroplasts were checked by looking for the appearance of ‘ghost’ cells upon treatment of cells with 1% SDS on a microscope slide. Zymolyase was removed by

spinning down at 3000 rpm for 1 min. Spheroplasts were gently re-suspended in 1% low melting point agarose (Cambrex Seaplaque GTG Agarose) in TSE (10 mM Tris-HCl (pH 7.5), 0.9 M sorbitol, 45 mM EDTA) and then immediately placed into 100  $\mu$ l plugs so that each plug consisted of  $1 \times 10^8$  cells  $\text{ml}^{-1}$ . Plugs were allowed to solidify at 4°C for 10 mins. Plugs were transferred to tubes containing 7 ml 0.25M EDTA, 50mM Tris-HCl (pH 7.5), 1% SDS and incubated at 55°C for 90 min. The solution was removed and replaced with 0.5M EDTA (pH 9.5), 1% lauryl sarcosine, 1 mg  $\text{ml}^{-1}$  proteinase K and incubated at 55°C for 48 hr, changing the solution after 24 hr. Plugs were washed in at least 12 ml volume of 10xTE (10 mM EDTA, 10mM Tris-HCl (pH 7.5)) for 30 mins at 25°C a second overnight wash was carried out and plugs left in the 25°C incubator. The following day plugs were incubated in 10xTE containing 0.04 mg  $\text{ml}^{-1}$  PMSF at 50°C, then twice more for 30 mins in 10xTE at 25°C. Plugs were stored at 4°C in 0.5M EDTA, 10 mM Tris-HCl (pH 9.5) until required.

#### **2.6.6.2 Gel electrophoresis**

Plugs were pre-equilibrated in 1xTAE (40 mM Tris-acetate, 1mM EDTA) on ice for 1 hr and loaded into a 0.8% agarose gel (Biorad Pulsed Field Certified Agarose) in 1 x TAE. PFGE was performed on a BioRad CHEF DR-III, which prior to use was washed using the following, 0.1% SDS wash 22°C overnight, two washes using  $\text{dH}_2\text{O}$  at 14°C, and another overnight wash with 1 x TAE (pH 8) at 14°C.

The next day the gel was ran in fresh 1 x TAE (pH 8) at 14°C using the following program: step 1, 24 hr at 2  $\text{vcm}^{-1}$ , 96° angle, 1200 sec switch time; step 2, 24 hr at 2  $\text{vcm}^{-1}$ , 100° angle, 1500 sec switch time; step 3, 24 hr at 2  $\text{vcm}^{-1}$ , 106° angle, 1800 sec switch time. *S. pombe* chromosomal DNA (Biorad) was loaded as a control. The gel was stained in ethidium bromide for 30 mins, followed by destaining in  $\text{dH}_2\text{O}$  until required.

#### **2.6.7 Comet assay – single cell gel electrophoresis**

An initial standard procedure for alkaline yeast comet assay was applied (Miloshev *et al.*, 2002). Yeast cells were grown to mid log phase and collected by centrifugation - 3 mins for 3000 rpm. Cells were washed with 1 ml S buffer (1 M Sorbitol, 25 mM Phosphate buffer, pH 6.5). Supernatant was poured away and the cells re-suspended in S buffer to give approximately  $10^4$  cells  $\text{ml}^{-1}$ . Aliquots of cells were mixed with low melting point agarose (Cambrex Seaplaque GTG Agarose) made in PBS pH 6.5 to give a final



concentration of 0.7%, the gel contained spheroplasting enzyme Zymolyase ( $10 \text{ mg ml}^{-1}$ ). The cells with low melting agarose and Zymolyase were spread as micro gels on standard microscope slides or comet slides obtained from Trevigen. These were covered with cover slips and placed at  $4^{\circ}\text{C}$  for 5 mins. The cover slips were gently removed. Micro gels were then incubated at  $37^{\circ}\text{C}$  for 20 mins for spheroplasting. After this incubation step the slides were submerged in lysis solution (1M NaCl, 50 mM EDTA, pH 8, 30 mM NaOH, 0.1% N-Lauryl sarcosine) for 1 hr at  $4^{\circ}\text{C}$ . Following lysis the slides were submerged in denaturing solution final pH 12.4 (30 mM NaOH, 10 mM EDTA pH 8) for 20 mins at  $4^{\circ}\text{C}$ , this denaturation step was repeated three times. The denaturing solution is also the alkaline running buffer and electrophoresis was carried out for 15 mins at  $0.6 \text{ vcm}^{-1}$ . After electrophoresis, the micro gels were neutralised by submerging the slides in 10 mM Tris-HCl pH 7.5 for 10 mins, and then for 10 mins in 75% ethanol and 95% ethanol. The micro gels were left to air dry, stained with ethidium bromide ( $1 \text{ mg ml}^{-1}$ ) and then visualised using a fluorescent microscope.

## **2.7 RNA materials**

### ***2.7.1 RNA chemicals***

Specialised chemicals such as Aqua Phenol were obtained from Qbiogene. Salmon sperm DNA was obtained from Sigma. Dextran sulphate was obtained from Amersham Pharmacia Biotech. Genescreen membrane was purchased from NEB Life Science and bovine serum albumin (BSA) was bought from Helena Biosciences.

### ***2.7.2 Photographic materials***

X-ray film was purchased from Fujifilm. The X-Omat 100 processor was supplied by Kodak.

## **2.8 RNA Methods**

### ***2.8.1 Preparation of total RNA***

RNA extraction from fission yeast required the growth of a 200 ml culture of cells to mid-exponential phase and harvested at 3000 rpm for 5 mins. Supernatant was discarded and

cells could be stored at  $-70^{\circ}\text{C}$  at this stage. The cell pellet was re-suspended in 1 ml STE (0.32 M sucrose, 20 mM Tris-HCl pH 7.5, 10 mM EDTA pH 8.0) and transferred to a screw cap microfuge tube and centrifuged at 13000 rpm for 1 min. The cell pellet was re-suspended in 200  $\mu\text{l}$  STE followed by 0.3g acid washed glass beads. 600  $\mu\text{l}$  NTES (100 mM NaCl, 5 mM EDTA, 50 mM Tris-HCl, 1% (w/v) SDS) was then added and then 500  $\mu\text{l}$  of water saturated hot phenol at  $65^{\circ}\text{C}$ . Cells were lysed using a Hybaid Ribolyser for 3 x 40 sec bursts at setting 4. After centrifugation at 13000 rpm for 5 mins the upper aqueous phase and protein interface was transferred into a fresh microfuge tube containing 400  $\mu\text{l}$  hot phenol. The mixture was again ribolysed for a 1 x 40 sec burst and again spun for 5 mins. The aqueous phase was transferred to 400  $\mu\text{l}$  of phenol:chloroform (1:1) at room temperature and ribolysed. After spinning, the aqueous phase was transferred to a second aliquot of phenol:chloroform and ribolysed. Again a 5 mins spin was applied and the aqueous phase transferred to a 300  $\mu\text{l}$  aliquot of phenol:chloroform and ribolysed. After spinning, the aqueous phase was transferred to a 300  $\mu\text{l}$  aliquot of chloroform and ribolysed. Finally after spinning for 5 mins the aqueous phase was transferred to a fresh microfuge tube where 3 volumes of 100% ethanol and one tenth volume of 3 M sodium acetate pH 5.2 were added. The RNA was precipitated overnight at  $-20^{\circ}\text{C}$ . The following day RNA was pelleted by centrifugation at 13000 rpm for 10 mins and supernatant was discarded. The pellet was washed in 200  $\mu\text{l}$  70% ethanol in RNase-free  $\text{dH}_2\text{O}$  and centrifuged for 1 min. Ethanol was then carefully removed and the pellet was re-suspended in 55  $\mu\text{l}$  RNase-free  $\text{dH}_2\text{O}$  and dissolved by incubating at  $65^{\circ}\text{C}$  with frequent pipetting. 5  $\mu\text{l}$  of RNA was diluted in 500  $\mu\text{l}$  of  $\text{dH}_2\text{O}$  to measure quality and quantity by spectrophotometry

### ***2.8.2 Preparation of RNA probe***

RNA probes were made by PCR corresponding in each case to  $\sim 1$  kb of the ORF of each gene. DNA probes were labelled with  $[\alpha\text{-}^{32}\text{P}]$  dCTP using the random hexa-nucleotide labelling procedure of Feinberg & Volgelstein, (1984).

### ***2.8.3 Purification of radiolabelled RNA probes***

Sephadex G-50 (Amersham Pharmacia-Biotech) was prepared by adding two volumes of TE and autoclaving. The plunger was removed from a 1 ml syringe (Plastipak) and a small wad of siliconized glass wool (Supelco) was used to plug the end before the end was

placed inside a microfuge tube. Sephadex G-50 was added to the syringe, the syringe and the microfuge tube were placed inside a 50 ml centrifuge tube and then centrifuged at 2000 rpm for 2 mins to remove the TE. This was repeated until ~7 ml Sephadex G-50 remained in the syringe. A fresh screw-top microfuge tube was then placed at the bottom of the syringe and the radio-labelled probe added to the Sephadex G-50 column. This was spun again at 2000 rpm for 2 mins and the syringe was monitored with a geiger counter to confirm that unincorporated nucleotides had been removed from the probe. The screw-top microfuge tube containing the purified probe was then heated for 5 mins at 95°C and chilled on ice for 5 mins to denature the probe before use.

## **2.8.4 Northern analysis**

### **2.8.4.1 Running of RNA samples on a formaldehyde gel**

Samples containing 10 µg of RNA were prepared in 20 µl of RNA buffer (600 µl formaldehyde, 200 µl formamide, 240 µl 5x MNE buffer, 160 µl) H<sub>2</sub>O. 1 µl of a 1 in 50 dilution of ethidium bromide was added to each sample and they were incubated at 65°C for 5 mins before loading. A 1% agarose gel containing 0.65 % formaldehyde and 20% 5 x MNE buffer was used and run in 1 x MNE buffer at either 70 volts for 3-4 hr or overnight at 15 volts. (5 x MNE buffer: 0.12 M MOPS, 25 mM NaOAc, 5 mM EDTA (pH 7)).

### **2.8.4.2 Transfer of RNA to nitrocellulose membrane**

Before blotting, the RNA gel was soaked in 50 mM NaOH for 15 mins to facilitate transfer of the larger transcripts and then in 10 mM Na<sub>2</sub>HPO<sub>4</sub>/NaH<sub>2</sub>PO<sub>4</sub> (pH 6.5) for 15 mins to remove the formaldehyde from the gel. The nylon membrane (Genescreen) was also briefly soaked, firstly in water, then in 10 mM Na<sub>2</sub>HPO<sub>4</sub>/NaH<sub>2</sub>PO<sub>4</sub> (pH 6.5) before blotting. The capillary method was then used to blot the gel overnight. Transferred RNA was fixed to the membrane by cross linking in a UV oven for 1 min and baking at 80°C for 3 hr.

### **2.8.4.3 Probing membrane**

The membrane was prepared for hybridisation of the probe by incubation at 42°C for 1–2 hr in pre-hybridisation buffer (10 ml formamide, 4 ml P buffer, 4 ml 33% (w/v) Dextran sulphate, 1.16 g NaCl, 200 µl denatured salmon sperm DNA. (P buffer: 1% BSA,

1% pyrrolidine, 1% Ficoll, 250 mM Tris-HCl, 0.5% Na Pyrophosphate, 5% SDS)). The appropriate radiolabelled DNA probes were denatured and added directly to the pre-hybridisation buffer and left to probe overnight.

#### **2.8.4.4 Membrane washing**

The membrane was washed twice for 5 mins in 2 x SSPE at 42°C and then in 0.2 x SSPE, 0.5% SDS at 65°C for periods of 15 mins until an acceptable radioactive signal was obtained. (20 x SSPE; 3 M NaCl, 0.2 M NaH<sub>2</sub>PO<sub>4</sub>, 0.02 M EDTA (pH 7.4)).

#### **2.8.4.5 Membrane stripping**

When required for re-hybridisation, membranes were stripped by washing for 30 mins 2 h in strip solution at 70°C until no radioactivity could be detected. (Strip solution; 5 mM TrisCl (pH 8.0), 0.2 mM EDTA, 0.05% Na pyrophosphate, 0.002% polyvinyl pyrrolidine, 0.002 % BSA, 0.002% Ficoll).

### ***2.8.5 Quantification of northern analysis using Image J***

Intensity of radioactive signals was quantified using the Image J programme and normalised to the corresponding rRNA gels. Triplicate measurements were made for each time point and the results were averaged to give the results shown.

### ***2.8.6 Quantification of nucleic acids by spectrophotometry***

DNA and RNA samples were diluted 100-fold in dH<sub>2</sub>O and the concentration estimated by measuring the absorbance (A) at 260 nm on a spectrophotometer. The following equation was then used to calculate DNA and RNA concentrations in  $\mu\text{g } \mu\text{l}^{-1}$ :

$A_{260}$ of 1.0 = 40 $\mu\text{g } \text{ml}^{-1}$ RNA or 50 $\mu\text{g } \text{ml}^{-1}$ DNA
---

Therefore multiple  $A_{260}$  by dilution factor (x 100)

Then x 40 (RNA) or x 50 (DNA)

/ 1000 to get  $\mu\text{g } \mu\text{l}^{-1}$

A useful indication of the purity of DNA and RNA samples is provided by the ratio of absorbances at 260/280 nm. Pure DNA samples have a 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.

## **2.9 Statistical Analysis**

Quantitative data shown in Chapter 4 are representative of results obtained from three separate experiments. Paired and unpaired *t-test* statistical analysis of the data were undertaken, where statistical significance is indicated by three values;  $p < 0.05\%$ ,  $p < 0.01$  and  $p < 0.001$ , indicating increased sensitivity in comparison to untreated cells within a strain followed by analysis between strains.

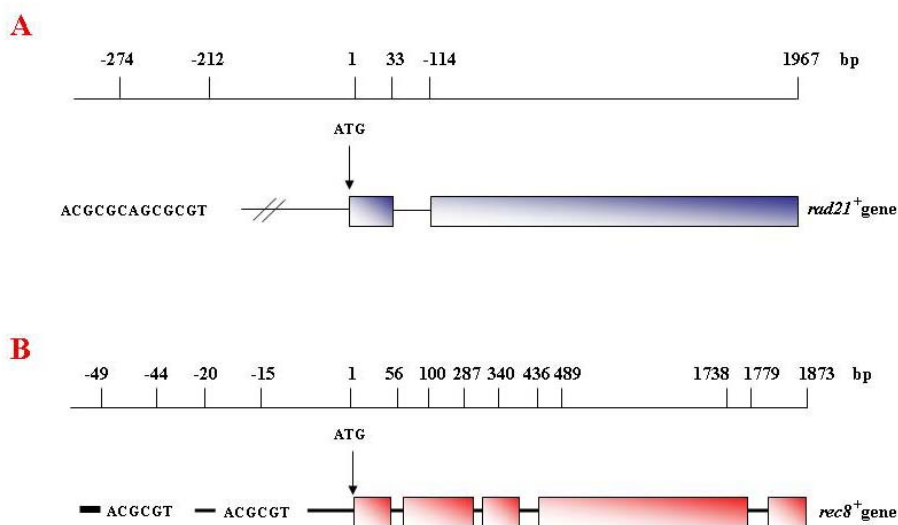
## Chapter 3

### 3.1 Introduction

As introduced in Chapter 1, cohesin is an evolutionary conserved multi-subunit complex composed of at least four proteins, which binds to chromosomes during S phase and is essential for the association of sister chromatids after their synthesis (Watanabe & Nurse, 1999; Jones & Sgouros 2001; Uhlmann, 2001; Guacci, 2007). This complex plays a critical role in the successful and accurate separation of sister chromatids during both mitosis and meiosis for which there are specific cohesin proteins. Defective sister chromatid cohesion leads to abnormal chromosome segregation during M phase, and results in the generation of aneuploid products.

The cohesin subunits of interest in this project are encoded by the *S. pombe* *rad21*<sup>+</sup> and *rec8*<sup>+</sup> genes. *rad21*<sup>+</sup> was first identified in a genetic screen for DNA repair mutants, and was found to be involved in the DNA double strand break (DSB) repair pathway, with mutants being sensitive to  $\gamma$ -irradiation (Birkenbihl & Subramani, 1992). *rec8*<sup>+</sup> was instead first identified by screening for mutants with reduced meiotic recombination at the *ade6*<sup>+</sup> locus (De Veaux *et al.*, 1992).

The *rad21*<sup>+</sup> and *rec8*<sup>+</sup> genes are both cell cycle regulated with peak transcripts and protein levels occurring at the G1-S boundary, with the proteins binding to DNA during replication and in turn preventing precocious chromosome segregation (Watanabe *et al.*, 2001). In their promoter regions, both genes have a conserved DNA motif, which has been named the *Mlu* I cell cycle box (MCB) (Figure 3.1). This motif is bound by DNA synthesis control 1 (DSC1), a transcription factor complex controlling periodic transcription at the G1-S boundary (Cunliffe *et al.*, 2004; McNerny, 2004). The differences in position and arrangement of MCB elements in the *rad21*<sup>+</sup> and *rec8*<sup>+</sup> promoters may reflect cell cycle specific differences in their transcriptional regulation (Cunliffe *et al.*, 2004; Figure 3.1).



**Figure 3-1: Chromosomal locations of *S. pombe rad21*<sup>+</sup> and *rec8*<sup>+</sup>.**

**A**, The *S. pombe rad21*<sup>+</sup> gene is on chromosome III and contains two exons (■) and one intron, encoding an mRNA of 2.6 kb. MCB sequences occur as a doublet (ACGCGCAGCGCGT) in the *rad21*<sup>+</sup> promoter region 274 bp upstream of the ATG. **B**, The *S. pombe rec8*<sup>+</sup> gene is on chromosome II and contains five exons (■) with four introns, encoding an mRNA of 1.7 kb. Two single consensus MCB sequences (ACGCGT) are found 49 bp and 20 bp upstream of the ATG (position 1).

In terms of their cohesive roles the two cohesins share many similarities, but they also have specific properties (Table 2). It has been suggested that these functions are an indirect consequence of the cohesins primary role in sister chromatid cohesion. For example, *rad21*<sup>+</sup> is involved in the DSB repair pathway (Birkenbihl & Subramani, 1992), and is also known to interact with tubulin genes indicating a role in microtubule function (Tatebayashi *et al.*, 1998). The *rec8*<sup>+</sup> gene has roles instead in linear element formation and alignment of homologous chromosomes (Molnar *et al.*, 1995; Parisi *et al.*, 1999).

	<i>rad21</i> <sup>+</sup>	<i>rec8</i> <sup>+</sup>
Properties	DNA double strand repair pathway	linear element formation
	microtubule function	alignment of homologous chromosomes
Similarities	Expression at G1/S	Expression at pre-meiotic S phase
	dense packing at outer centromeres	dense packing at outer and inner centromeres
	regulated by DSC1	regulated by DSC1
	regulate chromosome segregation	regulate chromosome segregation
	targeted proteolysis at anaphase	targeted proteolysis at anaphase

**Table 2: Properties and similarities of *S pombe* SpRad21p and SpRec8p cohesins.**

Table compiled using Parisi *et al.*, 1999; Tomonaga *et al.*, 2000; Tatebayashi *et al.*, 1998; Uhlmann 2001.

To summarise, *S. pombe* has two genes encoding cohesins that have related function whose expression appears to be regulated by similar transcriptional mechanisms. The *rad21*<sup>+</sup> gene is expressed during G1 of the mitotic cycle (Birkenbihl & Subramani, 1995), whereas *rec8*<sup>+</sup> expression is triggered upon induction of meiosis (Lin *et al.*, 1992). 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 first stage of this study was to determine and confirm the expression profiles of *rad21*<sup>+</sup> and *rec8*<sup>+</sup> genes during the mitotic and meiotic life cycles.



### 3.2 *rad21*<sup>+</sup> is periodically transcribed during the mitotic cell cycle

Previous published experiments studying the expression of *rad21*<sup>+</sup> through the mitotic cell cycle used *cdc25-22* temperature arrested and released cells to create synchronously dividing cells (Birkenbihl & Subramani, 1995). This method, although widely used, has the potential to introduce artefacts. Therefore, we created synchronously dividing cells using elutriation, which allowed us to use a wild-type strain.

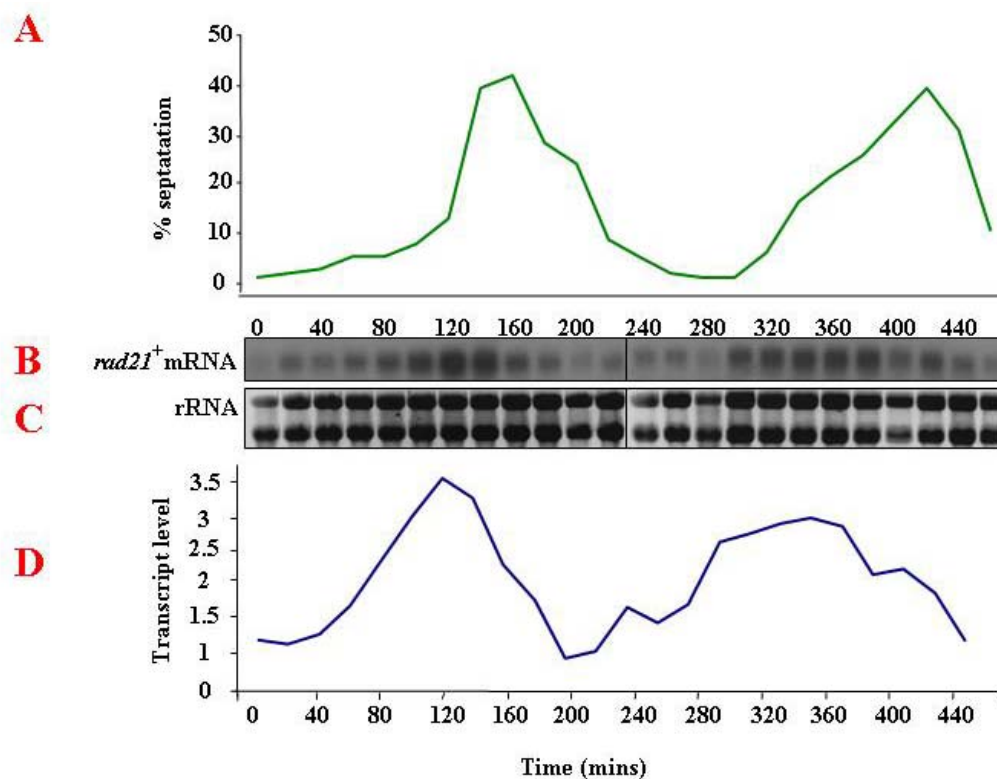
Wild-type (GG 218) cells were cultured in minimal media and grown to early log phase ( $1.5 \times 10^6$  cells ml<sup>-1</sup>) at 30°C. To obtain a synchronous population of dividing cells, a population of small cells was size selected by centrifugal elutriation using a Beckman JM/E and JE-6B rotor; these were grown at 30°C and samples collected at 20 minute intervals.

To monitor cell synchrony, the septation index of a small aliquot of each sample was 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 counted and this plotted against time (Figure 3.2A). In an exponentially growing *S. pombe* culture, G1 is so short that when septa form, the cells are already in S phase of the following cell cycle. Therefore, a peak in septation serves as a convenient S phase marker in this organism.

Total RNA was purified from each sample, and separated by electrophoresis under denaturing conditions and transferred to a nylon membrane by northern blotting (Figure 3.2B). Equal loading of RNA was confirmed by ethidium bromide staining of ribosomal RNA (rRNA) (Figure 3.2C). A DNA probe was made by amplifying a region of the open reading frame of *rad21*<sup>+</sup> by PCR using primers GO 118 and GO 119 (Appendix III). This DNA fragment was then labelled with <sup>32</sup>P by random hexanucleotide priming and hybridised to the messenger RNA (mRNA). mRNA levels were quantified by densitometry using the NIH image J program, accounting for minor differences in RNA loading (Figure 3.2D). The expected *rad21*<sup>+</sup> transcript size is 1.8 kb.

The mitotic northern blot analyses indicated that the *rad21*<sup>+</sup> gene encoded a single transcript of expected size that is specifically expressed during mitosis at the beginning of

S phase, in agreement with published observations. A similar experiment was conducted to examine the meiotic expression profile of *rec8*<sup>+</sup>.



**Figure 3-2: *rad21*<sup>+</sup> is periodically transcribed during the mitotic cell cycle.**

A synchronous population of wild-type (GG 218) cells was size selected by centrifugal elutriation, and samples collected at 20 minute intervals. **A**, Cells were fixed in formaldehyde and viewed by microscopy to monitor septation. The degree of synchrony is indicated by the septation index shown. **B**, Total RNA was prepared from each sample and 10  $\mu$ g separated on a formaldehyde gel, transferred to a nylon membrane and hybridized with a <sup>32</sup>P-labelled DNA probe for *rad21*<sup>+</sup> mRNA. **C**, Equal loading of RNA was confirmed by ethidium bromide staining of rRNA. **D**, Transcript levels were quantified by densitometry using NIH image software; units given are arbitrary.

### 3.3 *rec8*<sup>+</sup> expression is induced during *pat1-114* synchronous meiosis

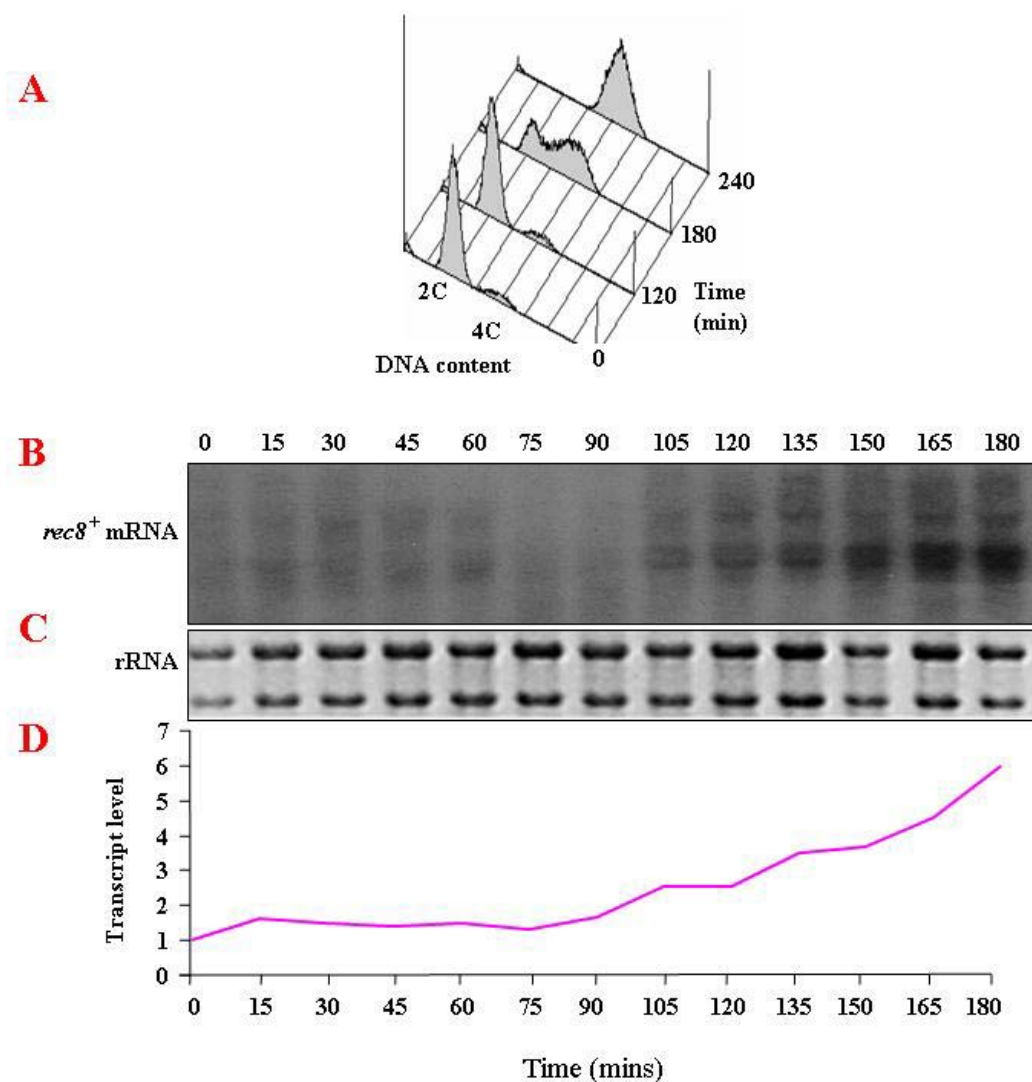
In wild-type *S. pombe*, meiosis is triggered during the G1 phase in response to nitrogen starvation and mating pheromones from cells of opposite mating type. Because once a cell passes START during G1 it is committed to complete the next mitotic cell cycle, entry into meiosis can only be triggered from G1 (Hayles & Nurse, 1992).

To induce synchronous entry into meiosis, a diploid *pat1-114* (GG 375) strain was used to mimic the natural induction of meiosis as closely as possible. To maximise synchrony, a diploid strain was used so that the conjugation step could be bypassed. However, *S. pombe* diploids are highly unstable, so this was overcome by using a diploid strain homozygous for mating type (*h*<sup>-</sup>/*h*<sup>-</sup>). SpPat1p is a tyrosine kinase that functions during G1 phase to prevent entry into meiosis by inhibiting SpSte11p and SpMei3p (Watanabe *et al.*, 1997). Incubation of the *pat1-114* mutant at restrictive temperature of 36°C during G1 leads to inactivation of SpPat1p, triggering synchronous entry into the meiotic cell cycle (Bähler *et al.*, 1991).

Cells were cultured to early log phase ( $1-5 \times 10^6$  cells ml<sup>-1</sup>) in minimal media at the permissive temperature of 25°C, before being harvested, washed and transferred to nitrogen-free media (EMM-N). Cells were then incubated for 16 hours 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. A nitrogen source (0.1 M NH<sub>4</sub>Cl) was added at the same time to enhance cell cycle synchrony. Samples were collected at 15 minute intervals and RNA extracted for northern blot analysis.

Duplicate samples were also taken, fixed in ethanol and stained with 2 µg ml<sup>-1</sup> propidium iodide for flow cytometric analysis. Propidium iodide is a fluorescent dye that labels DNA by intercalating into the double helix. Detection of the levels of fluorescence in cell samples by flow cytometry, was used 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 3.3A).

RNA was separated by electrophoresis and transferred to a nylon membrane for northern blotting. A DNA probe corresponding to a fragment of the *rec8*<sup>+</sup> coding region was generated by PCR using primers GO 694 and GO 695, followed by radio-labelling with <sup>32</sup>P by random hexanucleotide priming (Figure 3.3B). Equal loading of RNA was confirmed by ethidium bromide staining of ribosomal RNA (rRNA), with mRNA levels quantified by densitometry using an NIH Image programme (Figures 3.3C and 3.3D)



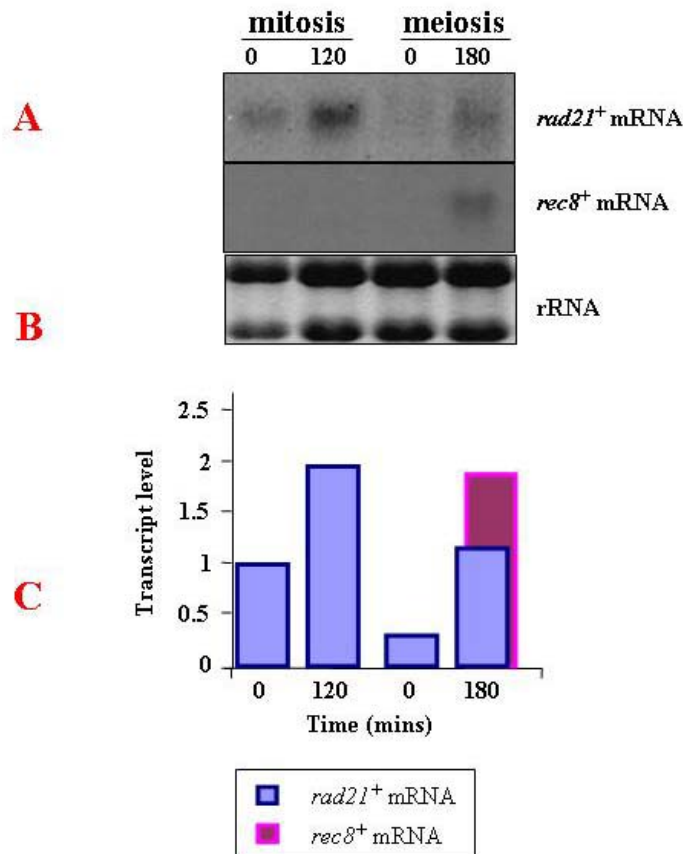
**Figure 3-3: *rec8*<sup>+</sup> is expressed following induction of meiosis.**

*pat-114* diploid.(GG 375) cells were arrested in G1 by nitrogen starvation for 16 hr at 25°C. By transferring the culture to restrictive temperature (36°C) synchronous entry into meiosis was induced. Cell samples were collected at 15 minute intervals. **A**, S phase progression was monitored using flow cytometry by staining cells with propidium iodide to measure DNA content. **B**, Total RNA was prepared from each sample and 10 µg separated on a formaldehyde gel, transferred to a nylon membrane and hybridized with a <sup>32</sup>P-labelled DNA probe for *rec8*<sup>+</sup> mRNA. **C**, Equal loading of RNA was confirmed by ethidium bromide staining of rRNA. **D**, Transcript levels were quantified by densitometry using NIH image software; units given are arbitrary.

Northern blot analysis of synchronously induced meiotic samples indicated at least two transcripts expressed during meiosis for *rec8*<sup>+</sup> which were specifically regulated. The transcripts first appeared at around 100 minutes after SpPat1p induction corresponding to the start of pre-meiotic S phase.

### 3.4 Comparison of *rad21*<sup>+</sup> and *rec8*<sup>+</sup> mRNA in mitosis and meiosis

To allow the direct comparison of the levels of *rad21*<sup>+</sup> and *rec8*<sup>+</sup> mRNA in the synchronous mitotic and meiotic cell cycle experiments (Figures 3.2 and 3.3), and to confirm that the expression of the two cohesins genes was specific to mitosis and meiosis, RNA samples corresponding to the peak mRNA levels were run on the same formaldehyde gel for northern blotting. The blot was then hybridised consecutively with both *rad21*<sup>+</sup> and *rec8*<sup>+</sup> probes.



**Figure 3-4: Comparison of mRNA levels of meiotic *rec8*<sup>+</sup> and mitotic *rad21*<sup>+</sup> from peak and trough mitotic cell cycle and meiotic samples.**

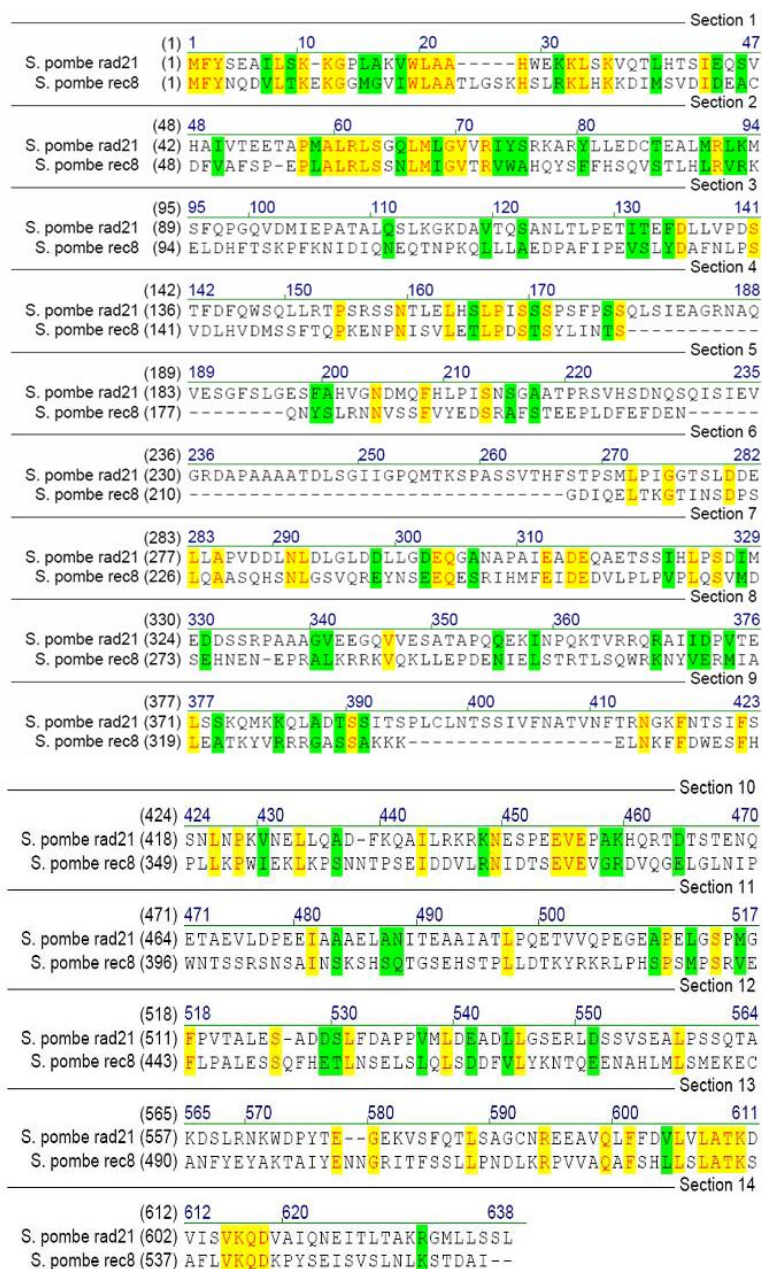
Mitotic samples at time 0 (minimal expression), 120 mins (maximum *rad21*<sup>+</sup> expression), from Figure 3.2; and meiotic samples at time 0 with a peak sample taken 180 mins after *pat1-114* induction from Figure 3.3. **A**, 10 µg total RNA was separated on a formaldehyde gel, transferred to a nylon membrane and hybridized with <sup>32</sup>P-labelled DNA probes for *rad21*<sup>+</sup> and *rec8*<sup>+</sup> mRNA. **B**, Equal loading of RNA was confirmed by ethidium bromide staining of rRNA. **C**, Transcript levels were quantified by densitometry using NIH image software; units given are arbitrary.

Hybridisations of *rec8*<sup>+</sup> probes with mitotic RNA and *rad21*<sup>+</sup> probe with meiotic RNA northern blot analysis confirmed that the two cohesins are specifically expressed in either mitosis or meiosis, respectively. However, a non-specific band at 180 minutes was detected in the meiotic samples with the *rad21*<sup>+</sup> probe which is an artefact of this experiment. This observation is confirmed by the microarray data available online (Mata *et al.*, 2002; <http://www.genedb.org/>).

### **3.5 *S. pombe* SpRad21p and SpRec8p**

SpRad21p is a 628 amino acid, 67 kDa nuclear protein essential for mitotic growth with an additional DNA DSB repair role (Birkenbihl & Subramani 1992). SpRec8p is a 561 amino acid, 64 kDa protein synthesised at the onset of meiosis and has been implicated to have a role during meiotic homologous recombination (Molnar *et al.*, 1995; Parisi *et al.*, 1999). It has been suggested that these functions are an indirect consequence of their primary role in sister chromatid cohesion (see also Table 1).

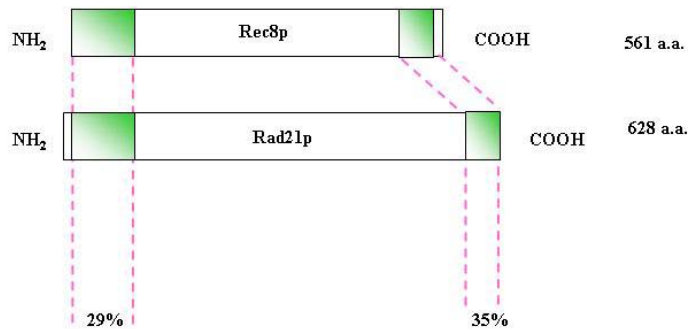
The *S. pombe* SpRad21p and SpRec8p protein sequences were aligned using the align X program in Vector NTI advanced, (Figure 3.5). Although low levels of similarity are seen across the entire open reading frame (ORF), sequence homology is present in the N and C-termini; 27% and 35%, respectively (Figure 3.6; Watanabe & Nurse, 1999),



**Figure 3-5: Sequence alignment of *S. pombe* SpRad21p and SpRec8p.**

Sequences shown are *S. pombe* SpRad21p (628 aa), and SpRec8p (561 aa). The black residues on the white colour are non similar residues, red on yellow regions are identical residues between the four proteins and finally black on green regions are similar residues between the two proteins. These proteins share 26% similarity with 15% identity. Areas of highest similarity are in the N and C termini. See also Figure 1.6.





**Figure 3-6: Sequence conservation between SpRad21p and Rec8p.**

*S. pombe* SpRec8p and SpRad21p share 29% and 35% similarity in the amino and carboxy-terminal regions respectively (Watanabe & Nurse, 1999).

Further alignments were carried out for homologues of *rad21*<sup>+</sup> and *rec8*<sup>+</sup> with protein sequences from *S. cerevisiae*, human and mouse, (Figures 3.7 and 3.8, respectively). These alignments emphasise that the same regions are also conserved between species (McKay *et al.*, 1996; Guacci *et al.*, 1997; Bhatt *et al.*, 1999; Parisi *et al.*, 1999), indicating that they are important for cohesin function. Low level similarity between the proteins is distributed across the entire ORF region, however more highly conserved regions are mainly at the N and C terminal ends. Functional domains are yet to be characterised for these proteins and the precise functions of these conserved domains remains to be elucidated.

Both SpRad21p and SpRec8p 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 (Alexandru *et al.*, 2001; Kaitna *et al.*, 2002; Rogers *et al.*, 2002).

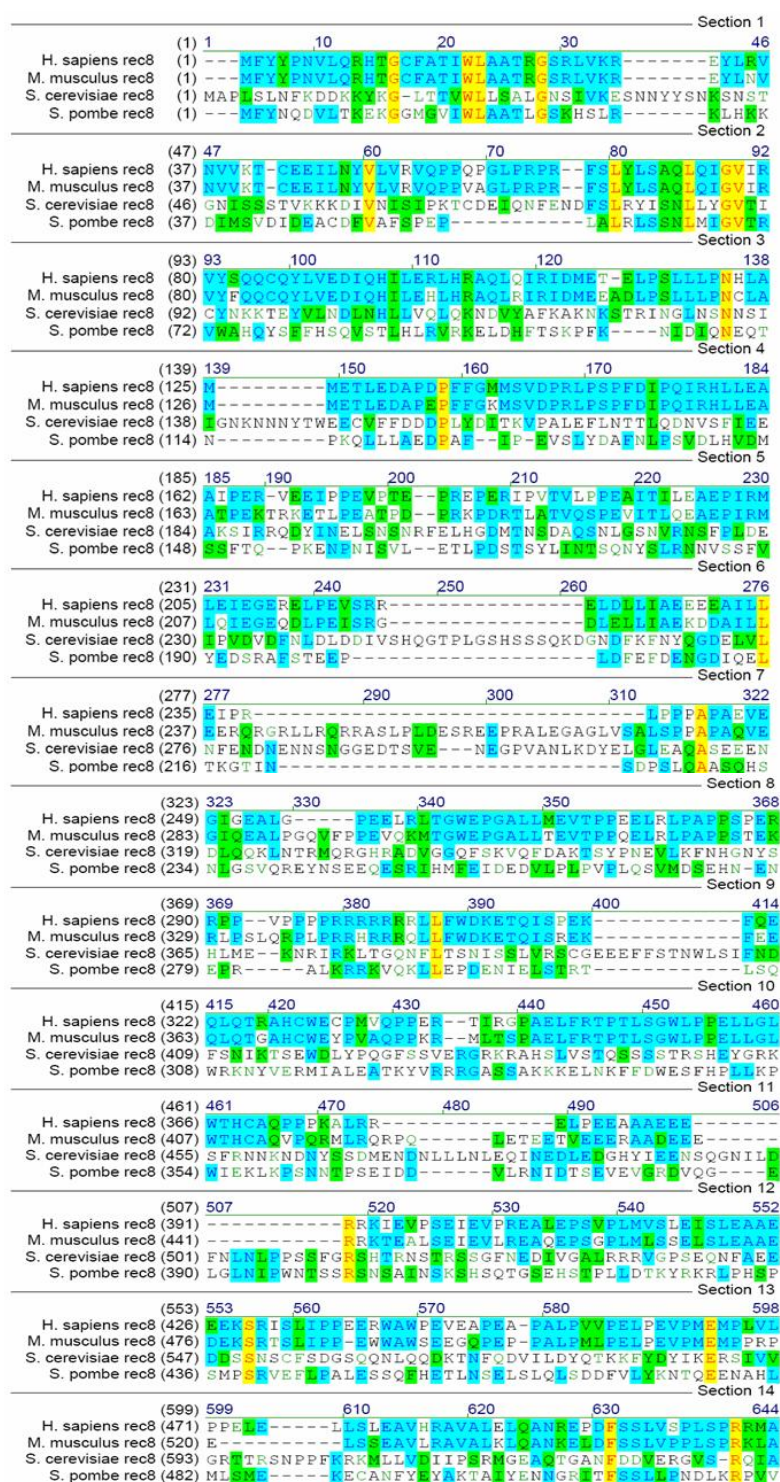
Although *rad21*<sup>+</sup> is periodically transcribed, levels of chromatin-bound SpRad21p are relatively constant throughout the cell cycle, with cleavage of only 5% being required for dissolution of sister chromatid cohesion during anaphase (Tomonaga *et al.*, 2000). Cleavage of both SpRad21p and SpRec8p require APC activity, and both proteins contain highly conserved separin cleavage sites (Uhlmann *et al.*, 1999), indicating they are both regulated during M phase by the APC-securin-separin pathway.

		Section 1				
	(1)	10	20	30	45	
H. sapiens rad21	(1)	-----	MFYAHEVLSKRGFLAKIWLAAHWDRK	-----	LTKAHVFE	
M. musculus rad21	(1)	-----	MFYAHEVLSKRGFLAKIWLAAHWDRK	-----	LTKAHVFE	
S. cerevisiae rad21	(1)	MVTENFQRLTVLRKATNRGFLAQCWLASNMSNIPRGS	VIQTHLAE			
S. pombe rad21	(1)	-----	MFYSSEALLSKRGFLAKVWLAAHWDRK	-----	LSRVQTLH	
		Section 2				
	(46)	46	60	70	80	90
H. sapiens rad21	(35)	CNLESSVESIISPKV	---MALRFTSGHLLIGVVRVYHRRKAVYLL			
M. musculus rad21	(35)	CNLESSVESIISPKV	---MALRFTSGHLLIGVVRVYHRRKAVYLL			
S. cerevisiae rad21	(46)	SAKEIAKAKAGCDDSEGDNEY	TLRFTSGHLLIGVVRVYHRRKAVYLL			
S. pombe rad21	(35)	TSEDCSVHATVTEETAP	---MALRFTSGHLLIGVVRVYHRRKAVYLL			
		Section 3				
	(91)	91	100	110	120	135
H. sapiens rad21	(76)	ADCNEAFIKIKMAFRPGVVDLPE	-----	ENFEAAYNAITG		
M. musculus rad21	(76)	ADCNEAFIKIKMAFRPGVVDLPE	-----	ENFEAAYNAITG		
S. cerevisiae rad21	(91)	TDIKRITTKISMLFKTSQKMTSTVN	-----	RLNTVTRVHQLM		
S. pombe rad21	(77)	EDCTEALMRTKMSRQFQGVDMIT	PATALQSLKGGKDAVT	QSANLIT		
		Section 4				
	(136)	136	150	160	170	180
H. sapiens rad21	(111)	FEERHEDQFLPDLDDIDVAQQFSLNQS	---VEEITMRREV			
M. musculus rad21	(111)	FEERHEDQFLPDLDDIDVAQQFSLNQS	---VEEITMRREV			
S. cerevisiae rad21	(129)	EDAVTERVLTVEGLFLDDTITPVLMAQ	---ENSMERKVVQ			
S. pombe rad21	(122)	FEETREFDLLVPESTFDFQWSQLLRTPSRSSNTLE	HSFSSSP			
		Section 5				
	(181)	181	190	200	210	225
H. sapiens rad21	(150)	GNISILQENIFGDFGMDDREIMR	---EGSAFEDDDMLVSTITSNLI			
M. musculus rad21	(150)	GNISILQENIFGDFGMDDREIMR	---EGSAFEDDDMLVSTITSNLI			
S. cerevisiae rad21	(168)	GAAPWDTLAVGRFSPFEEFEHNNLSMNLIFDEEGFIISKVW				
S. pombe rad21	(167)	SFPFSQLSITAGRNAQVSGGFSLG	---FAHVGNDMQFHLPISSNG			
		Section 6				
	(226)	226	240	250	260	270
H. sapiens rad21	(193)	LESFQSTSNLNEKLNHLEYEDVVKDDNFEGEM	-----			
M. musculus rad21	(193)	LESFQSTSNLNEKLNHLEYEDVVKDDNFEGEM	-----			
S. cerevisiae rad21	(213)	EEGTQSSRNFDHENYIQDDDFLLDASTIG	-----			
S. pombe rad21	(211)	AAEPRSVHSDNQGISVGRDAFAAAATLSGIIGFQMTKSPAS	-----			
		Section 7				
	(271)	271	280	290	300	315
H. sapiens rad21	(225)	-----	DGGIISDKLISNNDGGIIFDPPFALSEAGVMVF			
M. musculus rad21	(225)	-----	DGGIISDKLISNNDGGIIFDPPFALSEAGVMVF			
S. cerevisiae rad21	(245)	-----	WDLGTEKNQNDIDDDNVEEQFRRLG			
S. pombe rad21	(256)	SVTHFSTPSMLPIGTS	---DDELEAPVVDLIDLGLDLDLDEQGA			
		Section 8				
	(316)	316	330	340	350	360
H. sapiens rad21	(257)	EQPAHDDMDEDNVSNGGDESDSVDFVPEPMTMTDQTTLVNPEE				
M. musculus rad21	(257)	EQPAHDDMDEDNVSNGGDESDSVDFVPEPMTMTDQTTLVNPEE				
S. cerevisiae rad21	(272)	-----	SSMSDPTDFGFDLDIEKBAFAGNDITITAMIESQPF			
S. pombe rad21	(301)	NAFAISADHQAHTSSHLHSDIMEDSSRFPAAGVREGQVVE	---			
		Section 9				
	(361)	361	370	380	390	405
H. sapiens rad21	(302)	BAFALEPIDITVKETRAKRRKRLIVDSVKELDSKTIRAQLSDYSD				
M. musculus rad21	(302)	BAFALEPIDITVKETRAKRRKRLIVDSVKELDSKTIRAQLSDYSD				
S. cerevisiae rad21	(310)	-----	KQIGRNSKLNNTKSIQLDEEENSSIASNS			
S. pombe rad21	(344)	---TARQQEKINPQKAVRQCAATDFWTELSRQMKKQADTSS				
		Section 10				
	(406)	406	420	430	440	450
H. sapiens rad21	(347)	IVTTLDLAFPTKLLMMWKEGGVEKLFSLPAQPLWNNRLLKLFTR				
M. musculus rad21	(347)	IVTTLDLAFPTKLLMMWKEGGVEKLFSLPAQPLWNNRLLKLFTR				
S. cerevisiae rad21	(343)	TYKEERS-----	NLITQPNNFTKR---LWHELTESMSYLPDEPI			
S. pombe rad21	(386)	ITFPCLN---	TSSIVFNATVNFTRNGKENTIFSSNLNPKVNEI			
		Section 11				
	(451)	451	460	470	480	495
H. sapiens rad21	(392)	CLTFLVPEDLRKRRKGGEGADNLDSEFLKBFENPEVPREDDQQQ---				
M. musculus rad21	(392)	CLTFLVPEDLRKRRKGGEGADNLDSEFLKBFENPEVPREDDQQQ---				
S. cerevisiae rad21	(381)	LKNFLSYSLKRRK-----	IHNREGREGSIEPELVVS			
S. pombe rad21	(428)	LQADFQKAIIRKRRK-----	NSSPEVFEPAHQHTTSTEN---			
		Section 12				
	(496)	496	510	520	530	540
H. sapiens rad21	(434)	-HQQRDVIDEPIIEEFSRLQESVMBASRTNIDESAMFPPFPQGVK				
M. musculus rad21	(437)	FQFQRDVIDEPIIEEFSRLQESVMBASRTNIDESAMFPPFPQGVK				
S. cerevisiae rad21	(412)	LNLIDDVISNAGTNNSFNELIDNMSDFVPIIAGLNEA	FE---			
S. pombe rad21	(463)	-QEAASVLDPEETAA--	AELANTFAAIAITFPQETVQ	FE---		
		Section 13				
	(541)	541	550	560	570	585
H. sapiens rad21	(478)	RKAGQIDPEEVFPFQQVQMBIPFVPELFPPEEFPNICQLIPELEI				
M. musculus rad21	(482)	RKAGQIDPEEVFPFQQVQMBIPFVPELFPPEEFPNICQLIPELEI				
S. cerevisiae rad21	(454)	ENIDAKTRNEQTTIATKRRFTTGEAS-----	KAVIQMAKLE			
S. pombe rad21	(500)	GEAPEGSPMGFVTAESAIDSLFLAEE-----	VMLDADL			
		Section 14				
	(586)	586	600	610	620	630
H. sapiens rad21	(523)	BEKEKEKEKSEKDEEEDEEDASGGDQDQEEERRWNKRTQQLHLGI				
M. musculus rad21	(527)	BEKEKEKEKSEKDEEEDEEDASGGDQDQEEERRWNKRTQQLHLGI				
S. cerevisiae rad21	(493)	RKELS---SEKVIFTLVKSTNTEP				
S. pombe rad21	(538)	GS-----	ELLSSVSEAFSSQTAKSLRNKWDPYTE			

**Figure 3-7: Sequence alignment of *S. pombe* SpRad21p with related primary amino acid sequences from other organisms.**

Sequences shown are as follows; *H. sapiens* (631 aa), *M. musculus* (635 aa), *S. cerevisiae* (566 aa) *S. pombe* (628 aa). Black on white are non similar residues, green on white are weakly similar residues, red on yellow regions are identical residues in all four organisms whilst blue on cyan indicate identical residues not conserved in all four organisms. Finally, black on green are similar residues existing between the four organisms. Sequence alignment of the SpRad21p share 37% similarity with 7% identity.





**Figure 3-8: Sequence alignment of *S. pombe* SpRec8p with related primary amino acid sequences from other organisms.**

Sequences shown are as follows; *H. sapiens* (631 aa), *M. musculus* (635 aa), *S. cerevisiae* (566 aa), *S. pombe* (628 aa). Black on white are non similar residues, green on white are weakly similar residues, red on yellow regions are identical residues in all four organisms whilst blue on cyan indicate identical residues not conserved in all four organisms. Finally, black on green are similar residues existing between the four organisms. Sequence alignment of the SpRec8p share 78% similarity with 3% identity.

### 3.6 Summary

Data shown in this chapter demonstrate that during a wild-type synchronous mitosis, the cohesin *rad21*<sup>+</sup> is periodically expressed, with mRNA levels peaking during G1-S phase, while no *rec8*<sup>+</sup> transcript was detected throughout the mitotic cell cycle. Published findings indicate that *S. pombe* has two cohesins, one specific to mitosis and the other specific to meiosis (Lin *et al.*, 1992; Birkenbihl & Subramani, 1995). Although, the meiotic experiment in this chapter showed the presence of a *rad21*<sup>+</sup> in meiosis this is an artefact of this experiment since both published findings and the available microarray data (Mata *et al.*, 2002) have revealed that there is no *rad21*<sup>+</sup> transcript in meiosis.

Sequence alignments of the SpRad21p and SpRec8p indicated highest similarity between the two proteins in their N and C termini. To date, these are the only known cohesin domains, although their exact role in cohesin function has not been ascertained. Thus, at present, it cannot be stated which regions of the proteins are functionally significant for one cohesin and not the other.

When the alignments were compared between the different eukaryotic species, SpRad21p showed 7% identical amino acid residues, whereas SpRec8p showed less at 3%. In contrast, SpRec8p had a higher percentage similarity at, 78%, with SpRad21p at 37%. In addition to this the alignments of both SpRad21p and SpRec8p within organisms revealed varying evolutionary conservation, with *S. pombe* most similar to the higher eukaryotes *H. sapiens* and *M. musculus* than *S. cerevisiae*.

## Chapter 4

### 4.1 Introduction

The *S. pombe rad21*<sup>+</sup> gene was initially characterised as a radiation sensitive mutant with a role in DNA DSB repair (Birkenbihl and Subramani, 1992, 1995). Homologous genes were subsequently identified in other species, including *S. cerevisiae* ScMcd1p, which was found to share sequence similarity with ScSmc1p and ScSmc3p, 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* ScMcd1p mutants that underwent premature separation of sister chromatids during mitosis, led to the proposition of a role for ScMcd1p in chromosome cohesion. It is now known that SpRad21p associates with other subunits, forming part of a multi-subunit complex called cohesin (Tomonaga *et al.*, 2000). SpRad21p also interacts genetically and physically with several other molecules involved in both DNA replication and kinetochore function (Hartsuiker *et al.*, 2001; Takeda *et al.*, 2001; Toyuda *et al.*, 2002). Indeed, it is now becoming increasingly clear that SpRad21p plays a central role in the regulation of chromosome dynamics, and is required for several different processes occurring during various phases of the mitotic cell cycle.

SpRec8p, a meiotic homologue of SpRad21p, was first discovered in *S. pombe* (Molnar *et al.*, 1995), and also later identified in *S. cerevisiae* (Klein *et al.*, 1999). *S. pombe* SpRec8p was found to be essential for normal meiotic homologous recombination. Analysis of *rec8* mutants further revealed roles for this meiotic gene in accurate meiotic chromosome segregation and spore formation (Parisi *et al.*, 1999; Watanabe & Nurse, 1999; Watanabe *et al.*, 2001).

Chapter 3 describes how the expression of the two *S. pombe* cohesins *rad21*<sup>+</sup> and *rec8*<sup>+</sup> is cell cycle specific, with *rec8*<sup>+</sup> being transcribed only during sexual development and *rad21*<sup>+</sup> being expressed solely during the mitotic cycle. Apart from the DSC1 transcription factor co-activator *rep2*<sup>+</sup>, all known genes that are expressed during the mitotic cell cycle in *S. pombe*, are also expressed during the meiotic cell cycle (White *et al.*, 2001; Cunliffe *et al.*, 2004; McNerny, 2004). Even more intriguing is that *rad21*<sup>+</sup> encodes a protein that is essential for viability, yet appears not to be required during sexual development; instead the non-essential *rec8*<sup>+</sup> gene is the functional cohesin subunit in

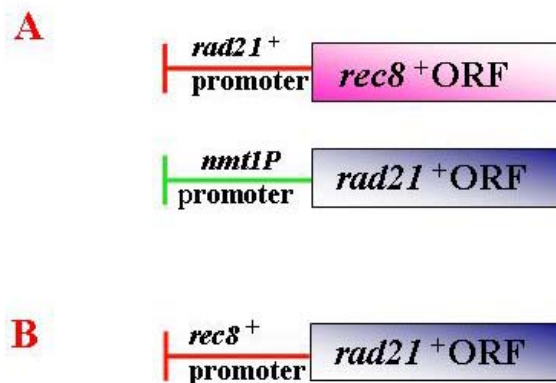
meiosis. A possible explanation for the absence of the essential function of the mitotic cohesin in meiosis, could be a consequence of the DNA specific repair role of the *rad21*<sup>+</sup> gene. This DSB repair task may be required in mitosis but not the alternative life cycle, since in meiosis DSBs are repaired via an alternative mechanism involving the SpRad32p.SpRad50p.SpNbs1p nuclease complex which is also involved in checkpoint responses (Ellermier & Smith., 2005; Section 1.17.3).

This observation suggests that the life cycle specific expression of the two cohesins has an important biological role, and it is a major aim of the work presented in this thesis to discover what these specific roles are.

To address the issue of specificity of the two fission yeast cohesins, Watanabe and Nurse (1999) examined the effects of switching the expression of the two genes so that they would be expressed during the alternative life cycle. They reported that by placing *rec8*<sup>+</sup> under *nmt1*<sup>+</sup> promoter control, so that it was ectopically and constitutively over expressed during the mitotic cell cycle, the lethal effect of a *rad21*<sup>+</sup> disruption (*rad21Δ*) could be partially rescued, although this was accompanied by a 20% decrease in growth rate and some inviable cells were formed. In contrast, *rad21*<sup>+</sup>, when ectopically over-expressed, was unable to support sexual development in the absence of *rec8*<sup>+</sup> (*rec8Δ*). When *rad21*<sup>+</sup> was over-expressed during the meiotic cell cycle in *rec8Δ* cells, meiotic defects such as abnormal spore development and precocious separation of sister chromatids during MI were observed. These findings suggested two possibilities: firstly, that the essential function of SpRad21p is partially conserved in the meiotic cohesin SpRec8p; secondly, that SpRec8p has functions that are specifically required for sexual development, that are not conserved in the mitotic cohesin SpRad21p.

In this thesis we sought to confirm and extend these observations by examining the ability of the two cohesins to complement each other. Significantly, however, we swapped the wild-type promoters of the two genes, so that cohesin expression was switched with each transcribed during the alternative life cycle. Importantly, each cohesin was expressed at physiological levels, and not over-expressed, and also at the correct cell cycle time at the beginning of S phase during the two life cycles. Using these constructs we hoped to more precisely delimitate the functional differences between the two cohesins, which might otherwise be masked by high levels of over-expression.

Earlier work in the laboratory set out to investigate whether SpRec8p in the mitotic cycle would allow normal mitotic growth (Riddell, 2003). These analyses revealed that expression of *rec8*<sup>+</sup> under the control of the *rad21*<sup>+</sup> promoter was able to support mitotic growth when SpRad21p is absent, with these cells having a similar division time to wild-type. Furthermore, FACs analysis exhibited that replacing SpRad21p with SpRec8p did not cause a significant increase in total cell cycle length, or a delay in S phase. These experiments demonstrated that *rec8*<sup>+</sup>, when expressed at the correct time and at physiological levels, can functionally replace *rad21*<sup>+</sup> during normal mitotic growth, suggesting that at least under these conditions, in an unperturbed mitosis, the *rec8*<sup>+</sup> gene could replace the gene in the *rad21*<sup>+</sup> mitotic cycle.



**Figure 4-1: Swapping cohesin promoters for alternative life cycle expression.**

Cohesin promoters were switched to enable expression in the alternative life cycle. **A**, Since *rad21*<sup>+</sup> is an essential gene it was put under the control of the no message in thiamine promoter (*nmt1P*) and *rec8*<sup>+</sup> under the control of the *rad21*<sup>+</sup> promoter. **B**, *rad21*<sup>+</sup> under the control of the *rec8*<sup>+</sup> allowing expression of SpRad21p in the meiotic cycle. For details of oligonucleotides used for the constructs see Appendix III.

These initial observations established that some functions of *rad21*<sup>+</sup> are conserved in *rec8*<sup>+</sup> which can sustain vegetative growth. Despite *rad21*<sup>+</sup> being an essential gene, its functions are involved in checkpoint repair mechanisms, which normally only manifest after DNA damage, and not during normal growth. This study aims to further investigate the functional conservation between *rad21*<sup>+</sup> and *rec8*<sup>+</sup>, and to look at the precise roles of these two cohesins. Specifically, we wished to identify whether the DNA damage repair function of *rad21*<sup>+</sup> is specific to this cohesin, and is not possessed by *rec8*<sup>+</sup>.



To achieve this, three strains were used: wild-type (GG 218) with functional SpRad21p; *rad21-45* (GG 921) in which a single base pair change makes the strain sensitive to DNA damage; and a *rad21P:rec8<sup>+</sup> nmt1P:rad21<sup>+</sup>* (GG 358) repressible strain grown in the presence (*nmt1P* “off”) or absence of thiamine (*nmt1P* “on”). In the latter strain, this resulted in cells which either contained only SpRec8p present instead of SpRad21p, or both SpRec8p and SpRad21p being present.

### **The *rad21-45* mutant**

The *rad21-45* mutant strain, a gift from Professor S Subramani (University of California, San Diego), is a radiation sensitive mutant deficient in the DSB repair system (Birkenbihl & Subramani, 1995). The inability of the mutant to perform efficient DNA repair is caused by a single base substitution, which changes wild-type isoleucine at position 67 into threonine. Also, *rad21-45* cells arrest in G2 after irradiation, but proceed after a cell cycle delay into mitosis, without having repaired the DNA lesions.

### **The *rad21P:rec8<sup>+</sup> nmt1P:rad21<sup>+</sup>* repressible strain**

The *rad21<sup>+</sup>* gene was shown to be essential for *S. pombe* survival, as disrupting or deleting the gene results in cell death (Birkenbihl & Subramani, 1995). Therefore, to overcome this the *rad21<sup>+</sup>* was placed under the control of the repressible *nmt1<sup>+</sup>* promoter (*nmt1P*), so that its expression could be regulated.

In its normal genomic locus the *nmt1* promoter controls expression of the *S. pombe nmt1<sup>+</sup>* gene, which encodes an intermediate in the biochemical pathway that synthesises the amino acid thiamine (Maundrell, 1990). In media lacking thiamine the *nmt1<sup>+</sup>* gene is expressed stimulating thiamine biosynthesis. However, in conditions where thiamine is present, cells no longer need to synthesise thiamine, and the *nmt1<sup>+</sup>* promoter completely and rapidly represses transcription of *nmt1<sup>+</sup>*. This feature of the *nmt1<sup>+</sup>* promoter allows it to be exploited to regulate expression of heterologous genes placed under its control.

The repressible strain was created by using an *nmt1P:rad21<sup>+</sup>* DNA construct, also a gift from Professor S. Subramani. This construct was made by replacing the 1.5 kb of DNA immediately upstream of the *rad21<sup>+</sup>* ORF with the *S. pombe ura4<sup>+</sup>* gene (for purposes of selecting the construct), and the *nmt1<sup>+</sup>* promoter. *nmt1P:rad21<sup>+</sup>* was integrated into the chromosomal *rad21<sup>+</sup>* locus via homologous recombination as the construct incorporates part of the *rad21<sup>+</sup>* 5' untranslated region and part of the 5' region of the *rad21<sup>+</sup>* open reading frame (Birkenbihl & Subramani, 1995). By this method, this DNA construct was integrated into various *S. pombe* strain backgrounds generating *rad21<sup>+</sup>* repressible strains.

To place the *rec8<sup>+</sup>* gene under the control of the *rad21<sup>+</sup>* promoter, the entire *rec8<sup>+</sup>* ORF and *rad21<sup>+</sup>* promoter region were amplified by PCR, using oligonucleotides listed in Appendix III, and the DNAs confirmed by sequencing (Riddell, 2003). Of direct relevance

to the creation of this construct *rec8*<sup>+</sup> contains a ‘determinant of selective removal’ (DSR) overlapping the 3’-UTR and ORF which controls its mRNA stability in mitosis (Harigaya *et al.*, 2006). By cloning the *rec8*<sup>+</sup> ORF precisely, the DSR was disrupted, thus preventing degradation of *rec8*<sup>+</sup> mRNA during mitosis. Restriction sites were incorporated in the PCR oligonucleotides to permit the convenient ligation of *rad21P* and *rec8*<sup>+</sup> DNAs into cloning vectors. The *rad21P:rec8*<sup>+</sup> double construct was then cloned into the integrating plasmid pJK148, creating pJK148/*rad21P:rec8*<sup>+</sup>, which was subsequently integrated into the *rad21*<sup>+</sup>-repressible strain creating GG 358, the strain used throughout this study to investigate life cycle specific cohesin function.

Four different damaging agents were used to examine the damage response function of the two fission yeast cohesins: ultra-violet (UV-C), methyl methane sulfonate (MMS), phleomycin, and  $\gamma$ -irradiation using a <sup>60</sup>Co source. Initially, viability assays to obtain qualitative and quantitative data were carried out with each of these damaging agents.

## 4.2 Viability assays

Qualitative and quantitative data were obtained by treating cells with a chronic dose of each damaging agent.

### 4.2.1 Qualitative data

Cells were grown to a concentration of  $5 \times 10^6$  cells ml<sup>-1</sup> in the presence of thiamine, with 200  $\mu$ l of culture serially diluted six times by a factor of five in 96 well microtiter plates. 5  $\mu$ l of each dilution was spotted on solid EMM with thiamine and irradiated with UV-C light of 0-200 J m<sup>-2</sup>, or spotted on solid EMM with thiamine containing varying concentrations of MMS or phleomycin. After five days of growth at 25°C, sensitivity was determined by the direct visual comparison of wild-type and *rad21-45* cells. All experiments were completed in triplicate.

### 4.2.2 Quantitative data

Cells were grown to a concentration of  $5 \times 10^6$  cells ml<sup>-1</sup> and a <sup>1</sup>/<sub>1000</sub> dilution plated on solid EMM with and without thiamine. The cells were then irradiated with increasing doses of UV-C light, or plated on EMM with and without thiamine, and with either MMS

or phleomycin, at varying concentrations. These were left to grow for 5 days at 25°C, after which colonies were counted. Viability was displayed as the percentage of colonies counted under experimental conditions, compared with colonies counted under optimal conditions.

### 4.3 Exposure of *S. pombe* to increasing doses of UV-C results in decreased cell survival

Exponentially growing cultures of the three strains were grown with thiamine; but the *nmt1* repressible strain was grown in the presence and absence of thiamine, to either repress or permit the expression of *nmt1P: rad21<sup>+</sup>*. For UV-C irradiation, an Amersham UV-C 500 Crosslinker emitting primarily 254 nm light was used. This method is advantageous over the more conventional method of UV-C emission using a germicidal lamp, because the Crosslinker produces uniform, consistent results. The Crosslinker has an internal photo-feedback system which automatically adjusts to account for the variation in UV-C intensity that occurs as the light tubes age. Similarly, there is no need to time the reaction because energy is continually measured within the chamber, automatically deactivating the UV-C light after the appropriate energy dose has been reached.

As described in Section 1.17.2.1 the UV radiation spectrum has been subdivided into three wavelength bands, the wavelength used in damage experiments is UV-C falling into the range of 100-290 nm. The absorption maximum of DNA is ~260 nm; thus exposure to wavelengths in this range can cause damage. Absorption of UV-C which has a wavelength of 254 nm causes adjacent pyrimidine dimers to become covalently linked.

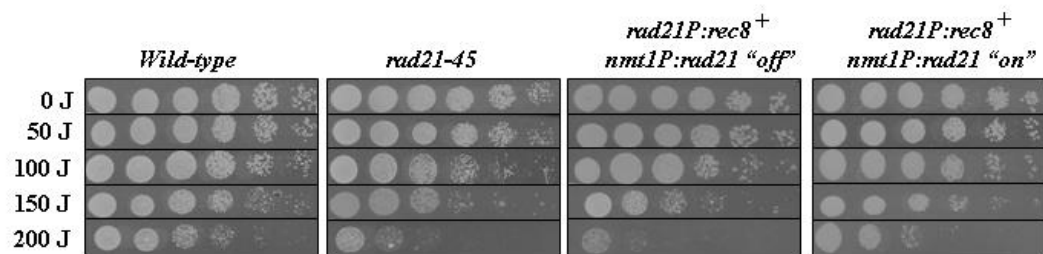
There are two common UV-C products arising from DNA as a result of UV induced damage these are, cyclobutane pyrimidine dimers (CPD), most commonly being thymine dimers and 6-4 photodimers formed with C-6 of the 5' pyrimidine and C-4 of the 3' pyrimidine, which in the 6-4 lesion is typically cytosine rather than thymine (Friedberg *et al.*, 2006). This product is also responsible for maximum mutagenicity induced by UV-C (Mitchell *et al.*, 1991). Both are bulky helix-distorting lesions that result in the arrest of replication and transcription, and are the major causes of cell death by UV-C. In comparison to *S. cerevisiae*, *S. pombe* is not as sensitive to UV-C radiation, possibly because it has the extended G2 phase allowing for any damage to be repaired before mitotic entry (Osman *et al.*, 2002). It also has an efficient checkpoint pathway, and highly developed recombination mechanisms with two pathways for excision repair of UV damage. The first of these pathways is called the nucleotide excision repair (NER) pathway, which excises an oligomer of approximately 29 bases in length, leaving a single-stranded template for synthesis (McCready *et al.*, 2000). Secondly, an alternative excision

repair pathway involving UV endonucleases has been identified in *S. pombe* not present in *S. cerevisiae* or humans (Yonemasu *et al.*, 1997; Yasuhira & Yasui, 2000).

### 4.3.1 Qualitative data

Although *S. pombe* does not appear to show photo reactivation (Schupbach, 1971) the work described in this thesis with irradiated cells was carried out under yellow light and the plates were incubated in the dark.

Qualitative data after exposure to UV-C were obtained as described, and are shown in Figure 4.2. For all three strains it was found that as higher doses were administered, decreased cell survival was observed.

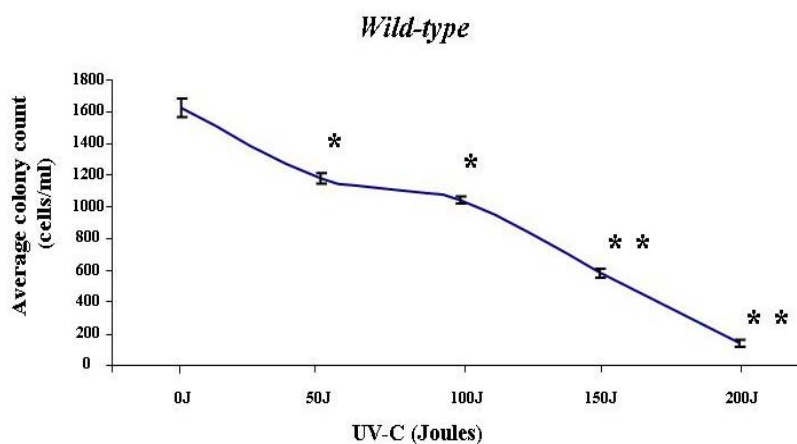


**Figure 4-2: Qualitative data: *S. pombe* strains exposed to increasing doses of UV-C results in decreased cell survival.**

Cells of three strains, wild-type (GG 218), *rad21-45* (GG 921) and *rad21P:rec8<sup>+</sup> nmt1P:rad21<sup>+</sup> "off"* (GG 358) were grown at 25°C to mid-exponential stage in the presence of thiamine, GG 358 was also grown in the absence of thiamine *rad21P:rec8<sup>+</sup> nmt1P:rad21<sup>+</sup> "on"*. Serial dilutions were then made in a 96 well plate and 5  $\mu$ l spotted onto EMM + thiamine plates before exposure to increasing doses of UV-C light. Plates were incubated at 25°C for five nights. Experiments were carried out in triplicate.

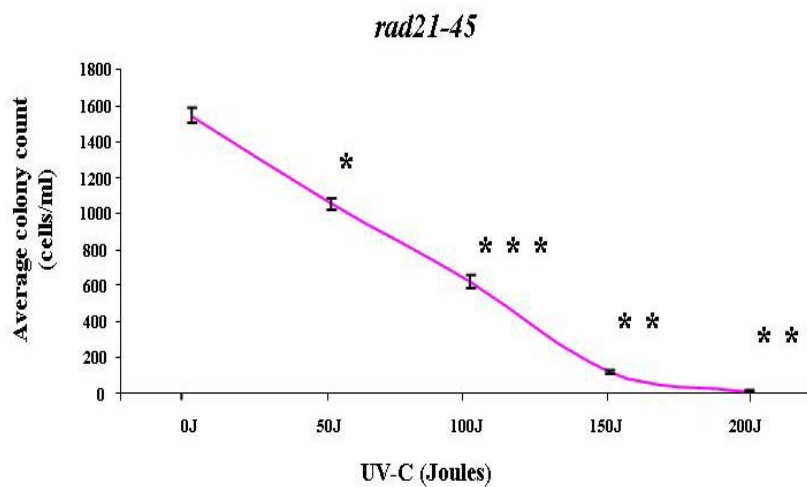
### 4.3.2 Quantitative data

Quantitative data were obtained as described and are shown in Figures 4.3 to 4.6. These were consistent with qualitative data, as for all three strains when higher doses of UV-C were administered, decreased cell survival was observed.



**Figure 4-3: Quantitative data: Wild-type cells exposed to increasing doses of UV-C results in decreased cell survival.**

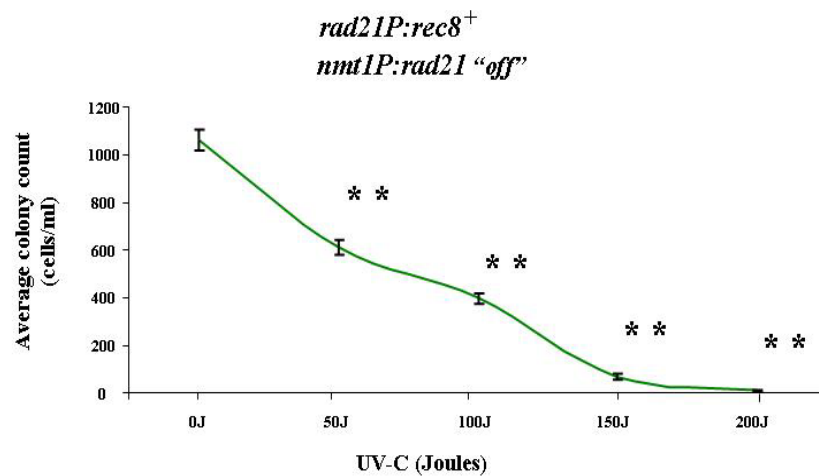
Wild-type (GG 218) cells, were grown at 25°C in minimal medium in the presence of thiamine to  $5 \times 10^6$  cells ml<sup>-1</sup>, and subsequently plated at the appropriate dilution of  $10^{-3}$  onto EMM + thiamine plates. The plates were then exposed to increasing doses of UV-C light and incubated at 25°C for five nights. Experiments were carried out in triplicates of five to ensure reproducibility and to allow for efficiency of plating. The average number of colonies counted was taken from the triplicates and plotted. Bars represent the standard error of the mean, with \*p < 0.05%, and \*\*p < 0.01, indicating increased sensitivity in comparison to untreated cells.



**Figure 4-4: Quantitative data: *rad21-45* cells exposed to increasing doses of UV-C results in decreased cell survival.**

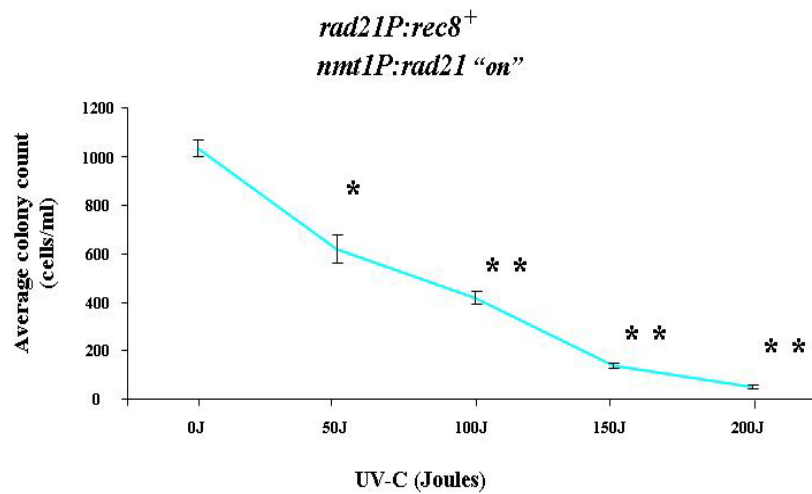
*rad21-45* (GG 921) cells, were grown at 25°C in minimal medium in the presence of thiamine to  $5 \times 10^6$  cells ml<sup>-1</sup>, and subsequently plated at the appropriate dilution of  $10^{-3}$  onto EMM + thiamine plates. The plates were then exposed to increasing doses of UV-C light and incubated at 25°C for five nights. Experiments were carried out in triplicates of five to ensure reproducibility and to allow for efficiency of plating. The average number of colonies counted was taken from the triplicates and plotted. Bars represent the standard error of the mean, with \*p < 0.05%, \*\*p < 0.01 and \*\*\*p < 0.001 indicating increased sensitivity in comparison to untreated cells.





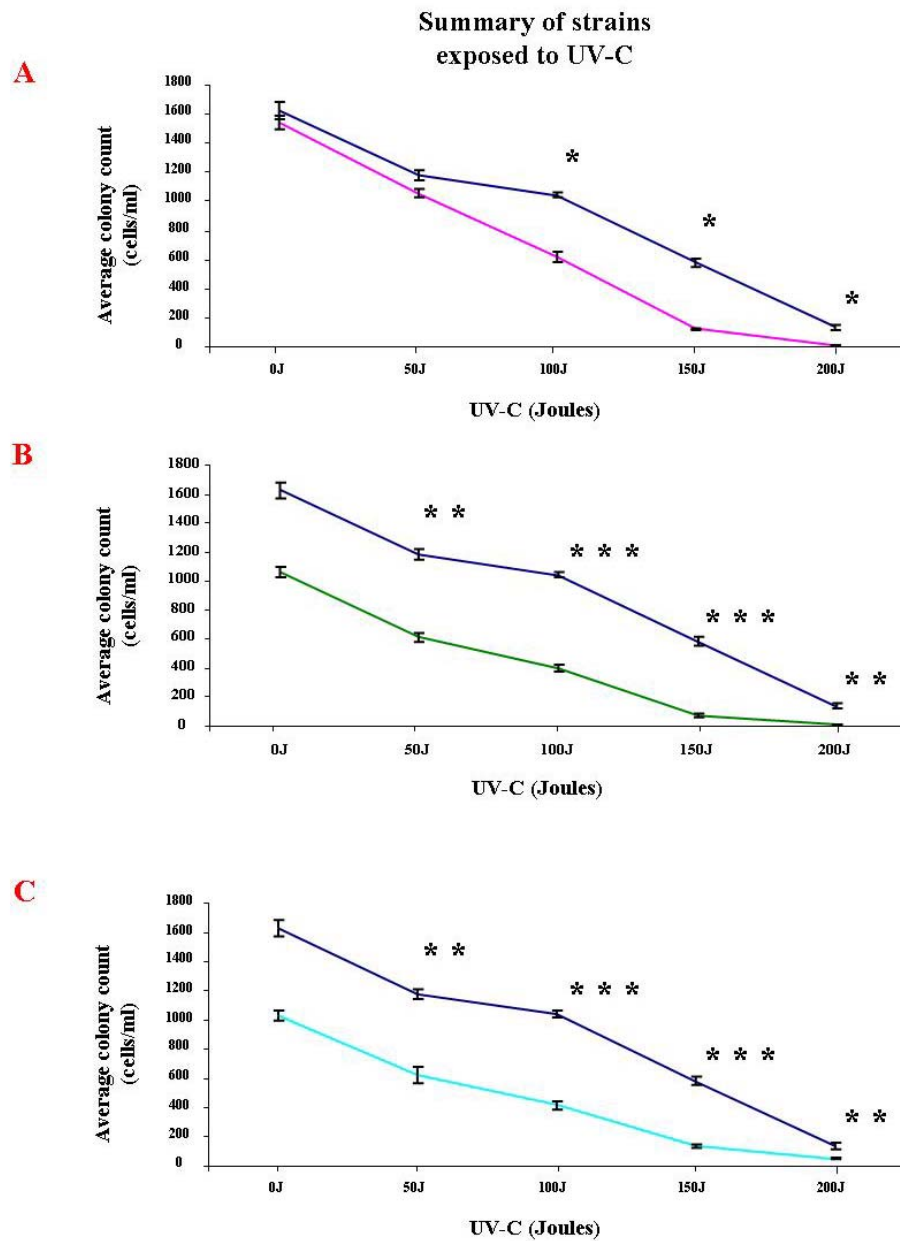
**Figure 4-5: Quantitative data: *rad21P:rec8<sup>+</sup> nmt1P:rad21<sup>+</sup> "off"* cells exposed to increasing doses of UV-C results in decreased cell survival.**

*rad21P:rec8<sup>+</sup> nmt1P:rad21<sup>+</sup> "off"* (GG 358) cells, were grown at 25°C in minimal medium in the presence of thiamine to  $5 \times 10^6$  cells  $\text{ml}^{-1}$ , and subsequently plated at the appropriate dilution of  $10^{-3}$  onto EMM + thiamine plates. The plates were then exposed to increasing doses of UV-C light and incubated at 25°C for five nights. Experiments were carried out in triplicates of five to ensure reproducibility and to allow for efficiency of plating. The average number of colonies counted was taken from the triplicates and plotted. Bars represent the standard error of the mean, with \*\* $p < 0.01$  indicating increased sensitivity in comparison to untreated cells.



**Figure 4-6: Quantitative data: *rad21P:rec8<sup>+</sup>* *nmt1P:rad21<sup>+</sup>* "on" cells exposed to increasing doses of UV-C results in decreased cell survival.**

*rad21P:rec8<sup>+</sup>* *nmt1P:rad21<sup>+</sup>* "on" (GG 358) cells, were grown at 25°C in minimal medium in the absence of thiamine to  $5 \times 10^6$  cells ml<sup>-1</sup>, and subsequently plated at the appropriate dilution of  $10^{-3}$  onto EMM plates. The plates were then exposed to increasing doses of UV-C light and incubated at 25°C for five nights. Experiments were carried out in triplicates of five to ensure reproducibility and to allow for efficiency of plating. The average number of colonies counted was taken from the triplicates and plotted. Bars represent the standard error of the mean, with \*p < 0.05% and \*\*p < 0.01 indicating increased sensitivity in comparison to untreated cells.



**Figure 4-7: Summary of quantitative data following UV-C exposure: average survival and percentage cell survival curves for all strains.**

Summary of results obtained. For damage induced with UV-C light, data indicates that for all three strains increasing exposure results in decreased survival, average colonies counted are plotted. **A**, Wild-type compared with *rad21-45*, **B**, Wild-type compared with *rad21P:rec8+ nmt1P:rad21+ "off"*, **C**, Wild-type compared with *rad21P:rec8+ nmt1P:rad21+ "on"*. Bars represent the standard error of the mean, with \* $p < 0.05\%$ , \*\* $p < 0.01$  and \*\*\* $p < 0.001$  indicating increased sensitivity.

#### **4.4 Exposure of *S. pombe* to increasing doses of methylmethane sulfonate (MMS) results in decreased cell survival**

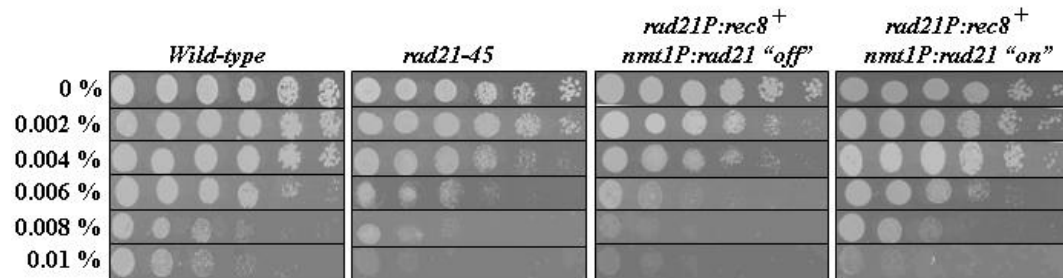
MMS induced DNA damaged leads to problems in S phase (Hartsuiker *et al.*, 2001). By introducing a methyl group (-CH<sub>3</sub>) onto DNA at a number sites, MMS has the ability to modify a single base, causing a chemical change. This in turn alters its base-pairing properties so that bases pair with the wrong base. After treatment with MMS, some guanines and some thymines in the DNA become methylated. A methylated guanine will pair with thymine rather than cytosine, giving GC to AT transitions, and a methylated thymine will pair with guanine rather than adenine, giving TA to CG transitions. Damage induced by DNA alkylation in living cells is repaired by two process; direct transfer of the modifying alkyl group to the repair protein, or the removal of the modified base by a glycosylase, leaving an apurinic or apyrimidinic site, which is then repaired by an excision-repair process.

Exponentially growing cultures of *S. pombe* were treated with increasing concentrations of MMS to assess viability both qualitatively and quantitatively. Cultures of the three strains were grown as for the UV-C experiments. For viability assays and qualitative assessment of cell survival, plates were poured on the day of use MMS was added directly to the media before pouring. Plates were made with the following concentrations: 0%, 0.002%, 0.004%, 0.006%, 0.008% and 0.01% (Figure 4.8).

Further plates were also made with concentrations of 0%, 0.1%, 0.3%, and 0.5%. However, MMS was added directly to the media (v/v), a 1:10 dilution made and cultures spotted immediately. These concentrations were used for quantitative as well as qualitative data (Figure 4.9). It was essential to have viability assessment in solid and liquid media, as the second concentration set was used for further experiments (Chapter 5) since cells had to be exposed to an acute MMS dose.

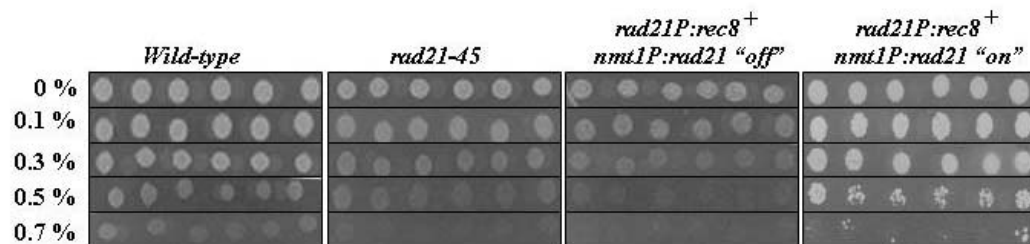
#### 4.4.1 Qualitative data

Qualitative data were obtained as described, and are shown in Figures 4.7 and 4.8. As increasing concentrations of MMS were administered decreased cell survival was seen for the three strains.



**Figure 4-8: Qualitative data (1): *S. pombe* strains exposed to increasing concentrations of MMS results in decreased cell survival.**

Wild-type (GG 218) cells, were grown at 25°C in minimal medium in the presence of thiamine to  $5 \times 10^6$  cells  $\text{ml}^{-1}$ . MMS was added to the liquid media in the required concentrations (v/v) of 0%, 0.1%, 0.3%, 0.5% and 0.7% and subsequently plated at the appropriate dilution of  $10^{-3}$  onto EMM + thiamine plates, which were then incubated for five nights at 25°C. Experiments were carried out in triplicates of five.

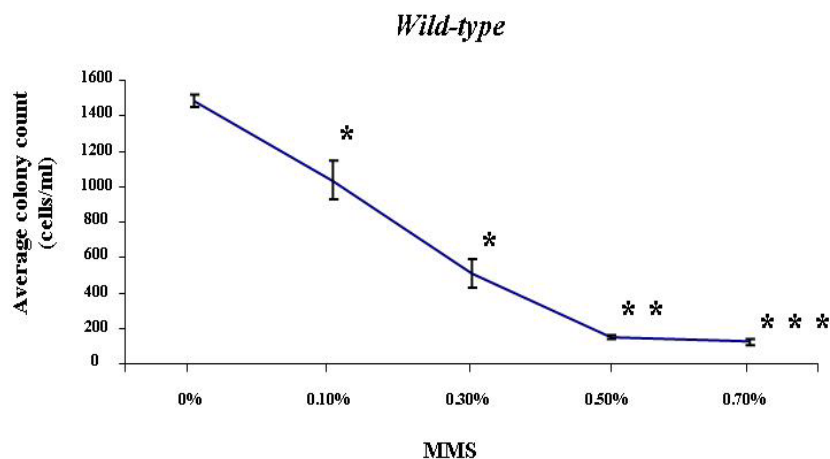


**Figure 4-9: Qualitative data (2): Concentrations used for further experiments: *S. pombe* strains exposed to increasing concentrations of MMS results in decreased cell survival.**

Cells of three strains, wild-type (GG 218), *rad21-45* (GG 921) and *rad21P:rec8+ nmt1P:rad21* "off" (GG 358) were grown at 25°C to mid-exponential stage in the presence of thiamine, GG 358 was also grown in the absence of thiamine *rad21P:rec8+ nmt1P:rad21* "on". Serial dilutions were then made in a 96 well plate and 5  $\mu\text{l}$  spotted onto solid EMM + thiamine plates containing increasing concentrations of MMS (v/w). Plates were incubated at 25°C for five nights. Experiments were carried out in triplicate.

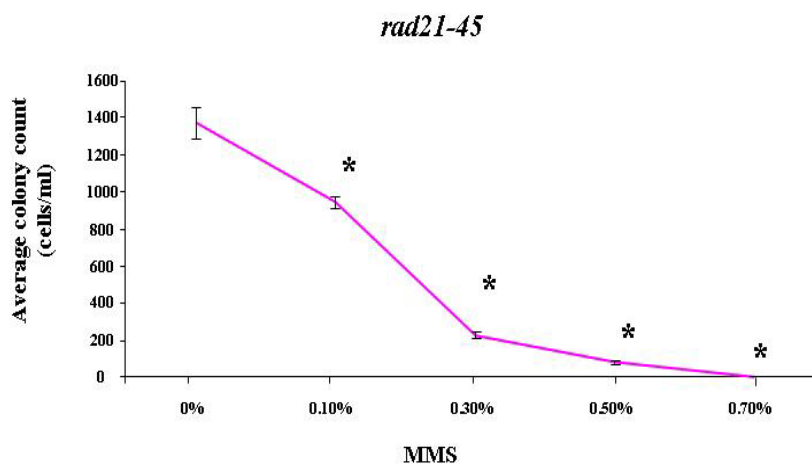
#### 4.4.2 Quantitative data

Quantitative data were obtained as described. Consistent with qualitative data, as increasing concentrations of MMS were administered decreased cell survival was seen for the three strains.



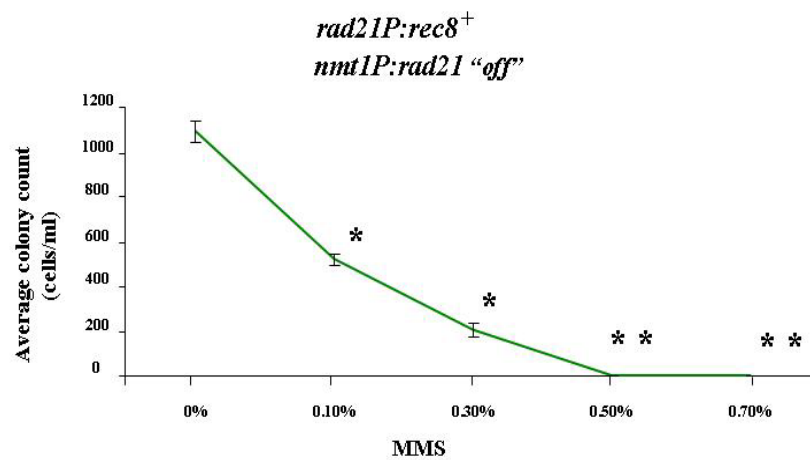
**Figure 4-10: Quantitative data: Wild-type cells treated with increasing concentrations of MMS results in decreased cell survival.**

Wild-type (GG 218) cells, were grown at 25°C in minimal medium in the presence of thiamine to  $5 \times 10^6$  cells ml<sup>-1</sup>, appropriate volumes of culture were taken to which MMS was added at a final concentration (v/v) of 0%, 0.1%, 0.3%, 0.5% and 0.7% and these plated at the appropriate dilution of  $10^{-3}$  onto EMM + thiamine plates which were incubated at 25°C for five nights. Experiments were carried out in triplicates of five to ensure reproducibility and to allow for efficiency of plating. The average number of colonies counted was taken from the triplicates and plotted. Bars represent the standard error of the mean, with \*p < 0.05%, \*\*p < 0.01 and \*\*\*p < 0.001 indicating increased sensitivity in comparison to untreated cells.



**Figure 4-11: Quantitative data: *rad21-45* cells treated with increasing concentrations of MMS results in decreased cell survival.**

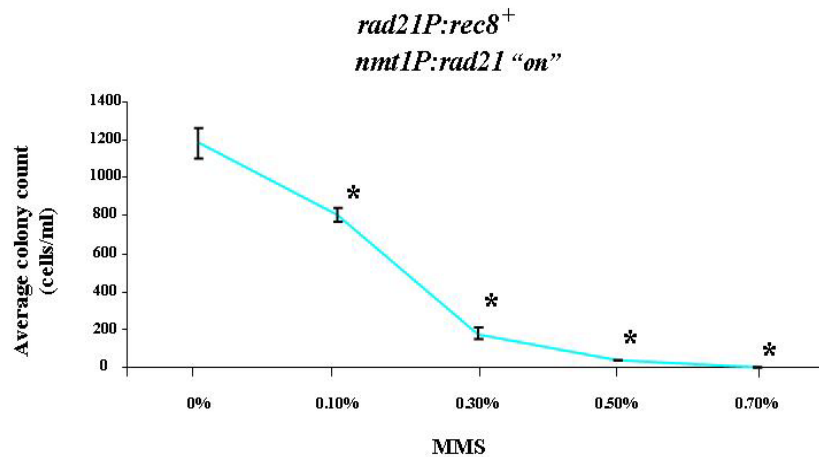
*rad21-45* (GG 921) cells, were grown at 25°C in minimal medium in the presence of thiamine to  $5 \times 10^6$  cells  $\text{ml}^{-1}$ , appropriate volumes of culture were taken to which MMS was added at a final concentration (v/v) of 0%, 0.1%, 0.3%, 0.5% and 0.7% and these plated at the appropriate dilution of  $10^{-3}$  onto EMM + thiamine plates which were incubated at 25°C for five nights. Experiments were carried out in triplicates of five to ensure reproducibility and to allow for efficiency of plating. The average number of colonies counted was taken from the triplicates and plotted. Bars represent the standard error of the mean, with \* $p < 0.05$  indicating increased sensitivity in comparison to untreated cells.



**Figure 4-12: Quantitative data: *rad21P:rec8<sup>+</sup> nmt1P:rad21<sup>+</sup> "off"* cells treated with increasing concentrations of MMS results in decreased cell survival.**

*rad21P:rec8<sup>+</sup> nmt1P:rad21<sup>+</sup> "off"* (GG 358) cells, were grown at 25°C in minimal medium in the presence of thiamine to  $5 \times 10^6$  cells  $\text{ml}^{-1}$ , appropriate volumes of culture were taken to which MMS was added at a final concentration (v/v) of 0%, 0.1%, 0.3%, 0.5% and 0.7% and these plated at the appropriate dilution of  $10^{-3}$  onto EMM + thiamine plates which were incubated at 25°C for five nights. Experiments were carried out in triplicates of five to ensure reproducibility and to allow for efficiency of plating. The average number of colonies counted was taken from the triplicates and plotted. Bars represent the standard error of the mean, with \* $p < 0.05$  and \*\* $p < 0.01$  indicating increased sensitivity in comparison to untreated cells.



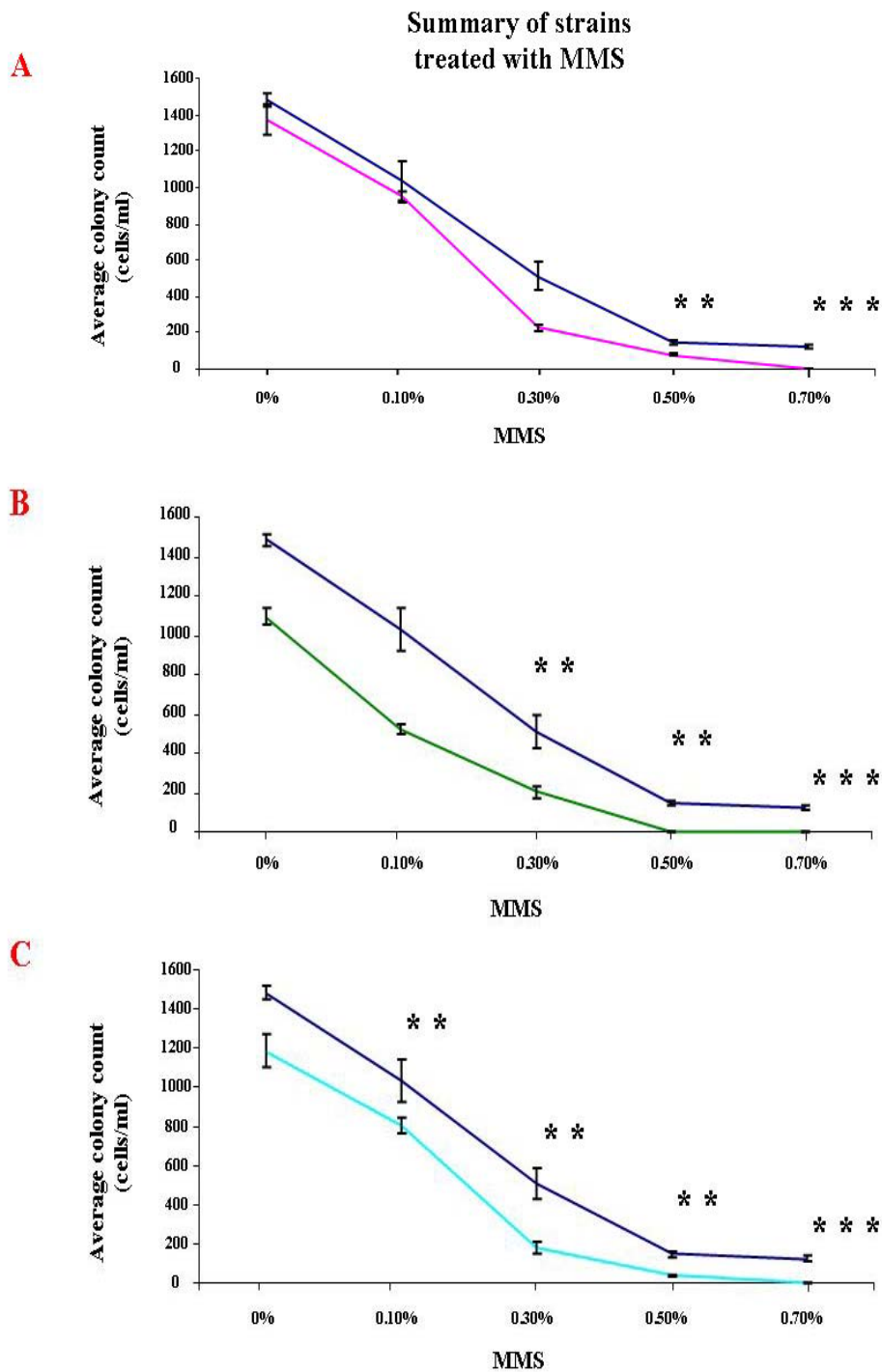


**Figure 4-13: Quantitative data: *rad21P:rec8<sup>+</sup> nmt1P:rad21<sup>+</sup>* “on” cells treated with increasing concentrations of MMS results in decreased cell survival.**

*rad21P:rec8<sup>+</sup> nmt1P:rad21<sup>+</sup>* “on” (GG 358) cells, were grown at 25°C in minimal medium in the absence of thiamine to  $5 \times 10^6$  cells ml<sup>-1</sup>, appropriate volumes of culture were taken to which MMS was added at a final concentration (v/v) of 0%, 0.1%, 0.3%, 0.5% and 0.7% and these plated at the appropriate dilution of  $10^{-3}$  onto EMM plates which were incubated at 25°C for five nights.

Experiments were carried out in triplicates of five to ensure reproducibility and to allow for efficiency of plating. The average number of colonies counted was taken from the triplicates and plotted.

Bars represent the standard error of the mean, with \*p < 0.05% indicating increased sensitivity in comparison to untreated cells.



**Figure 4-14: Summary of quantitative data following MMS treatment: average survival and percentage cell survival curves for all strains.**

Summary of results obtained. For damage induced with MMS, data indicates that for all three strains increased concentration results in decreased survival, average colonies counted are plotted. **A**, Wild-type compared with *rad21-45*, **B**, Wild-type compared with *rad21P:rec8+ nmt1P:rad21+ "off"*, **C**, Wild-type compared with *rad21P:rec8+ nmt1P:rad21+ "on"*. Bars represent the standard error of the mean, with \*\* $p < 0.01$  and \*\*\* $p < 0.001$  indicating increased sensitivity.

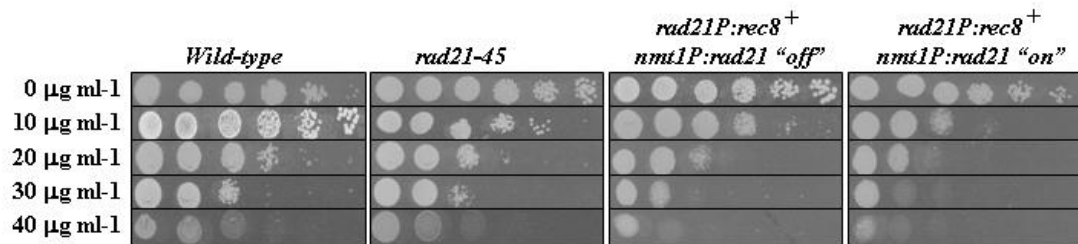
## 4.5 Exposure of *S. pombe* to increasing doses of phleomycin results in decreased cell survival

Phleomycin, a glycopeptide antibiotic, is a bleomycin-like compound isolated from a mutant strain of *Streptomyces verticillus*. These drugs bind to DNA, blocking S phase entry during the cell cycle, producing DNA base-loss and single and DSB (Steighner & Povrik, 1990). The action of phleomycin is similar to that of its structurally related analogue, bleomycin, although phleomycin is more cytotoxic. Both produce internucleosomal cleavage and chromosomal degradation. At higher doses, unrepaired breaks are substantially more abundant with phleomycin than with bleomycin, and thus contribute to more lethal effects. Such DSBs can be repaired by homologous recombination (HR), or by non-homologous end joining.

Exponentially growing cultures of *S. pombe* were treated with increasing concentrations of phleomycin to assess viability, both qualitatively and quantitatively. Cultures of the three strains were grown with thiamine: wild-type, *rad21-45*, *rad21P:rec8<sup>+</sup> nmt1P:rad21<sup>+</sup>* “off”, and a culture with the strain *rad21P:rec8<sup>+</sup> nmt1P:rad21<sup>+</sup>* “on” was grown without thiamine. For viability assays and qualitative assessment of cell survival on solid media phleomycin was added at the following concentrations: 0  $\mu\text{g ml}^{-1}$ , 10  $\mu\text{g ml}^{-1}$ , 20  $\mu\text{g ml}^{-1}$ , 30  $\mu\text{g ml}^{-1}$  and 40  $\mu\text{g ml}^{-1}$ .

### 4.5.1 Qualitative data

Qualitative data were obtained as described, and are shown in Figure 4.15. As increasing concentrations of phleomycin were administered decreased cell survival for all three strains was observed. However, the main difference encountered with this damaging agent was the surprising result that when the *rad21P:rec8<sup>+</sup> nmt1P:rad21* “on” was treated with phleomycin, it had the lowest cell viability off all the strains tested.

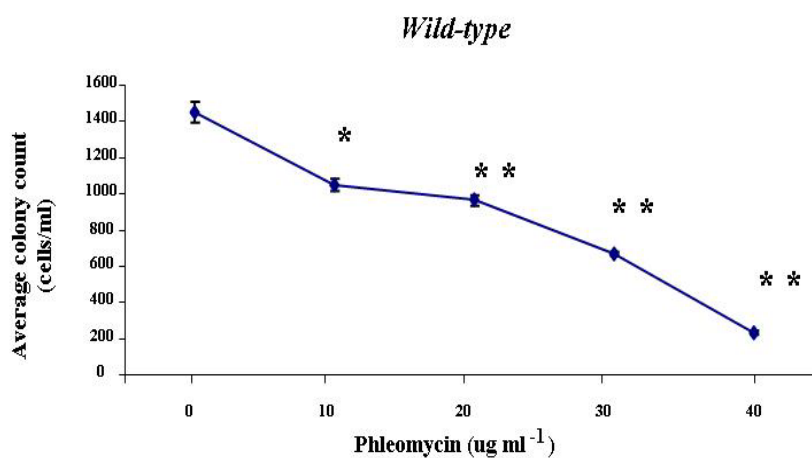


**Figure 4-15: Qualitative data: *S. pombe* strains exposed to increasing concentrations of phleomycin result in decreased cell survival.**

Cells of three strains, wild-type (GG 218), *rad21-45* (GG 921) and *rad21P:rec8<sup>+</sup> nmt1P:rad21<sup>+</sup> "off"* (GG 358) were grown at 25°C to mid-exponential stage in the presence of thiamine, GG 358 was also grown in the absence of thiamine *rad21P:rec8<sup>+</sup> nmt1P:rad21<sup>+</sup> "on"*. Serial dilutions were then made in a 96 well plate and 5 µl spotted onto solid EMM + thiamine plates containing increasing concentrations of phleomycin (v/w). Plates were incubated at 25°C for five nights. Experiments were carried out in triplicate.

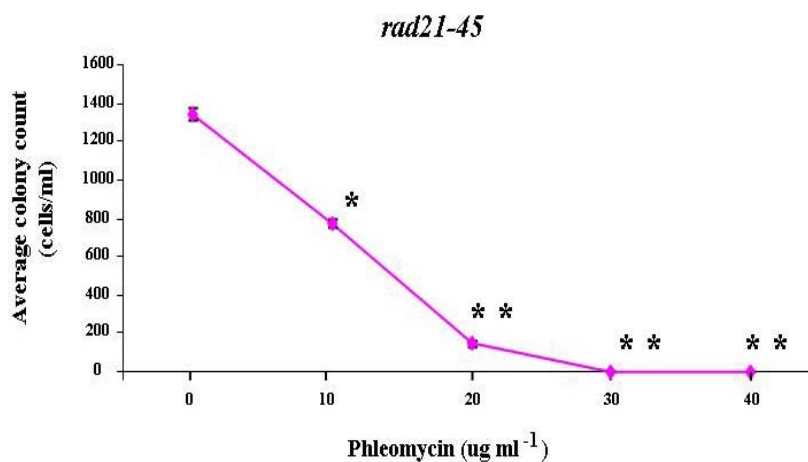
### 4.5.2 Quantitative data

Quantitative data were obtained as described, and are shown in Figures 4.16 to 4.20. Consistent with the qualitative data, it was observed that increasing concentrations of phleomycin resulted in decreased cell survival for all three strains. However, again the same unexpected observation for the *rad21P:rec8<sup>+</sup> nmt1P:rad21* “on” strain was seen, with it showing the most sensitivity to phleomycin.



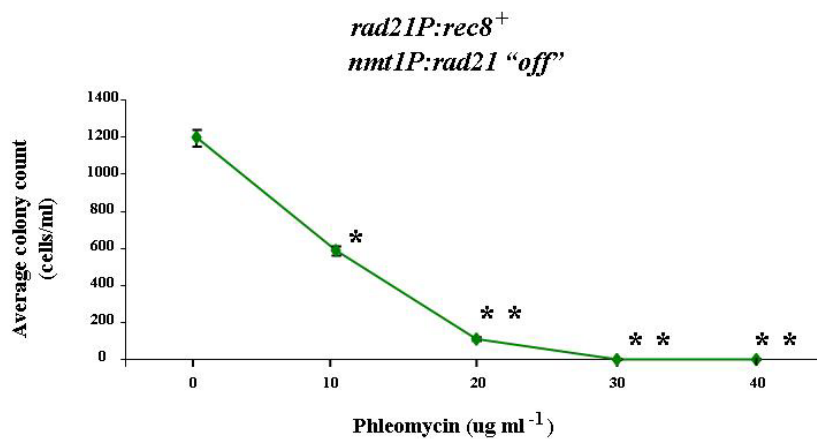
**Figure 4-16: Wild-type cells treated with increasing concentrations of phleomycin results in decreased cell survival.**

Wild-type (GG 218) cells, were grown at 25°C in minimal medium in the presence of thiamine to  $5 \times 10^6$  cells ml<sup>-1</sup>, and were subsequently plated at the appropriate dilution of  $10^{-3}$  onto EMM + thiamine plates, containing increasing concentrations of the toxic glycopeptide antibiotic phleomycin. Concentrations used were 0 µg ml<sup>-1</sup>, 10 µg ml<sup>-1</sup>, 20 µg ml<sup>-1</sup>, 30 µg ml<sup>-1</sup> and 40 µg ml<sup>-1</sup>. The plates were then incubated at 25°C for five nights. Experiments were carried out in triplicates of five to ensure reproducibility and to allow for efficiency of plating. The average number of colonies counted was taken from the triplicates and plotted. Bars represent the standard error of the mean, with \*p < 0.05% and \*\*p < 0.01 indicating increased sensitivity in comparison to untreated cells.



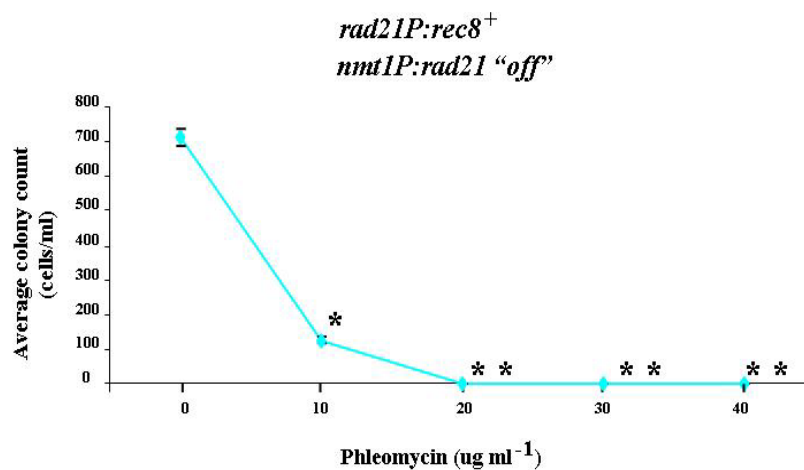
**Figure 4-17: Quantitative data: *rad21-45* cells treated with increasing concentrations of phleomycin results in decreased cell survival.**

*rad21-45* (GG 921) cells, were grown at 25°C in minimal medium in the presence of thiamine to  $5 \times 10^6$  cells ml<sup>-1</sup>, and were subsequently plated at the appropriate dilution of  $10^{-3}$  onto EMM + thiamine plates, containing increasing concentrations of the toxic glycopeptide antibiotic phleomycin. Concentrations used were 0 µg ml<sup>-1</sup>, 10 µg ml<sup>-1</sup>, 20 µg ml<sup>-1</sup>, 30 µg ml<sup>-1</sup> and 40 µg ml<sup>-1</sup>. The plates were then incubated at 25°C for five nights. Experiments were carried out in triplicates of five to ensure reproducibility and to allow for efficiency of plating. The average number of colonies counted was taken from the triplicates and plotted. Bars represent the standard error of the mean, with \*p < 0.05% and \*\*p < 0.01 indicating increased sensitivity in comparison to untreated cells.



**Figure 4-18: Quantitative data: *rad21P:rec8<sup>+</sup> nmt1P:rad21* "off" cells treated with increasing concentrations of phleomycin results in decreased cell survival.**

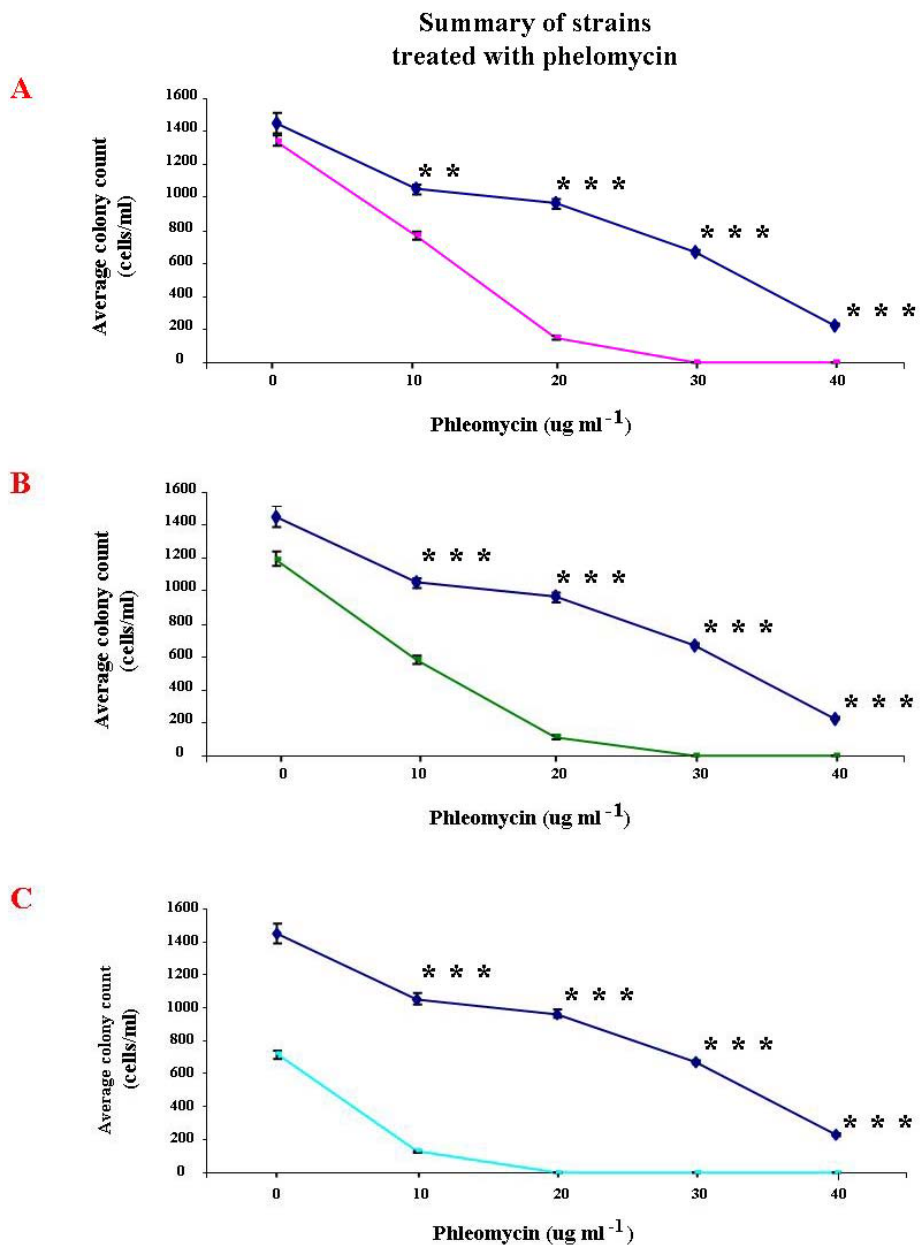
*rad21P:rec8<sup>+</sup> nmt1P:rad21* "off" (GG 358) cells, were grown at 25°C in minimal medium in the presence of thiamine to  $5 \times 10^6$  cells ml<sup>-1</sup>, and were subsequently plated at the appropriate dilution of  $10^{-3}$  onto EMM + thiamine plates, containing increasing concentrations of the toxic glycopeptide antibiotic phleomycin. Concentrations used were 0  $\mu\text{g ml}^{-1}$ , 10  $\mu\text{g ml}^{-1}$ , 20  $\mu\text{g ml}^{-1}$ , 30  $\mu\text{g ml}^{-1}$  and 40  $\mu\text{g ml}^{-1}$ . The plates were then incubated at 25°C for five nights. Experiments were carried out in triplicates of five to ensure reproducibility and to allow for efficiency of plating. The average number of colonies counted was taken from the triplicates and plotted. Bars represent the standard error of the mean, with \*p < 0.05% and \*\*p < 0.01 indicating increased sensitivity in comparison to untreated cells.



**Figure 4-19: Quantitative data: *rad21P:rec8<sup>+</sup> nmt1P:rad21<sup>+</sup>* “on” cells treated with increasing concentrations of phleomycin results in decreased cell survival.**

*rad21P:rec8<sup>+</sup> nmt1P:rad21<sup>+</sup>* “on” (GG 358) cells, were grown at 25°C in minimal medium in the presence of thiamine to  $5 \times 10^6$  cells ml<sup>-1</sup>, and were subsequently plated at the appropriate dilution of  $10^{-3}$  onto EMM + thiamine plates, containing increasing concentrations of the toxic glycopeptide antibiotic phleomycin. Concentrations used were 0  $\mu\text{g ml}^{-1}$ , 10  $\mu\text{g ml}^{-1}$ , 20  $\mu\text{g ml}^{-1}$ , 30  $\mu\text{g ml}^{-1}$  and 40  $\mu\text{g ml}^{-1}$ . The plates were then incubated at 25°C for five nights. Experiments were carried out in triplicates of five to ensure reproducibility and to allow for efficiency of plating. The average number of colonies counted was taken from the triplicates and plotted. Bars represent the standard error of the mean, with \* $p < 0.05$  and \*\* $p < 0.01$  indicating increased sensitivity in comparison to untreated cells.





**Figure 4-20: Summary of quantitative data following phleomycin treatment: average survival and percentage cell survival curves for all strains.**

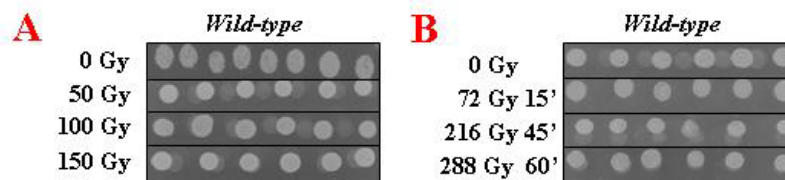
Summary of results obtained. For damage induced with phleomycin, data indicates that for all three strains increased concentration results in decreased survival, average colonies counted are plotted. **A**, Wild-type compared with *rad21-45*, **B**, Wild-type compared with *rad21P:rec8<sup>+</sup> nmt1P:rad21<sup>+</sup> "off"*, **C**, Wild-type compared with *rad21P:rec8<sup>+</sup> nmt1P:rad21<sup>+</sup> "on"*. Bars represent the standard error of the mean, with \*p < 0.05%, \*\*p < 0.01 and \*\*\*p < 0.001 indicating increased sensitivity.

Data obtained using UV-C and MMS as damaging agents revealed that cells containing *rec8*<sup>+</sup> instead of *rad21*<sup>+</sup> were more sensitive as decreased cell survival was seen with increasing concentrations of phleomycin. However, a significant difference seen with this mutagen and not with the other agents was that the strain in which SpRad21p was over-expressed *rad21P:rec8*<sup>+</sup> *nmt1P:rad21*<sup>+</sup> “on” had decreased cell survival than even the strain in which SpRec8p was expressed *rad21P:rec8*<sup>+</sup> *nmt1P:rad21*<sup>+</sup> “off”.

## 4.6 Ionizing radiation

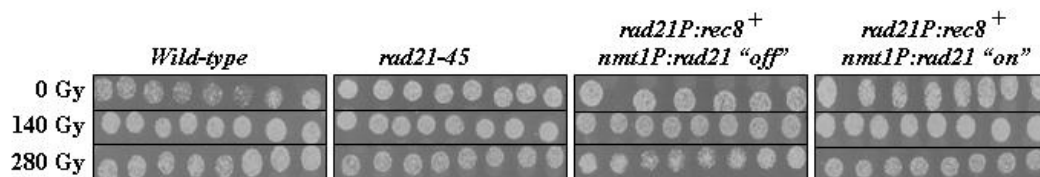
Ionizing radiation, known to create the most genotoxic of lesions, causes DNA DSBs. In the natural environment where cells are exposed continuously to radiation, radiation can exert its effect on the DNA duplex both directly and indirectly. Indirect damage occurs because in an aqueous environment, ionizing energy produces reactive species that interact with DNA, thus causing lesions.

Wild-type *S. pombe* cells were grown exponentially, diluted, and spotted onto solid medium before irradiation with  $^{60}\text{Co}$ . Two variations in irradiation protocols were completed, one with increasing doses and the other with increasing exposure times (Figure 4.21). As *S. pombe* proved to be very difficult to irradiate with this  $^{60}\text{Co}$  source all strains were spotted to see if there was any visible difference when the meiotic cohesin was expressed in the mitotic cycle. Unfortunately, even then no was difference seen (Figure 4.22). Although the dose rate was increased, the source at  $2\text{ Gy min}^{-1}$ , remained too weak, and as there was no other source available no further work could be carried out using ionizing radiation.



**Figure 4-21: Irradiation of wild-type SpRad21p with  $^{60}\text{Co}$ .**

Wild-type (GG 218) cells were grown at  $25^{\circ}\text{C}$  in the presence of thiamine and  $3\ \mu\text{l}$  spotted onto EMM + thiamine plates. These plates were then exposed to increasing doses of  $\gamma$  radiation and incubated for two nights at  $25^{\circ}\text{C}$ . **A**, 0 Gy to 150 Gy were administered. **B**, Doses were then administered relative to how long the plates were under the  $\gamma$  source. Times were 15 to 60 mins.



**Figure 4-22: Irradiation of all strains with  $^{60}\text{Co}$ .**

Cells of three strains, wild-type (GG 218), *rad21-45* (GG 921) and *rad21P:rec8<sup>+</sup> nmt1P:rad21<sup>+</sup> "off"* (GG 358) were grown at  $25^{\circ}\text{C}$  to mid-exponential stage in the presence of thiamine, GG 358 was also grown in the absence of thiamine *rad21P:rec8<sup>+</sup> nmt1P:rad21<sup>+</sup> "on"*. These were spotted onto EMM + thiamine plates. Doses of 140 Gy and 280 Gy were administered.

## 4.7 Analysis of the SpRad21p repressible strain in the absence of thiamine *rad21P:rec8<sup>+</sup> nmt1P:rad21<sup>+</sup>* “on”

The repressible strain *rad21P:rec8<sup>+</sup> nmt1P:rad21<sup>+</sup>* contains the *rad21<sup>+</sup>* gene under the control of the *nmt1<sup>+</sup>* promoter. There are three versions of the repressible thiamine *nmt1<sup>+</sup>* promoter, ranging from extremely high, to moderate or low transcription efficiency, respectively, all active when cells are grown in the absence of thiamine both in repressed and induced conditions (Forsburg, 1993). The three plasmid versions are pREP1/pREP-3X (300 fold expression), pREP-41X (25 fold expression) and pREP-81X (7 fold expression), with the pREP1/pREP-3X version containing the wild-type version of the promoter, and the other two derivative, attenuated versions (Maundrell 1990). For *nmt1<sup>+</sup>* promoter suppression cells are grown in the presence of thiamine at a concentration of 5  $\mu\text{g ml}^{-1}$ . However, even in the presence of thiamine, the *nmt1<sup>+</sup>* promoter does not repress completely, and thus the ability to construct a clean “shut off” plasmid depends on the *nmt1<sup>+</sup>* promoter used, the stability of the protein being expressed, and the sensitivity of the cell to levels of the protein.

In the specific context of the *rad21P:rec8<sup>+</sup> nmt1P:rad21<sup>+</sup>* strain, this construct used the wild-type *nmt1<sup>+</sup>* promoter, which allows maximum over-expression in the absence of thiamine. However, the DNA was integrated into the chromosome and so present in single copy, both of which resulted in reduced over-expression and cleaner shut off. When cells grown in the absence of thiamine were treated with UV-C and MMS they had only slightly more survival than when SpRec8p was being expressed, which was surprising as it had been expected that these cells would have either wild-type or near wild-type levels of viability. This decreased viability may be due to the presence of excessive levels of SpRad21p, which at high levels may be toxic for the cell.

Furthermore, the *rad21P:rec8<sup>+</sup> nmt1P:rad21<sup>+</sup>* strain, when grown in the absence of thiamine had the lowest viability when exposed to increasing concentrations of phleomycin. This may be due, not only to increased SpRad21p levels, but also the toxic effects of phleomycin resulting in an additive affect which is more detrimental to cell survival.

## 4.8 Summary

Both qualitative and quantitative data demonstrated that regardless of the DNA damaging agent used, or the type of damage caused to DNA, increasing concentrations results in decreased cell survival in all the three *rec8<sup>+</sup>* strains tested. Unfortunately, no data were obtained using ionizing data, as the only source available was not strong enough.

This chapter sets the basis for the next chapter where the transcriptional profiles of the cohesins in response to DNA damage are investigated, and chromosome integrity using pulsed-field gel electrophoresis is monitored. The effects of MMS are examined in more detail.

# Chapter 5

## 5.1 Introduction

The evolutionary conserved multi-subunit complex cohesin plays a critical role in the successful and accurate separation of sister chromatids during both mitosis and meiosis. Defects in cohesin function lead to abnormal chromosome segregation during M phase resulting in the generation of aneuploid products. This complex is composed of two SMC proteins, SpPsm1p (ScSmc1p) and SpPsm3p (ScSmc3) and two SCC proteins, SpRad21p, (ScScc1p/ScMcd1p) (mitosis) and SpRec8p (meiosis) and SpPsc3p (ScScc3p).

Chapter 3 introduced the two cohesin proteins studied in this thesis, and analysed their life cycle specific expression. The *rad21*<sup>+</sup> and *rec8*<sup>+</sup> genes represent a pair of genes, both encoding proteins with cohesin function, each expressed during mitosis and meiosis respectively. In Chapter 4, the specific function of *rad21*<sup>+</sup> with emphasis on its role in DNA damage repair was studied. Chronic exposure to three damaging agents; UV-C light, MMS and phleomycin revealed that cells containing *rec8*<sup>+</sup> instead of *rad21*<sup>+</sup> were more sensitive to DNA damaging agents and increasing doses and concentrations respectively.

In this Chapter this interesting observation is further studied, with the specific role of cohesin expression and chromosome integrity analysed.

## 5.2 Northern analysis

### 5.2.1 *Transcriptional profiles of rad21<sup>+</sup> and rec8<sup>+</sup> after DNA damage following treatment with MMS*

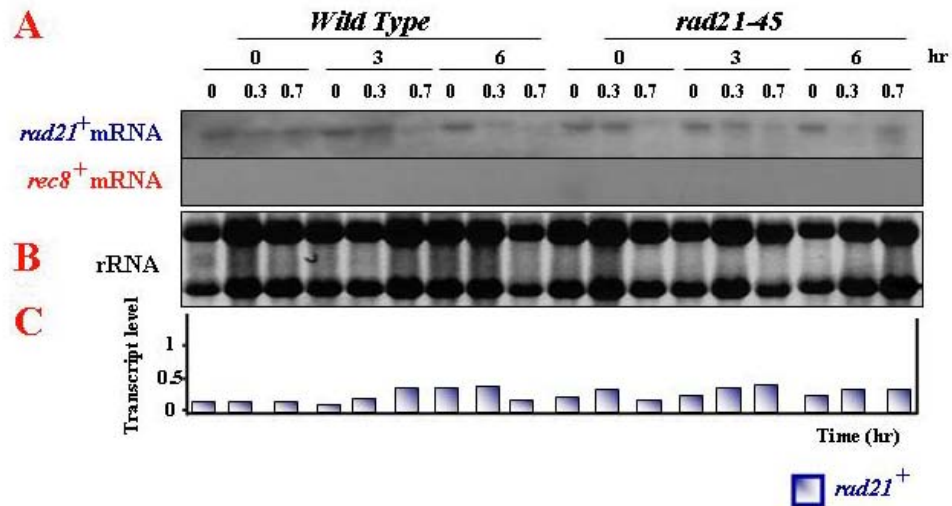
Initially, the expression profiles of *rad21<sup>+</sup>* and *rec8<sup>+</sup>* were examined after the addition of MMS, to explore if either is regulated by this DNA damaging agent. Wild-type and *rad21-45* strains were grown in the presence of thiamine, and the *rad21P:rec8<sup>+</sup> nmt1P:rad21<sup>+</sup>* repressible strain was grown in the presence and absence of thiamine, before the addition of varying concentrations of MMS. Cell number was monitored and, after treatment, samples were taken hourly over a 4 hour period, harvested, and subjected to northern blot analysis to study levels of *rad21<sup>+</sup>* and *rec8<sup>+</sup>* mRNA.

### 5.2.2 *rad21<sup>+</sup> but not rec8<sup>+</sup> is induced in response to MMS*

Initial MMS concentrations selected for the northern blot experiments were 0.3% and 0.7%. This was because the qualitative and quantitative data indicated cell survival at 0.3%, and complete cell death at the upper limit of 0.7%. In control samples with 0% MMS constant levels of *rad21<sup>+</sup>* mRNA were observed (Figure 5.1). However, at 0.3% MMS *rad21<sup>+</sup>* mRNA increased till 3 hours after which they decreased at 6 hours, likely due to cell death at this concentration. In contrast, at 0.7% MMS, *rad21<sup>+</sup>* mRNA decreased immediately following addition, with lower levels at 3 and 6 hours. At all MMS concentrations *rec8<sup>+</sup>* mRNA was not detected, showing this gene is not induced by DNA damage in mitotic cells.

Related observations were made with the *rad21-45* mutant following addition of MMS. Comparable to that seen in wild-type, *rad21-45* control samples with 0% MMS had constant levels of *rad21<sup>+</sup>* mRNA present (Figure 5.1). At 0.3 and 0.7% MMS, however, *rad21<sup>+</sup>* was not induced with constant levels of mRNA to 3 hours, and a reduction up to 6 hours.

### 5.2.3 Wild-type and *rad21-45* cells



**Figure 5-1: *S. pombe* cells treated with increasing concentrations of MMS results in cell death.**

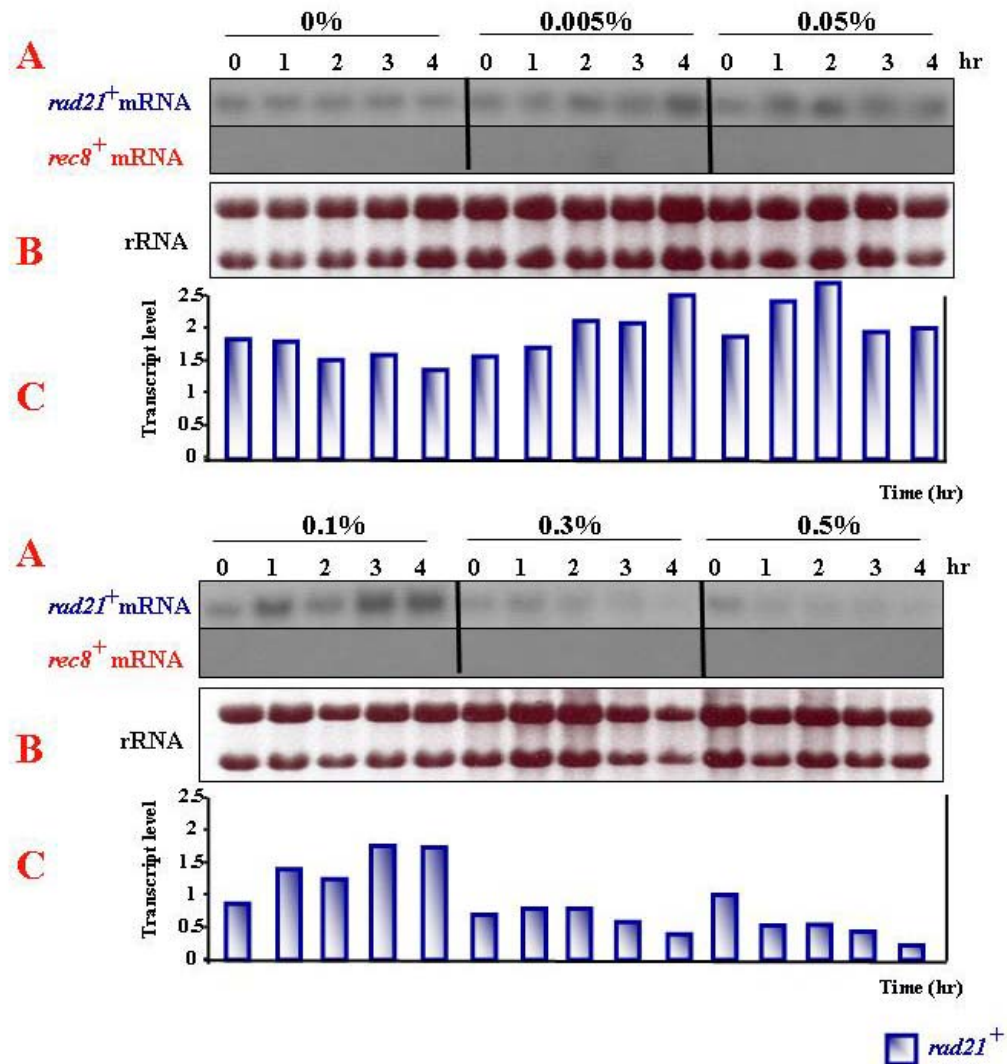
Wild-type (GG 218) and *rad21-45* (GG 921) cells were grown to mid-exponential phase in the presence of thiamine before treatment with increasing concentrations of MMS of 0%, 0.3% and 0.7%. For both strains cells were harvested for northern blot analysis after 0, 3 and 6 hr, **A**, 10  $\mu$ g total RNA was separated on a formaldehyde gel, transferred to a nylon membrane and the blot hybridized with radioactive probes for *rad21*<sup>+</sup> and *rec8*<sup>+</sup>. **B**, Equal loading of RNA samples was confirmed with ethidium bromide staining of rRNA. **C**, Transcript levels were quantified by densitometry using NIH image software; units given are arbitrary.

In the following experiment lower concentrations of MMS were thus used, and interestingly, it was found that in both wild-type and *rad21-45* cells *rad21*<sup>+</sup> mRNA increased 2 hours after MMS addition, but not *rec8*<sup>+</sup> mRNA (Figures. 5.2 and 5.3). This apparent induction of *rad21*<sup>+</sup> transcription occurred with MMS concentrations of 0.05% and 0.1%, but not at 0.5%, where almost complete cell death occurred (Figure 4.9).

To test whether this induction of *rad21*<sup>+</sup> expression was not an indirect effect due to the accumulation of cells in G1 caused by the MMS treatment, *cdc25-22* cells (GG 185) were grown to mid-exponential stage before half the culture was shifted to the restrictive temperature of 36°C for 3 hours, to cause a G2-M cell cycle arrest. This and the parallel asynchronous culture at 25°C were then treated with 0.05% MMS, with cell samples taken at 0, 1 and 2 hours for northern blot analysis of *rad21*<sup>+</sup> mRNA levels. Cells arrested at G2-M showed a similar response, with *rad21*<sup>+</sup> mRNA levels increasing after just 1 hour (Figure 5.4). This observation confirms that the rapid induction of *rad21*<sup>+</sup> was a direct result of the addition of the MMS.

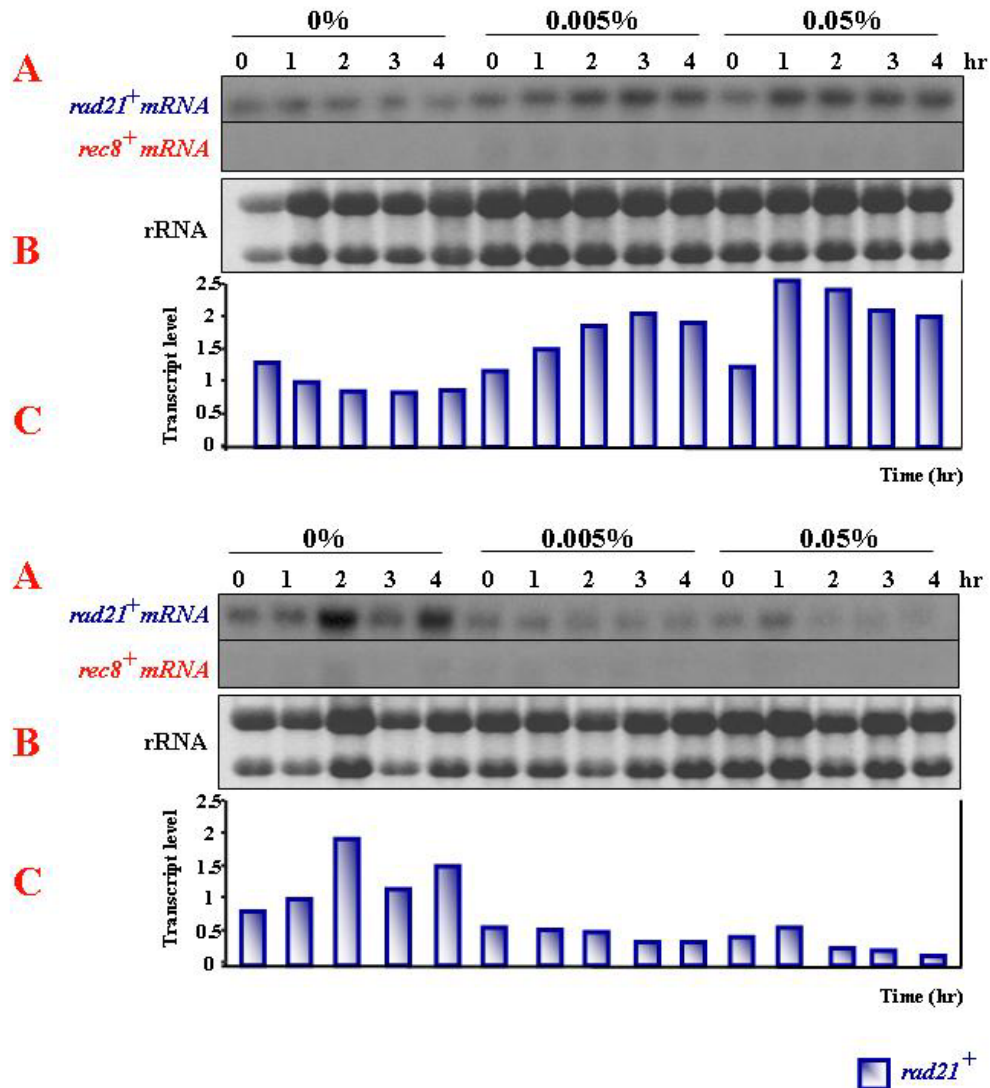


Furthermore, microarray data available online revealed that the other three cohesin subunits, *psm1*<sup>+</sup>, *psm3*<sup>+</sup> and *psc3*<sup>+</sup>, also show increased transcription in response to damage induced with MMS (<http://www.genedb.org/>); this observation could be confirmed and further explored using northern blot analysis.



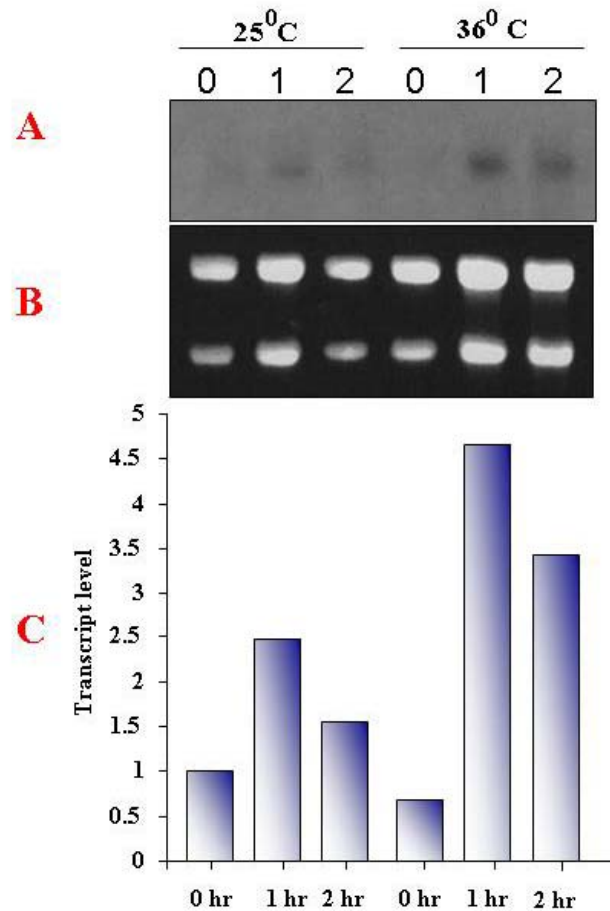
**Figure 5-2: Wild-type *S. pombe* cells treated with increasing concentrations of MMS result in an induction of expression of *rad21*<sup>+</sup> but not *rec8*<sup>+</sup>.**

Wild-type (GG 218) cells were grown to mid-exponential phase in the presence of thiamine before treatment with increasing concentrations of MMS 0%, 0.005%, 0.05%, 0.1%, 0.3% and 0.5%. Cells were harvested for northern blot analysis after 0, 2 and 4 hr. **A**, 10  $\mu$ g total RNA was separated on a formaldehyde gel, transferred to a nylon membrane and the blot hybridized with radioactive probes for *rad21*<sup>+</sup> and *rec8*<sup>+</sup>. **B**, Equal loading of RNA samples was confirmed with ethidium bromide staining of rRNA. **C**, Transcript levels were quantified by densitometry using NIH image software; units given are arbitrary.



**Figure 5-3: *rad21-45 S. pombe* cells treated with increasing concentrations of MMS result in an induction of expression of *rad21<sup>+</sup>* but not *rec8<sup>+</sup>*.**

*rad21-45* (GG 921) cells were grown to mid-exponential phase in the presence of thiamine before treatment with increasing concentrations of MMS 0%, 0.005%, 0.05%, 0.1%, 0.3% and 0.5%. Cells were harvested for northern blot analysis after 0, 2 and 4 hr. **A**, 10  $\mu$ g total RNA was separated on a formaldehyde gel, transferred to a nylon membrane and the blot hybridized with radioactive probes for *rad21<sup>+</sup>* and *rec8<sup>+</sup>*. **B**, Equal loading of RNA samples was confirmed with ethidium bromide staining of rRNA. **C**, Transcript levels were quantified by densitometry using NIH image software; units given are arbitrary.



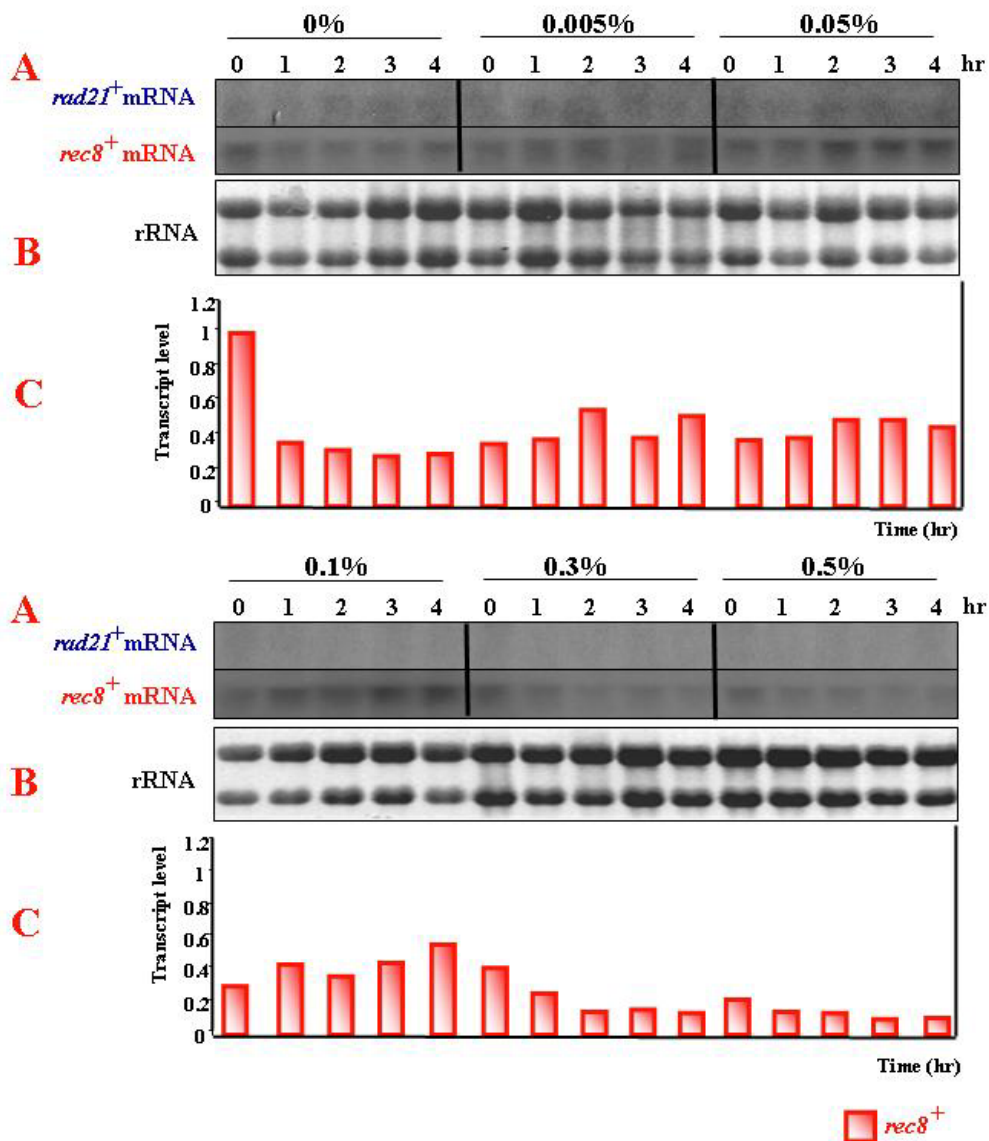
**Figure 5-4: MMS treated cells arrested at G2-M result in rapid induction of *rad21<sup>+</sup>*.**

*cdc25-22* (GG 185) cells were grown at 25°C before the culture was divided into equal volumes and MMS added at a concentration of 0.05%. One culture was kept at 25°C whilst the other was incubated at the restrictive temperature of 36°C to arrest cells at the G2 phase of the cell cycle. Samples were taken before addition of MMS and then at the 1 and 2 hr time points. **A**, 10 µg total RNA was separated on a formaldehyde gel, transferred to a nylon membrane and the blot hybridized with radioactive probes for *rad21<sup>+</sup>*. **B**, Equal loading of RNA samples was confirmed with ethidium bromide staining of rRNA. **C**, Transcript levels were quantified by densitometry using NIH image software; units given are arbitrary.

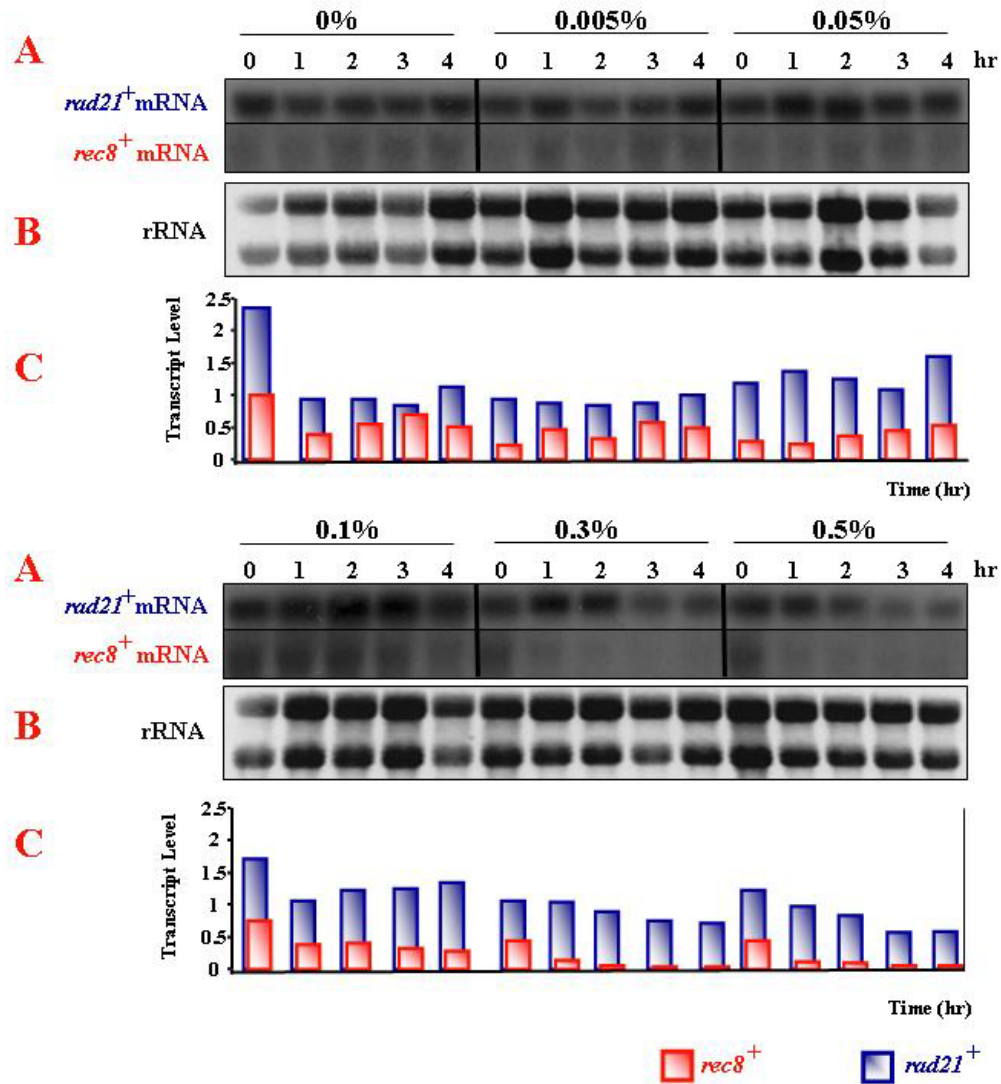
#### **5.2.4 Repressible strain in the presence of thiamine *rad21P:rec8<sup>+</sup>* *nmt1P:rad21<sup>+</sup>* “off” and absence of thiamine *rad21P:rec8<sup>+</sup>* *nmt1P:rad21<sup>+</sup>* “on”**

As expected, in the *rad21P:rec8<sup>+</sup> nmt1P:rad21<sup>+</sup>* repressible strain in the presence of thiamine, *rad21<sup>+</sup>* mRNA was not induced, but was replaced by *rec8<sup>+</sup>* transcript. This was only at MMS concentrations of up to 0.1% where a proportion of the cells survived. In contrast, at 0.5%, where most cells died, reduced *rec8<sup>+</sup>* mRNA levels were detected (Figure 5.5). These observations suggest that the specific induction of *rad21<sup>+</sup>* transcription is part of the mechanism by which SpRad21p mediates DNA damage repair. Furthermore, they confirm that *rec8<sup>+</sup>* mRNA, and by inference SpRec8p protein, despite being present in the repressible strain, do not enable this repair to occur.

When the *rad21P:rec8<sup>+</sup> nmt1P:rad21<sup>+</sup>* repressible strain was grown in the absence of thiamine, SpRad21p was expressed at a constant level, with levels of SpRec8p also apparent (Figure 5.6). However, although cell survival levels improved in comparison to when the same strain was grown in the presence of thiamine, survival levels were still less than wild-type. This may be due to the reasons described in Section 4.7.



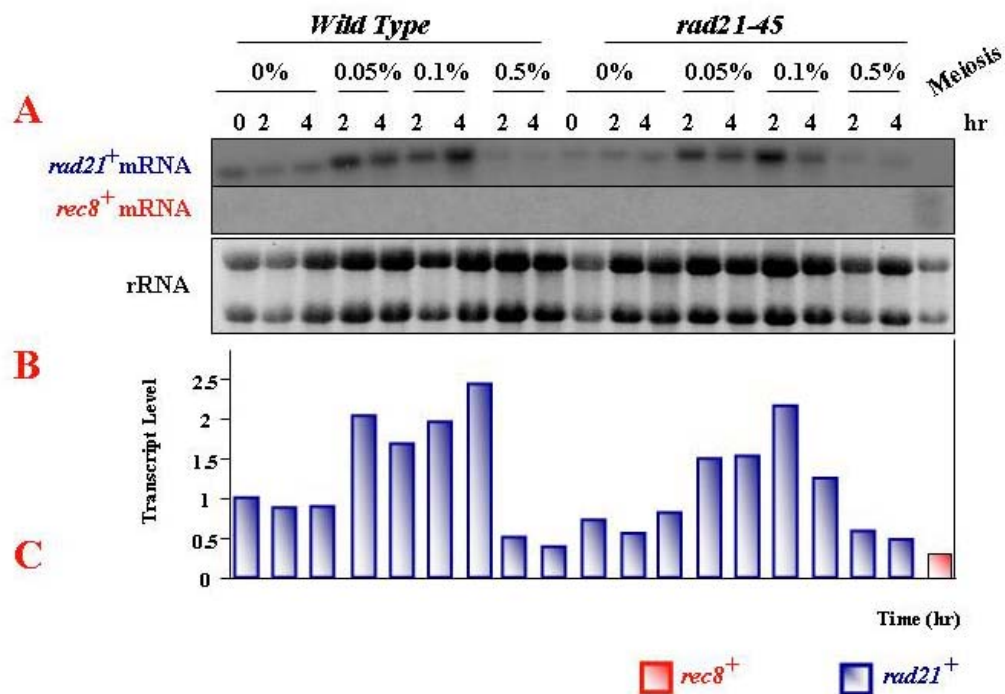
**Figure 5-5: *rad21P:rec8<sup>+</sup> nmt1P:rad21<sup>+</sup>* “off” *S. pombe* cells treated with increasing concentrations of MMS result in decreased cell survival despite the presence of *rec8<sup>+</sup>*.** *rad21P:rec8<sup>+</sup> nmt1P:rad21<sup>+</sup>* “off” (GG 358) cells were grown to mid-exponential phase in the presence of thiamine before treatment with increasing concentrations of MMS 0%, 0.005%, 0.05%, 0.1%, 0.3% and 0.5%. Cells were harvested for northern blot analysis after 0, 2 and 4 hr. **A**, 10  $\mu$ g total RNA was separated on a formaldehyde gel, transferred to a nylon membrane and the blot hybridized with radioactive probes for *rad21<sup>+</sup>* and *rec8<sup>+</sup>*. **B**, Equal loading of RNA samples was confirmed with ethidium bromide staining of rRNA. **C**, Transcript levels were quantified by densitometry using NIH image software; units given are arbitrary.



**Figure 5-6: *rad21P:rec8<sup>+</sup> nmt1P:rad21<sup>+</sup>* “on” *S. pombe* cells treated with increasing concentrations of MMS result in decreased cell survival despite the presence of *rad21<sup>+</sup>*.** *rad21P:rec8<sup>+</sup> nmt1P:rad21<sup>+</sup>* “on” (GG 358) cells were grown to mid-exponential phase in the absence of thiamine before treatment with increasing concentrations of MMS 0%, 0.005%, 0.05%, 0.1%, 0.3% and 0.5%. Cells were harvested for northern blot analysis after 0, 2 and 4 hr. **A**, 10  $\mu$ g total RNA was separated on a formaldehyde gel, transferred to a nylon membrane and the blot hybridized with radioactive probes for *rad21<sup>+</sup>* and *rec8<sup>+</sup>*. **B**, Equal loading of RNA samples was confirmed with ethidium bromide staining of rRNA. **C**, Transcript levels were quantified by densitometry using NIH image software; units given are arbitrary.

### 5.2.5 Summary northern blot of all strains

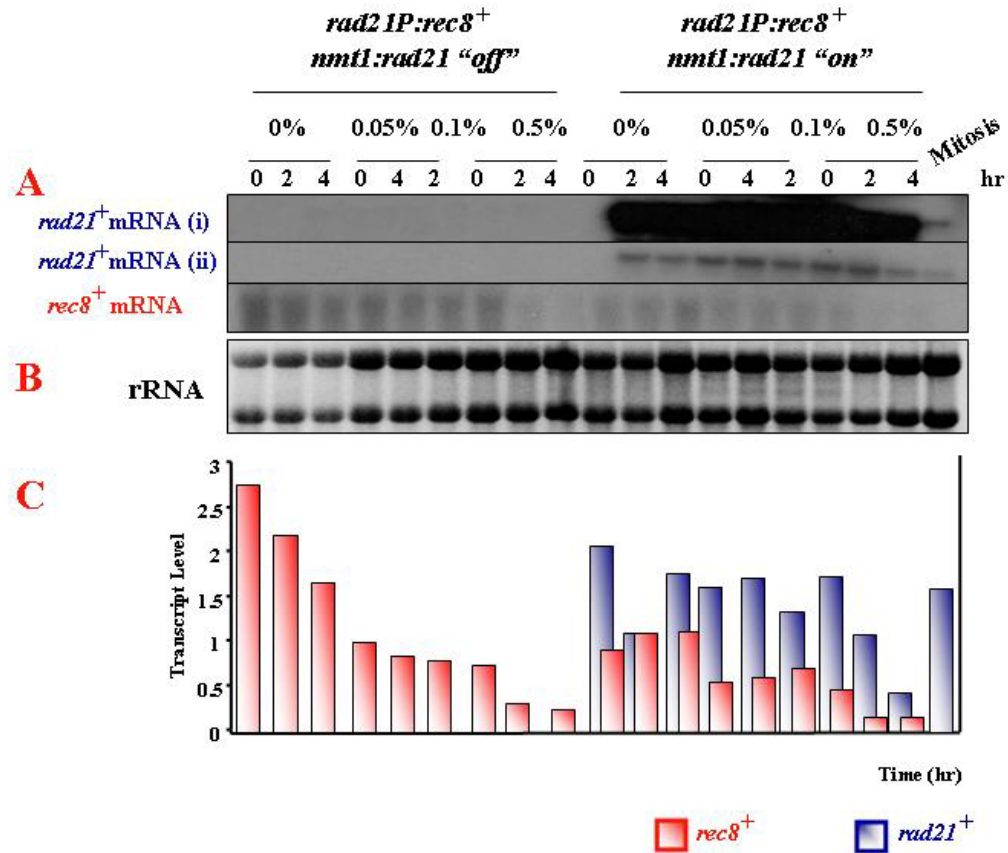
Northern blot analysis was carried out with cultures treated with four different concentrations of MMS for varying times on the membrane to permit direct comparison of *rad21*<sup>+</sup> mRNA levels. The four concentrations of MMS used were 0%, 0.05%, 0.1% and 0.5%, with samples taken at 0, 2 and 4 hours (Figures 5.7 and 5.8). As in the *rad21P:rec8*<sup>+</sup> *nmt1P:rad21*<sup>+</sup> repressible strain in the absence of thiamine, excessive *rad21*<sup>+</sup> mRNA were seen (Figure 5.8 A (i)); thus a shorter autoradiographic exposure was taken (Figure 5.8 A (ii)). These high levels of *rad21*<sup>+</sup> mRNAs did not result in higher cell viability than wild-type (Figure 4.8 qualitative data and Figure 4.14 quantitative data). This may be due to unnaturally high concentrations of SpRad21p being toxic for cells (Section 4.6).



**Figure 5-7: Summary of MMS treated wild-type and *rad21-45* *S. pombe*.**

Four MMS concentrations were selected, 0%, 0.05%, 0.1% and 0.5% over three time points of 0, 2 and 4 hr. Cells were harvested for northern blot analysis after 0, 2 and 4 hr. **A**, 10  $\mu$ g total RNA was separated on a formaldehyde gel, transferred to a nylon membrane and the blot hybridized with radioactive probes for *rad21*<sup>+</sup> and *rec8*<sup>+</sup> a peak meiotic (Meiosis) sample is included as a control at the end of the gel. **B**, Equal loading of RNA samples was confirmed with ethidium bromide staining of rRNA. **C**, Transcript levels were quantified by densitometry using NIH image software; units given are arbitrary.





**Figure 5-8: Summary of *rad21P:rec8<sup>+</sup> nmt1P:rad21<sup>+</sup>* "on" and "off" *S. pombe* treated with increasing concentrations of MMS.**

Four MMS concentrations were selected, 0%, 0.05%, 0.1% and 0.5% over three time points of 0, 2 and 4 hr. Cells were harvested for northern blot analysis after 0, 2 and 4 hr. **A**, 10  $\mu$ g total RNA was separated on a formaldehyde gel, transferred to a nylon membrane and the blot hybridized with radioactive probes for *rad21<sup>+</sup>* and *rec8<sup>+</sup>*, peak mitotic (Mitosis) sample is included as a control at the end of the gel. **B**, Equal loading of RNA samples was confirmed with ethidium bromide staining of rRNA. **C**, Transcript levels were quantified by densitometry using NIH image software; units given are arbitrary.



### 5.3 Role of cohesin in DNA damage repair

It has long been suspected that cohesion between sister chromatids is essential for efficient DSB repair in mitotic cells. In *S. cerevisiae* it has been discovered that cohesins can also mediate chromosome repair outside the S phase DNA replication period with SCC1 playing a part (Sjögren & Nasmyth, 2001; Ström & Sjögren, 2007) and that cohesion can be established also in G2-M in response to DSB induction (Ström *et al.*, 2004).

In humans, research on HsRad21p has sought to establish a possible link between the RAD21 repair gene and proliferation in tumour cells (Atienza *et al.*, 2005). The gene served as a target in breast cancer cells whereby down-regulation of RAD21 expression resulted in decreased cellular proliferation, increased apoptosis, and increased cell killing after exposure to chemotherapeutic agents. The expression of cohesin in response to DSB induction and as a target in tumour cells confirms further the role of the mitotic cohesin subunit in response to DNA damage.

The observation that *rad21*<sup>+</sup> expression is induced after MMS treatment (Figure 5.4) supports the idea that this may also be occurring in fission yeast. However, the fact that cells expressing *rec8*<sup>+</sup> instead of *rad21*<sup>+</sup> show strikingly reduced viability after DNA damage, and fail to repair their chromosomes, suggests that this function is specific to SpRad21p.

## 5.4 Chromosome integrity and repair after DNA damage

### 5.4.1 Fission yeast cells, containing either *SpRad21p* or *SpRec8p* grown in the presence of MMS contain fragmented chromosomes

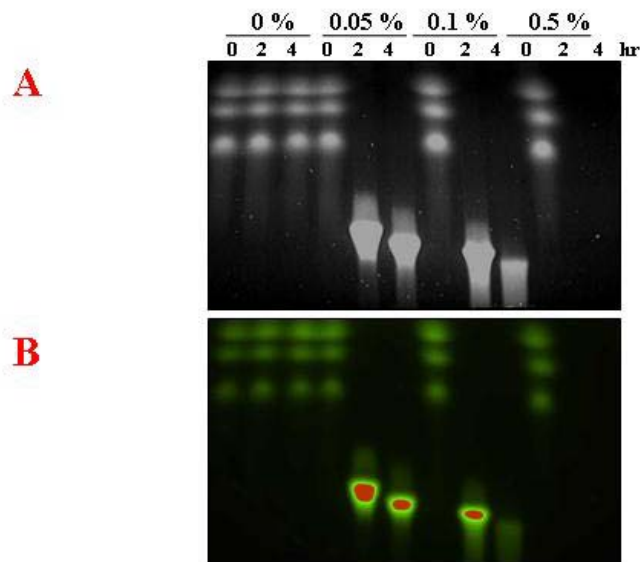
Linear DNA molecules are routinely separated using agarose gels, although all DNA molecules larger than ~40 kb migrate through a gel at the same rate. However, above this critical size the application of a constant electrical field to horizontal agarose gels becomes pointless since separation can no longer occur. Pulsed-field gel electrophoresis (PFGE) solves this problem, by switching the electric field periodically between two different directions; this method allows the separation of DNA molecules up to ~5 Mb in length. The interval at which the field direction is changed is termed the pulse time and may range from a few seconds to hours, with progressively larger DNA fragments resolving at larger pulse times (Smith *et al.*, 1987; Sambrook & Russell, 2001).

Fission yeast has three chromosomes of sizes 3.5, 4.7 and 5.7 Mb, and has an efficient DNA damage repair mechanism that *SpRad21p* appears to control (Birkenbihl & Subramani, 1992). The specificity of this function to *rad21*<sup>+</sup> was examined by monitoring the repair to damaged chromosomes by utilising PFGE as a repair assay which in combination with MMS treatment generates fragmented chromosomes (Lundin *et al.*, 2005).

Cultures of the three strains were grown to mid exponential phase at 25°C before the addition of MMS, wild-type (GG 218), *rad21-45* (GG 921), *rad21P:rec8*<sup>+</sup> *nmt1P:rad21* “off” (GG 358) with thiamine, and a culture with the repressible strain in the absence of thiamine *rad21P:rec8*<sup>+</sup> *nmt1P:rad21* “on” (GG 358). MMS was added in varying concentrations, with samples taken at 0, 2 and 4 hours after treatment for PFGE. The final agarose gel was stained with ethidium bromide and visualised using the UVISOFT analysis software, DNA intensity levels were quantified by densitometry using the NIH image J programme.

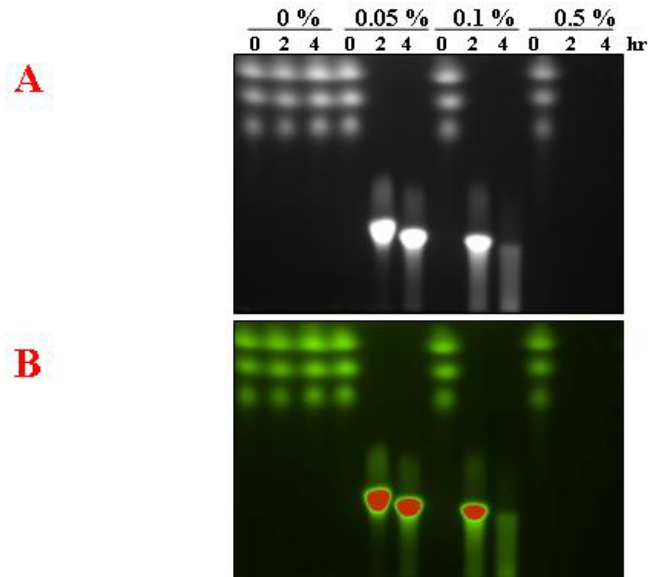
As expected, in untreated cells, the three fission yeast chromosomes were detectable (Figures 5.9 to 5.12). However, the addition of 0.05% MMS for 2 hours caused significant DNA damage to all strains. Chromosomes were sufficiently damaged so that most of the

intact chromosomes were no longer observed, and instead broken DNA ran as a low molecular weight higher mobility smear. When higher concentrations of MMS were added, 0.1% and 0.5%, increased DNA damage was detectable, again at similar levels in all three strains. These observations suggest that damage to DNA at the same MMS concentration is equivalent in mitotic cells containing SpRec8p or SpRad21p. Figure 5.13 shows a summary experiment for all strains with an MMS concentration of 0.05%.

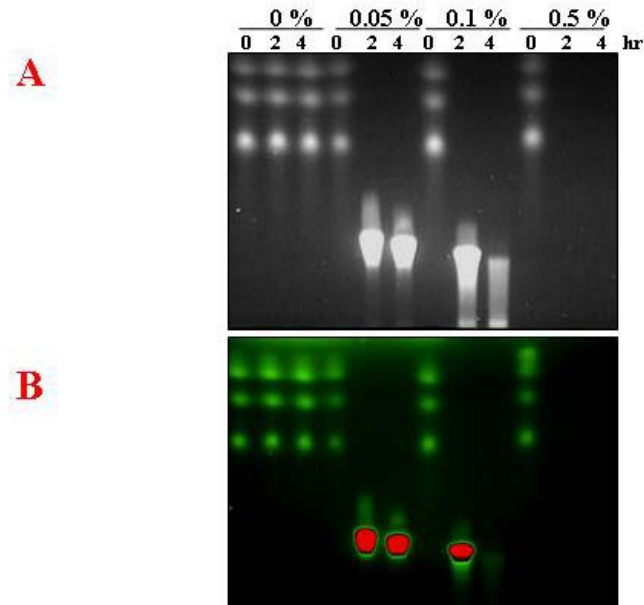


**Figure 5-9: Effects on chromosome integrity in wild-type following treatment with MMS.**

Wild-type (GG 218) cells were grown to mid-exponential stage in the presence of thiamine before treatment with varying concentrations of MMS of the range 0%, 0.05%, 0.1% and 0.5%. Cells were harvested after 0, 2 and 4 hr for analysis by pulse field gel electrophoresis. **A**, Agarose plugs containing  $1 \times 10^8$  cells  $\text{ml}^{-1}$  were prepared for each sample, loaded into an agarose gel and chromosomes separated by electrophoresis. **B**, Red/green colour intensity gel displaying extent of degradation with increasing concentrations of MMS.

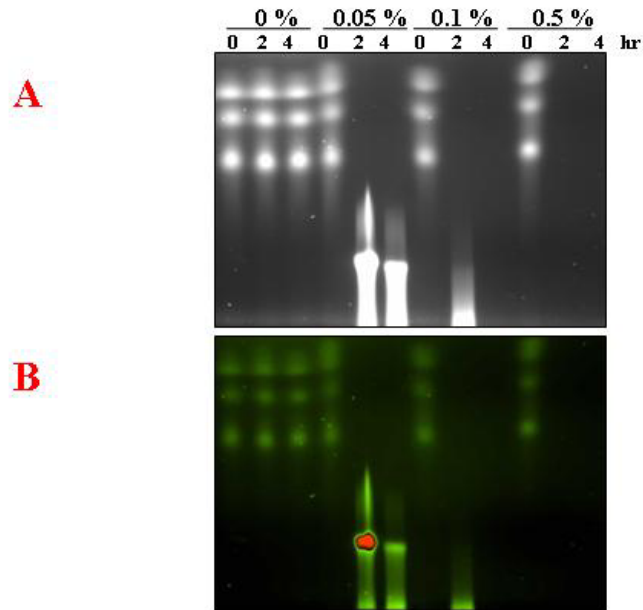


**Figure 5-10: Effects on chromosome integrity in *rad21-45* following treatment with MMS.** *rad21-45* (GG 921) cells were grown to mid-exponential stage in the presence of thiamine before treatment with varying concentrations of MMS of the range 0%, 0.05%, 0.1% and 0.5%. Cells were harvested after 0, 2 and 4 hr for analysis by pulse field gel electrophoresis. **A**, Agarose plugs containing  $1 \times 10^8$  cells  $\text{ml}^{-1}$  were prepared for each sample, loaded into an agarose gel and chromosomes separated by electrophoresis. **B**, Red/green colour intensity gel displaying extent of degradation with increasing concentrations of MMS.



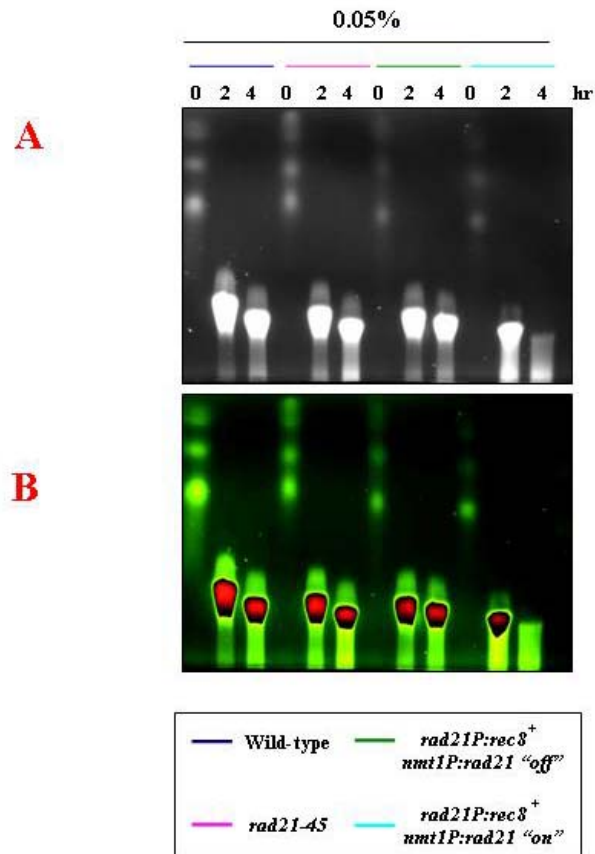
**Figure 5-11: Effects on chromosome integrity in *rad21P:rec8<sup>+</sup> nmt1P:rad21<sup>+</sup>* “off” following treatment with MMS.**

*rad21P:rec8<sup>+</sup> nmt1P:rad21<sup>+</sup>* “off” (GG 358) cells were grown to mid-exponential stage in the presence of thiamine before treatment with varying concentrations of MMS of the range 0%, 0.05%, 0.1% and 0.5%. Cells were harvested after 0, 2 and 4 hr for analysis by pulse field gel electrophoresis. **A**, Agarose plugs containing  $1 \times 10^8$  cells  $\text{ml}^{-1}$  were prepared for each sample, loaded into an agarose gel and chromosomes separated by electrophoresis. **B**, Red/green colour intensity gel displaying extent of degradation with increasing concentrations of MMS.



**Figure 5-12: Effects on chromosome integrity in *rad21P:rec8<sup>+</sup> nmt1P:rad21<sup>+</sup>* “on” following treatment with MMS.**

*rad21P:rec8<sup>+</sup> nmt1P:rad21<sup>+</sup>* “on” (GG 358) cells were grown to mid-exponential stage in the presence of thiamine before treatment with varying concentrations of MMS of the range 0%, 0.05%, 0.1% and 0.5%. Cells were harvested after 0, 2 and 4 hr for analysis by pulse field gel electrophoresis. **A**, Agarose plugs containing  $1 \times 10^8$  cells  $\text{ml}^{-1}$  were prepared for each sample, loaded into an agarose gel and chromosomes separated by electrophoresis **B**, Red/green colour intensity gel displaying extent of degradation with increasing concentrations of MMS.



**Figure 5-13: Summary of effects on *S. pombe* chromosome integrity following treatment with MMS.**

An MMS concentration of 0.05%, over three time points of 0, 2 and 4 hr was selected. Cells were harvested for pulse field gel analysis. **A**, Agarose plugs containing  $1 \times 10^8$  cells  $\text{ml}^{-1}$  were prepared for each sample, loaded into an agarose gel and chromosomes separated by electrophoresis. **B**, Red/green colour intensity gel displaying extent of degradation with increasing concentrations of MMS.

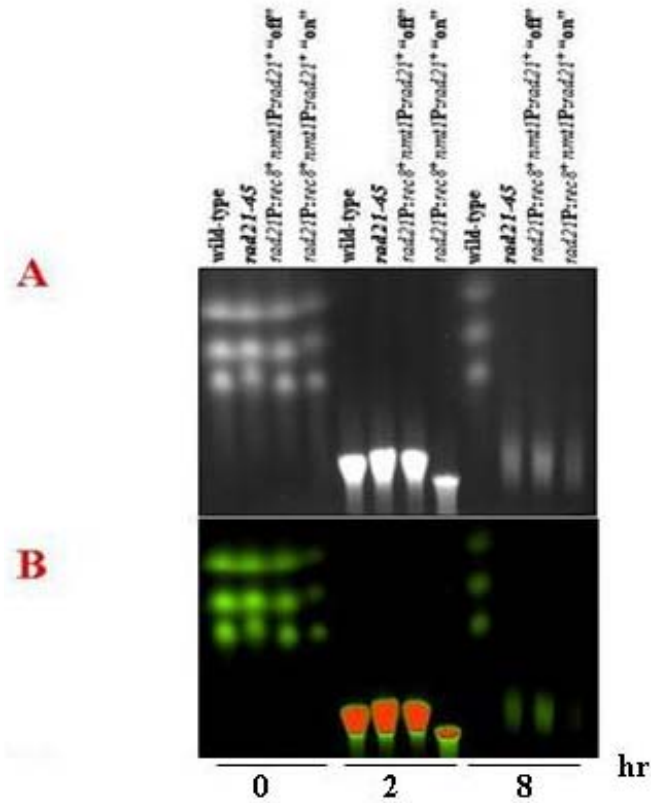
### **5.4.2 *S. pombe* cells expressing SpRec8p instead of SpRad21p in mitosis do not repair their chromosomes after an recovery period**

The previous PFGE experiments were repeated to allow the examination of the ability of the cohesin strains to repair DNA damage, and under these conditions interesting differences were observed between SpRec8p and SpRad21p.

Cells were treated with 0.05% MMS for 2 hours, a sample taken, and the remaining culture washed three times with fresh media, before being left to grow in fresh medium without MMS for 6 hours. Samples for each strain were taken at 0 hours before MMS addition, 2 hours after treatment, and 6 hours following removal of MMS. Cell samples for microscopic analysis were also taken, and cell counts were completed over a 9 hour period to monitor growth rate and viability.

This experiment revealed that although wild-type cells could repair the MMS DNA damage, as shown by the reappearance of the three intact chromosomes, and the disappearance of the smear of broken DNA, this did not occur in *rad21P:rec8<sup>+</sup>* cells (Figure 5.14). This phenotype is similar to that observed in *rad21-45* cells following  $\gamma$ -irradiation (Birkenbihl & Subramani, 1992). This inability to repair DNA damage also manifested in division rates and cell phenotypes. The division rate of all three strains slowed after treatment with MMS, but wild-type and *rad21-45* cells recovered to divide normally by 6 hours after MMS removal. In contrast, *rad21P:rec8<sup>+</sup>* cells had not resumed division by this time and did not divide by 9 hours, as shown by no increase in cell number. Both the repressible strain in the presence of thiamine (*rad21P:rec8<sup>+</sup> nmt1P:rad21<sup>+</sup>* “off”) and *rad21-45* cells showed an elongated phenotype, indicative of cell cycle arrest and checkpoint activation (Figures 5.15 to 5.18). Growth curves in the presence and absence of MMS are summarised in Figure 5.19.

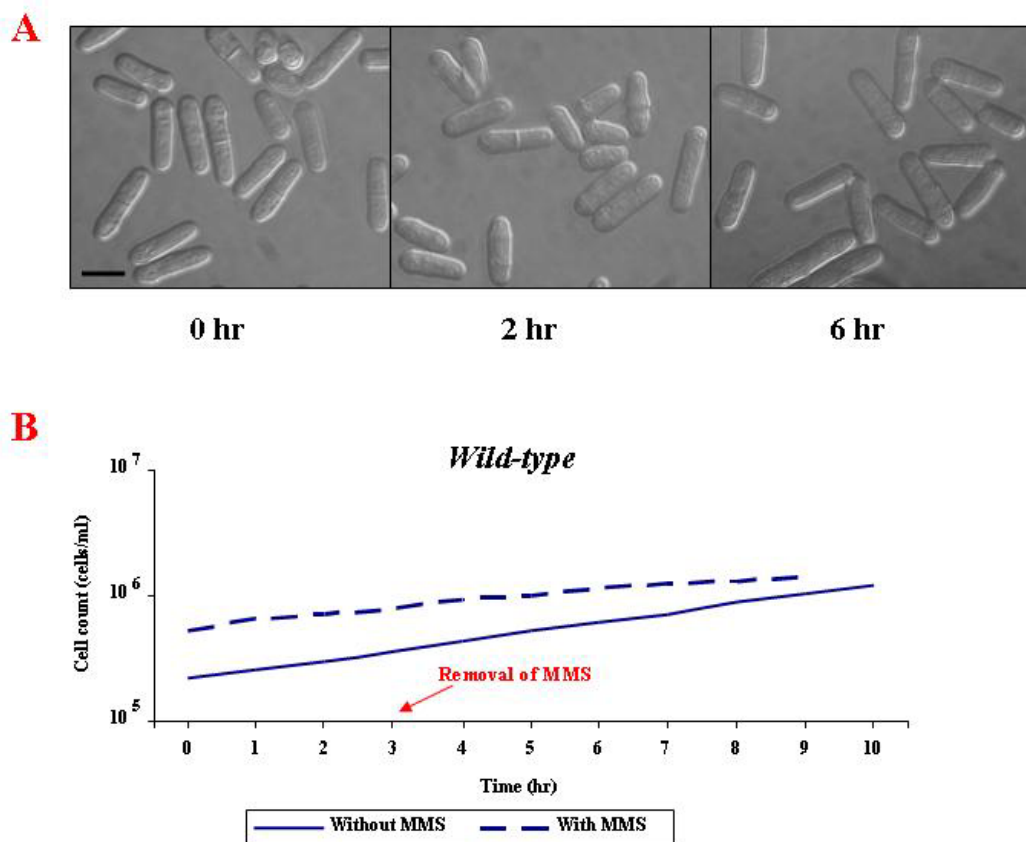




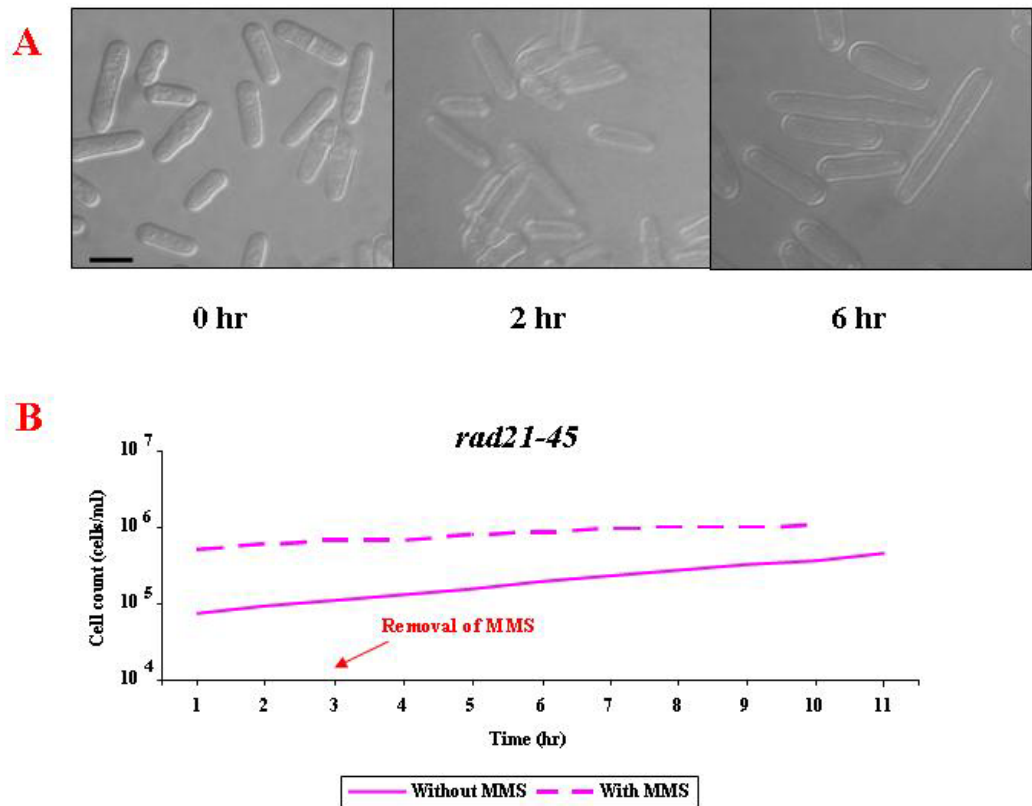
**Figure 5-14: Effect of *rec8*<sup>+</sup> replacing *rad21*<sup>+</sup> on the ability of cells to repair double strand breaks.**

Cells of three strains, wild-type (GG 218), *rad21-45* (GG 921) and *rad21P:rec8<sup>+</sup> nmt1P:rad21<sup>+</sup> "off"* (GG 358) were grown at 25°C to mid-exponential stage in the presence of thiamine; GG 358 was also grown in the absence of thiamine *rad21P:rec8<sup>+</sup> nmt1P:rad21<sup>+</sup> "on"*. Cultures were treated with MMS for 2 hr, before being re-suspended in fresh medium without MMS for 6 hr. Samples were taken before, during and after MMS treatment for analysis by pulse field gel electrophoresis.

**A**, Agarose plugs containing  $1 \times 10^8$  cells  $\text{ml}^{-1}$  were prepared for each sample, loaded into an agarose gel and chromosomes separated by electrophoresis. **B**, Red/green colour intensity gel displaying extent of degradation.

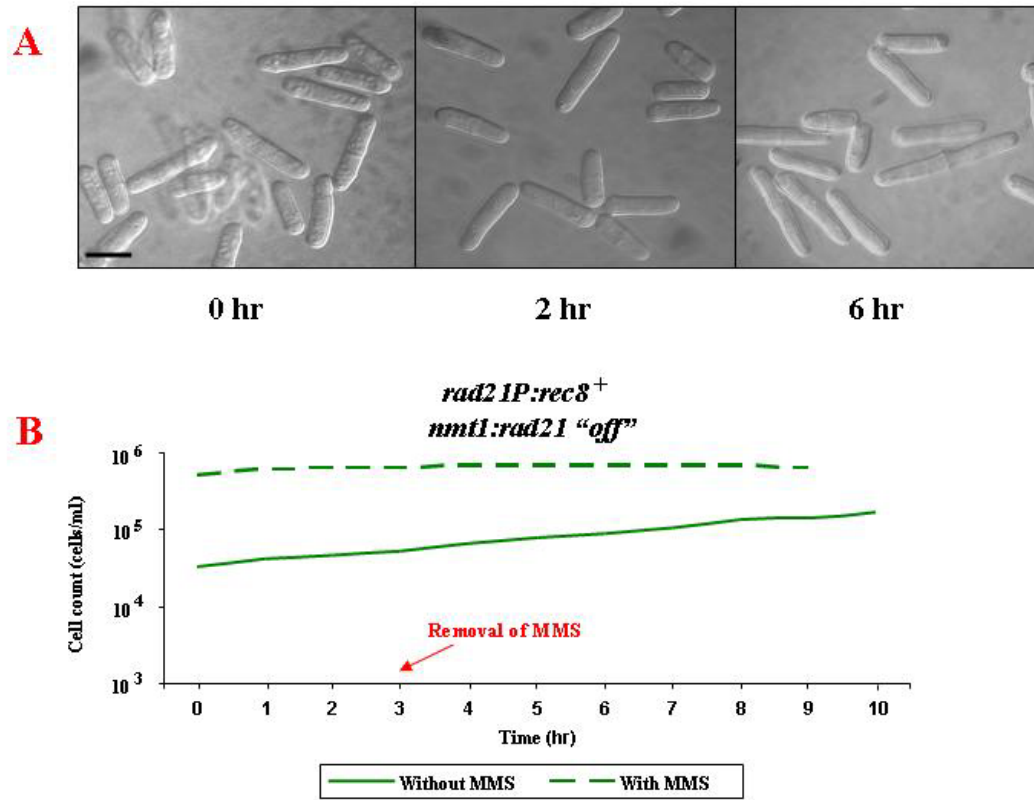


**Figure 5-15: Analysis of the recovery of wild-type cells after treatment with MMS.** Wild-type (GG 218) cells were grown to mid-exponential phase in the presence of thiamine before treatment with 0.05% MMS for 2 hr. Cells were then washed and re-suspended in fresh medium for 6 hr. Samples were taken hourly for cell count readings using a Z series Coulter Counter, and before (0 hr), during (2 hr) and after MMS treatment for microscopic analysis. **A**, Nomarski images, bar 5  $\mu$ m. **B**, Growth curves; for untreated samples (solid line) and MMS treated cells (dashed line). Time of removal of MMS is indicated by the red arrow.



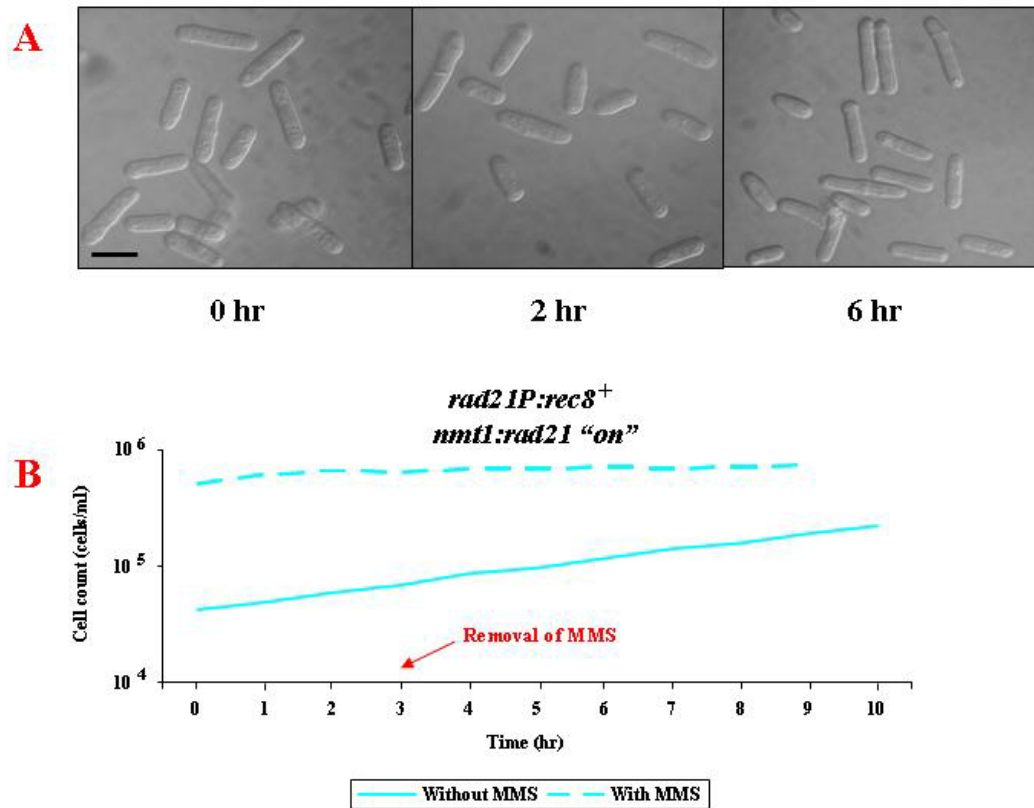
**Figure 5-16: Analysis of the recovery of *rad21-45* cells after treatment with MMS.**

*rad21-45* (GG 921) cells were grown to mid-exponential phase in the presence of thiamine before treatment with 0.05% MMS for 2 hr. Cells were then washed and re-suspended in fresh medium for 6 hr. Samples were taken hourly for cell count readings using a Z series Coulter Counter, and before (0 hr), during (2 hr) and after MMS treatment for microscopic analysis. **A**, Nomarski images, bar 5  $\mu$ m. **B**, Growth curves; for untreated samples (solid line) and MMS treated cells (dashed line). Time of removal of MMS is indicated by the red arrow.



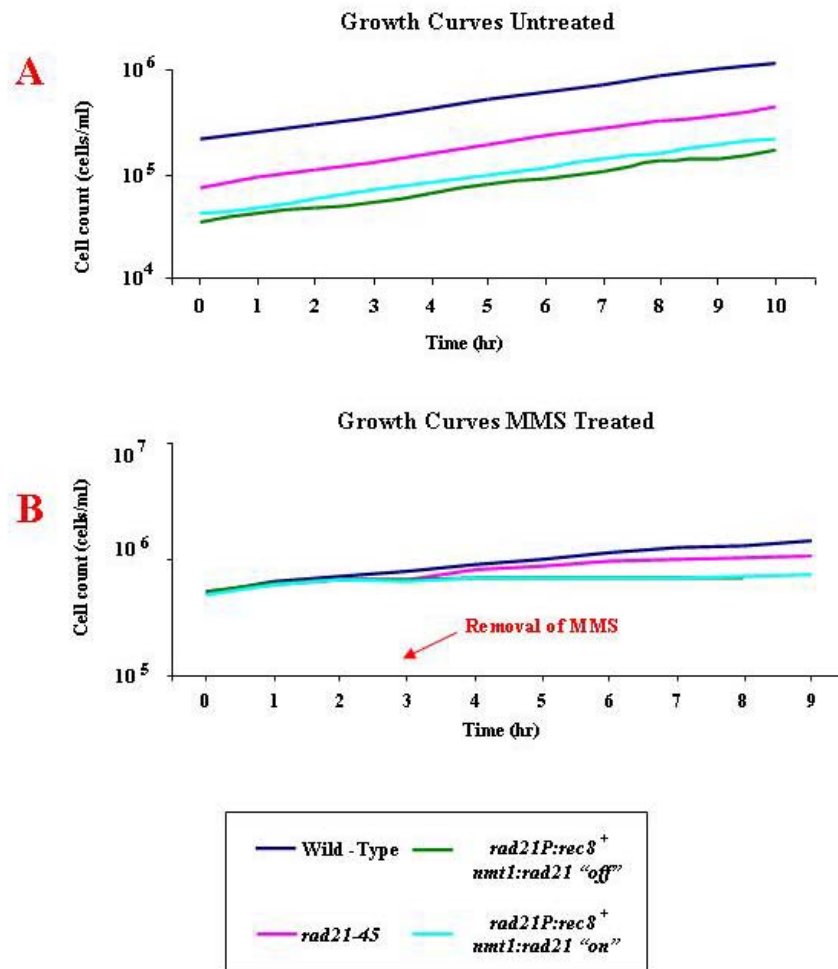
**Figure 5-17: Analysis of the recovery of *rad21P:rec8<sup>+</sup> nmt1P:rad21<sup>+</sup> "off"* after treatment with MMS.**

*rad21P:rec8<sup>+</sup> nmt1P:rad21<sup>+</sup> "off"* (GG 358) cells were grown to mid-exponential phase in the presence of thiamine before treatment with 0.05% MMS for 2 hr. Cells were then washed and re-suspended in fresh medium for 6 hr. Samples were taken hourly for cell count readings using a Z series Coulter Counter, and before (0 hr), during (2 hr) and after MMS treatment for microscopic analysis. **A**, Nomarski images, bar 5  $\mu$ m. **B**, Growth curves; for untreated samples (solid line) and MMS treated cells (dashed line). Time of removal of MMS is indicated by the red arrow.



**Figure 5-18: Analysis of the recovery of *rad21P:rec8<sup>+</sup> nmt1P:rad21<sup>+</sup>* "on" cells after treatment with MMS.**

*rad21P:rec8<sup>+</sup> nmt1P:rad21<sup>+</sup>* "on" (GG 358) cells were grown to mid-exponential phase in the absence of thiamine before treatment with 0.05% MMS for 2 hr. Cells were then washed and re-suspended in fresh medium for 6 hr. Samples were taken hourly for cell count readings using a Z series Coulter Counter, and before (0 hr), during (2 hr) and after MMS treatment for microscopic analysis. **A**, Nomarski images, bar 5  $\mu$ m. **B**, Growth curves; for untreated samples (solid line) and MMS treated cells (dashed line). Time of removal of MMS is indicated by the red arrow.



**Figure 5-19: Summary of *S. pombe* growth rates in the presence or absence of MMS.**

Three *S. pombe* strains, wild-type (GG 218), *rad21-45* (GG 921) *rad21P:rec8<sup>+</sup> nmt1P:rad21<sup>+</sup> "on"* (GG 358) were grown to mid-exponential phase in the presence of thiamine, and *rad21P:rec8<sup>+</sup> nmt1P:rad21<sup>+</sup> "off"* in the absence of thiamine, before treatment with 0.05% MMS for 2 hr. Cells were then washed and re-suspended in fresh medium for 6 hr. Samples were taken hourly to count cell numbers using a Z series Coulter Counter. **A**, Untreated cells. **B**, MMS treated cells. Time of removal of MMS is indicated by the red arrow.

## 5.5 Comet assay

The comet assay, or single cell gel electrophoresis, was first described by Ostling & Johanson, (1984) and provides a method for evaluating the extent of DNA damage within a single eukaryotic cell. The technique combines DNA gel electrophoresis with fluorescence microscopy, and is based upon the ability of denatured and fragmented DNA to migrate out of the cell under the influence of an electric field. Cells are immobilized in a bed of low melting point agarose and undamaged DNA migrates slower remaining within the confines of the nucleoid when a current is applied. The comet head contains the high-molecular weight DNA while the comet tail is composed of the smaller fragmented DNA. Following gentle cell lyses, samples are treated with alkali to unwind and denature the DNA and hydrolyse sites of damage. The samples are then electrophoresed and stained with a fluorescent DNA intercalating dye such as ethidium bromide before visualisation using a fluorescent microscope.

An attempt was made to apply this to cells to determine the extent of damage caused by MMS in wild-type fission yeast cells, with the intention of using the same method on *rad21-45* and *rad21P:rec8* cells, as another way to determine differences in the two fission yeast cohesins to repair damaged DNA. The significant advantage and primary motivation in attempting to use this method is that it allowed the quantification of DNA damage, which would allow quantifying the differences between the *rad21*<sup>+</sup> and *rec8*<sup>+</sup> cohesins. The comet assay would complement the northern experiments since it was expected that higher levels of DNA damage caused by increasing MMS concentrations, would result in induction of *rad21*<sup>+</sup> mRNA. In addition to this, it would also have confirmed that cell death was more prevalent in those cells where only *rec8*<sup>+</sup> mRNA was present. The PFGE experiments showed chromosome fragmentation, thus the comet assay in this context would have served as an accompanying tool to quantify the extent of damage caused with increasing MMS concentrations. Unfortunately, after several attempts and variations of protocol no results were obtained. The protocols used are described below.

### 5.5.1 Standard procedure

An initial standard procedure for alkaline yeast comet assay was applied (Miloshev *et al.*, 2002). Wild-type untreated cells were grown to mid-log phase and collected by centrifugation - 3 mins for 3000 rpm. Cells were washed with 1 ml S buffer (1 M sorbitol,

25 mM phosphate buffer, pH 6.5). Supernatant was discarded and the cells re-suspended in S buffer to give approximately  $10^4$  cells  $\text{ml}^{-1}$ . Aliquots of cells were mixed with low melting point agarose (Cambrex Seaplaque GTG Agarose) made in PBS pH 6.5 to give a final concentration of 0.7%, the gel contained spheroblasting enzyme Zymolyase ( $10 \text{ mg ml}^{-1}$ ). The cells with low melting agarose and Zymolyase were spread as micro gels on standard microscope slides. The slides were covered with cover slips and placed at  $4^\circ\text{C}$  for 5 mins. The cover slips were gently removed. Micro gels were then incubated at  $37^\circ\text{C}$  for 20 mins for spheroplasting. After incubation the slides were submerged in lysis solution (1M NaCl, 50 mM EDTA, pH 8, 30 mM NaOH, and 0.1% N-lauryl sarcosine) for 1 hr at  $4^\circ\text{C}$ . Following lysis the slides were submerged in denaturing solution final pH 12.4 (30 mM NaOH, 10 mM EDTA pH 8) for 20 mins at  $4^\circ\text{C}$ , with this denaturation step repeated three times. The denaturing solution is also the alkaline running buffer and electrophoresis was carried out for 15 mins at  $0.6 \text{ V cm}^{-1}$ . After electrophoresis, the micro gels were neutralised by submerging the slides in 10 mM Tris-HCl pH 7.5 for 10 mins, and then for a further 10 mins in 75% ethanol and 95% ethanol. The micro gels were left to air dry, stained with ethidium bromide ( $1 \text{ mg ml}^{-1}$ ) and visualised using a fluorescent microscope.

With this initial approach the agarose constricted after air drying and by the time the washes were completed the agarose had slid off the slide. As up to twelve slides were made to take agarose constricting and coming away from slides into account, those that did retain the agarose were subjected to electrophoresis. However, it appeared that the DNA did not leave the cell, thus raising three issues: (1) incomplete spheroblasting; (2) too many cells present to be electrophoresed and; (3) the gels were not electrophoresed long enough. To address the latter issue, slides were electrophoresed for longer. However, this could only be done for a certain period ( $\sim 35$  mins) before the micro gel melted in the tank buffer.

### **5.5.2 Variation one**

Since one of the problems was micro gels constricting, and also the gel sliding off the slide this issue was addressed first. Comet slides obtained from Trevigen were used; these have a coating to enhance agarose adherence, however even with the use of the specialised slides, the agarose still constricted and slid off.

An alternative approach was to use a standard microscope slide, which was completely submerged in a 50 ml centrifuge tube containing melted agarose; immediately after



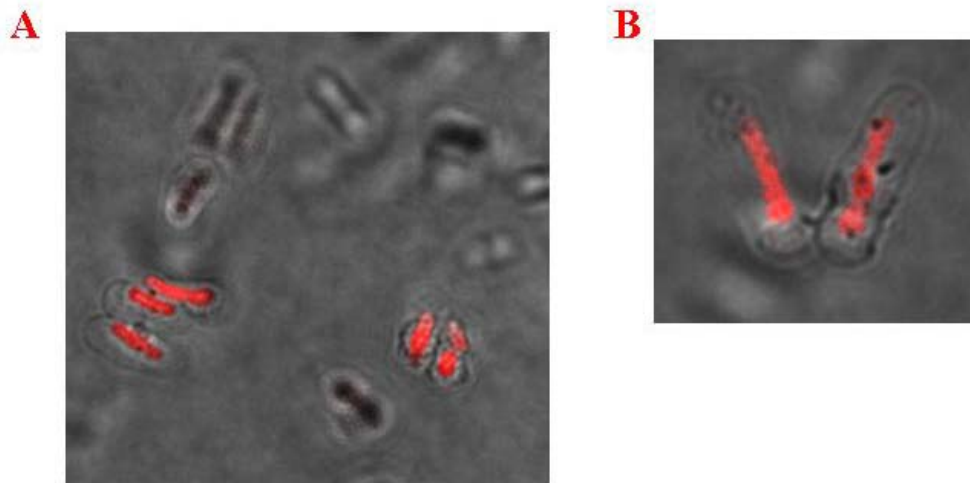
submersion the slide was taken out and left to air dry. This second approach solved the problem of constricting gels.

### **5.5.3 Variation two**

After making the slide with the agarose base, the remaining issues of incomplete spheroblasting and electrophoresis times were addressed. In this protocol multiple slides were prepared to take into consideration the various run times and whether spheroblasting should be carried out before or after application to the slide.

Two different cell counts were used, the recommended  $10^4$  cells  $\text{ml}^{-1}$  and a 1:10 dilution, to identify whether the number of cells electrophoresed posed a problem. For spheroblasting, two approaches were undertaken, firstly the standard protocol was applied to both cell populations and, secondly, cells were spheroblasted prior to application on the slides. Spheroblasting the cells before application to microscope slides may reduce the likelihood of incomplete spheroblasting.

Regardless of electrophoresis times or time of spheroblasting, no results were obtained. A standard image seen using the microscope with the variations in procedure can be seen in Figure 5.20. The resulting image appears to show that the DNA has not left the cell. This may be due to incomplete spheroblasting or the micro gel not being electrophoresed long enough. In view of these factors and taking into account the current differences in protocol further alterations could possibly include lower dilutions of cells being electrophoresed or using lower percentages of micro gels.



**Figure 5-20: Typical image obtained with comet assay.**  
A, Fluorescent image obtained after ethidium bromide staining of wild-type untreated cells. B, Dividing mitotic cell with DNA stained with ethidium bromide.

## 5.6 Summary

In this Chapter, northern blot analyses were carried out to examine the induction of *rad21*<sup>+</sup> and *rec8*<sup>+</sup> genes following MMS treatment. The results obtained coincided and agreed with those obtained from experiments shown in Chapter 4. For MMS, cell death became more pronounced at concentrations above 0.1%. As evident from the northern analyses at MMS concentrations of 0.05% and 0.1% *rad21*<sup>+</sup> induction was seen, and PFGE revealed increased DNA damage at the higher concentrations. The concentration of 0.5% MMS selected as an upper limit for experiments consistently showed cell death as seen across all the experiments carried out from the qualitative/quantitative data, decreased mRNA levels and PFGE.

An MMS concentration of 0.05% was chosen for further analysis as this caused increased level of *rad21*<sup>+</sup> mRNA as revealed in the northern analyses. This induction of *rad21*<sup>+</sup> transcription was shown to be a direct effect of the addition of MMS, as mRNA levels increased as early as 1 hour in cells arrested at G2-M.

0.05% MMS was selected for the PFGE repair assay and, as expected, at this concentration wild-type cells repaired their chromosomes after a six hour recovery period, with cells restoring a wild-type appearance and growth rate. In contrast phenotypic analysis of the *rad21-45* mutant after MMS treatment, and after the same recovery period, showed that cells were unable to repair their chromosomes and displayed an elongated phenotype, characteristic of cell cycle arrest and G2 checkpoint activation. These observations are consistent with the fact that *rad21-45* activates a checkpoints and is capable of cell cycle arrest but proceeds into the next stage of the cell cycle without repairing its DNA (Birkenbhil & Subramani, 1995). Similarly, the repressible *rad21*<sup>+</sup> strain in the presence of thiamine expressing only *rec8*<sup>+</sup> was unable to repair its chromosomes after the same recovery period; and phenotypic analysis revealed an elongated phenotype and that growth in culture was not maintained with cell numbers decreasing constantly over the 6 hour recovery period.

Thus, all the presented data showed that the DNA repair specific function of SpRad21p is not conserved in the meiotic cohesin SpRec8p. Even when *rec8*<sup>+</sup> mRNA levels are present in the cell after DNA damage, SpRec8p is not utilised for repairing damaged DNA.

## Chapter 6

### 6.1 Introduction

The fission yeast *rad21*<sup>+</sup> and *rec8*<sup>+</sup> genes are a pair of homologous genes that encode proteins which form part of a four subunit complex called cohesin; expression of each is restricted to either mitosis or meiosis respectively, with peak transcript levels occurring during DNA replication in both life cycles. Before a cohesive role was assigned to *rad21*<sup>+</sup>, it was identified and recognized for its role in DNA repair (Birkenbihl & Subramani, 1992); later sequence data analysis showed it to be similar to the budding yeast MCD1/SCC1 gene and it was shown to encode a component of the cohesin complex (Guacci *et al.*, 1997; Michaelis *et al.*, 1997). The *rec8*<sup>+</sup> gene in comparison was first identified in a screen for mutants with decreased recombination at the *ade6*<sup>+</sup> locus, and subsequently shown to be the meiotic equivalent of the *rad21*<sup>+</sup> gene (De Veaux *et al.*, 1992; Parisi *et al.*, 1998).

To address the mitotic and meiotic-specific functions of *rad21*<sup>+</sup> and *rec8*<sup>+</sup>, the upstream promoter regions of each gene were interchanged so that they would be ectopically expressed during the alternative life cycle, both at physiological levels and at the appropriate cell cycle time. As a result, *rad21*<sup>+</sup> was expressed in the meiotic cycle and *rec8*<sup>+</sup> expressed in the mitotic cycle. This thesis focussed primarily on the mitotic cohesin SpRad21p in the mitotic cycle although some experiments were carried out studying SpRec8p function in meiosis (Appendix I).

Previous experiments in the laboratory revealed that ectopically expressed SpRec8p in mitosis was able to maintain normal physiological growth with no affect on S phase progression (Riddell, 2003). Similarly, expression of SpRad21p in meiosis at physiological levels, enhanced spore viability and ascospore morphology when compared to a *rec8Δ*, also with no affect on S phase. These initial data indicated that, at least under these unperturbed conditions, the two cohesin genes have overlapping and related functions. This study expanded and focussed on these observations by examining the damage specific function of the mitotic cohesin SpRad21p, to elucidate whether the meiotic cohesin SpRec8p has these properties.

## 6.2 Chapter 3:- *rad21*<sup>+</sup> and *rec8*<sup>+</sup> have life cycle specific expressions

Chapter 3 introduced the *rad21*<sup>+</sup> and *rec8*<sup>+</sup> cohesin subunits, emphasising their similarities and life cycle specific expression patterns. Table 1 summarised the basic properties of the two cohesins: for example, SpRad21p has a role in DNA double strand break (DSB) repair and microtubule function whilst, in contrast, SpRec8p has roles in linear element formation, alignment of homologous chromosomes and homologous recombination. Their fundamental similarities are also listed, with both expressed at the start of S phase and under the control of the MBF-MCB (DSC1) transcription system which recognises the specific *Mlu I* sequence “ACGCGT”, a consensus DNA sequence motif present in the promoters of both *rad21*<sup>+</sup> and *rec8*<sup>+</sup> genes. Furthermore, within their respective cycles, both subunits have roles in regulating chromosome separation and are targeted for proteolysis at anaphase by the APC/C, thus enabling chromosome segregation.

Of particular focus in this chapter was to highlight the life cycle specific properties of the two subunits. To do this northern blot analysis was performed to study the transcriptional profiles of the two genes. Mitotic analysis indicated the presence of a single transcript for *rad21*<sup>+</sup> of 1.8 kb (Figure 3.2), whilst analysis of the meiotic samples indicated at least two transcripts expressed during meiosis for *rec8*<sup>+</sup> which were specifically regulated. The expected *rec8*<sup>+</sup> transcript is 1.7 kb; however, an additional smaller mRNA was detected which may be an isoform of the gene or reflect non-specific hybridization of the probe DNA (Figure 3.3). As another experiment to confirm that both subunits were life cycle specific, and to allow a direct comparison of mRNA levels in the synchronous cells, samples corresponding to peak mRNA levels of each gene were run on the same gel for northern blotting (Figure 3.4). The resulting blot was consecutively probed for both *rad21*<sup>+</sup> and *rec8*<sup>+</sup>. This analysis confirmed that the two cohesins are specifically expressed in either mitosis or meiosis, respectively. In addition, control hybridisations of mitotic RNA with the *rec8*<sup>+</sup> probe and meiotic RNA with the *rad21*<sup>+</sup> probe, following by prolonged exposure to an autoradiogram revealed no signal, thus confirming the specific expression of both genes to one life cycle and not the other.

### 6.3 Chapter 4:- Qualitative and quantitative analysis following treatment with UV, MMS and phleomycin

The data presented in Chapter 3 formed an important basis for this thesis confirming the life cycle specific expression of the two cohesin subunits in fission yeast. Following these observations this study aimed to investigate whether the specific DNA damage repair function of the *rad21*<sup>+</sup> gene was possessed by the *rec8*<sup>+</sup> gene. To accomplish this three strains were used: a wild-type strain containing the normal unaltered copy of the *rad21*<sup>+</sup> gene (GG 218), and thus the experimental control; a mutant, *rad21-45* (GG 921); and a *rad21P:rec8*<sup>+</sup> *nmt1P:rad21*<sup>+</sup> (GG 358) repressible strain grown in the presence (*nmt1P* “off”) or absence of thiamine (*nmt1P* “on”).

The *rad21-45* mutant strain has a single base pair change from wild-type isoleucine at position 67 into threonine making the mutant sensitive to DNA damage and deficient in the DSB repair system (Birkenbhil & Subramani, 1995). The *rad21P:rec8*<sup>+</sup> *nmt1P:rad21*<sup>+</sup> allowed the specific expression of *rec8*<sup>+</sup> during mitosis. As *rad21*<sup>+</sup> is an essential gene it was placed under the control of the regulatable *nmt1*<sup>+</sup> promoter so that its expression could be repressed allowing the sole expression of *rec8*<sup>+</sup> in this strain. .

Chapter 4 described a series of experiments that investigated the effect of different damaging agents when added to these three strains. Cell viability was studied after treatment with the pyrimidine cross-linker UV-C, the alkylating agent MMS, and the glycopeptide phleomycin, the latter to mimic the effects of ionizing radiation and induce DSBs. For each of the three strains, viability assays to obtain qualitative analysis and quantitative data were carried out.

The general trend seen by administering increasing concentrations of all three damaging agent was a decrease in cell viability in all three strains, but with the wild-type control most resistant, followed by the *rad21-45* mutant, and the *rad21P:rec8*<sup>+</sup> *nmt1P:rad21*<sup>+</sup> strain most sensitive. Cells were grown exponentially and either serial dilutions spotted on solid medium for qualitative analysis, or a <sup>1</sup>/<sub>1000</sub> dilution plated for quantitative analysis; under both conditions EMM with or without thiamine was used to either repress or induce *rad21*<sup>+</sup> in *nmt1* promoter strain.

For UV-C treatment, cells were spotted and plated on solid media, and data demonstrated highest cell death occurring at 150 J (Figures 4.2 and 4.7).

In MMS treated cells survival experiments were carried out at two concentrations: in the first experiments MMS was added directly to solid media and this revealed that cell viability was most affected at a concentration of 0.006%. MMS data were also collected when MMS was added to liquid media and this instead showed viability decreased at a higher concentration of 0.3% (Figures 4.8, 4.9 and 4.14).

In experiments studying the effect of phleomycin this was added directly to solid media for both qualitative and quantitative analyses, and these data showed highest cell death at  $20 \mu\text{g ml}^{-1}$ . However, a surprising result was seen when phleomycin was added to the SpRad21p repressible strain both in the presence (*nmt1P* “off” – SpRec8p expression) or absence of thiamine (*nmt1P* “on” SpRad21p and SpRec8p expression). The strain in which SpRad21p was expressed along with SpRec8p showed viability decreasing at  $10 \mu\text{g ml}^{-1}$ . This observation was only seen with phleomycin and not with the other DNA damaging agents. This unexpected result might be explained by considering the type of DNA damage that phleomycin induces, which is DSBs. *nmt1P* is the strongest of the thiamine promoters with 300 fold expression. Possibly, such high levels of SpRad21p in addition to DSBs are toxic for the cell, thus reducing cell viability (Figures 1.15 and 1.20).

The fourth damaging agent used was ionizing radiation which causes DNA DSBs. Serial dilutions of all strains were spotted onto solid EMM plates and irradiated with a  $^{60}\text{Co}$  source. Unfortunately, no difference was seen even after increasing doses were applied. This was because the only source of ionizing radiation available was at  $2 \text{ Gy min}^{-1}$  which was too weak; as there was no other convenient  $^{60}\text{Co}$  source to use, no further work could be carried out using this DNA damaging agent (Figure 4.22).

Whilst Chapter 4 looked at the effects of three damaging agents, determining the extent of damage caused and sensitivity of each strain, Chapter 5 examined in more detail the affect of MMS. The transcriptional profiles and chromosome integrity was investigated in each of the strains after MMS treatment.

## 6.4 Chapter 5:- Transcriptional profiles and chromosome integrity experiments following treatment with MMS

Chapter 5 sought to further investigate the affects of increasing MMS concentrations on *rad21*<sup>+</sup> and *rec8*<sup>+</sup>. Here, the affects were analysed by examining the transcriptional profiles of the two cohesin genes in response to MMS by northern blotting and monitoring chromosome integrity by PFGE.

### 6.4.1 Transcriptional profiles

Six concentrations of MMS at 0.005%, 0.05%, 0.1%, 0.3%, 0.5% in liquid medium were used with samples collected hourly over a four hour time period. Interestingly, data revealed that in both wild-type and *rad21-45* cells *rad21*<sup>+</sup> mRNA increased 2 hours after MMS addition, but not *rec8*<sup>+</sup> mRNA (Figures 5.2 and 5.3).

To further examine this interesting observation, and to show that induction was indeed a specific response to the addition of MMS and not due to an accumulation of cells at G1, the temperature-sensitive mutant *cdc25-22* (GG 185) was used to cause a cell cycle arrest at G2-M. 0.05% MMS was added to the cultures and samples taken before and after G2-M arrest, at 0, 1 and 2 hours. Cells arrested at G2-M revealed *rad21*<sup>+</sup> mRNA levels increasing after just 1 hour following the addition of MMS (Figure 5.4). This observation confirms that the rapid induction of *rad21*<sup>+</sup> was a direct result of the addition of the MMS and not due to cells accumulating at G1. This is a striking result, and so it would be important to repeat this experiment with phleomycin to examine if a similar rapid response is seen after the addition of a different DNA damaging agent that causes DSBs. If a similar response was observed this would suggest that this gene induction is due to the presence of damaged DNA. If this is the case this implies that fission yeast may have a transcriptional response mechanism that controls expression of *rad21*<sup>+</sup> under DNA damage conditions.

The *rad21P:rec8*<sup>+</sup> *nmt1P:rad21*<sup>+</sup> strain in the presence and absence of thiamine was then treated with MMS at the same concentrations. In the presence of thiamine *rad21*<sup>+</sup> mRNA was not induced, but instead was replaced with *rec8*<sup>+</sup> transcript. Here, despite the presence of *rec8*<sup>+</sup> mRNA and by inference SpRec8p protein, cell viability decreased, suggesting that SpRec8p was not repairing damaged DNA (Figure 5.5). When the strain was then cultured in the absence of thiamine and then treated with the same concentrations of MMS both



*rad21*<sup>+</sup> and *rec8*<sup>+</sup> transcripts were present, although *rad21*<sup>+</sup> was present at constant levels (Figure 5.6). Although viability did decrease under these conditions, cell survival levels improved in comparison to when the same strain was grown in the presence of thiamine, however survival levels were much lower when compared to wild-type. This may be due to the reasons mentioned earlier in this Discussion, described in detail in Section 4.7.

### **6.4.2 Chromosome integrity**

Northern blot analysis demonstrated that *rad21*<sup>+</sup> and not *rec8*<sup>+</sup> mRNA was induced when MMS was added. The second set of experiments in this chapter investigated by PFGE analysis whether increased expression of *rad21*<sup>+</sup> was utilised in DNA repair. Data from Chapter 4 showed cell viability decreasing at concentrations above 0.1% MMS in liquid culture and northern data revealed decreased *rad21*<sup>+</sup> transcript levels at this concentration. Therefore, four initial concentrations were selected: 0%, 0.05%, 0.1%, and an upper limit of 0.5%. An increase in *rad21*<sup>+</sup> transcript and levels of *rec8*<sup>+</sup> transcript were present up to 0.5%, however above this concentration no transcript for either gene was detectable. *S. pombe* has three chromosomes of 3.5, 4.7 and 5.7 Mb in size. Should any damage be encountered under normal conditions it is overcome due to the existence of an efficient DNA damage repair mechanism; SpRad21p appears to be a factor involved in controlling this process (Birkenbihl & Subramani, 1992). By employing PFGE the specific function of *rad21*<sup>+</sup> was investigated in more detail. However, a potential problem using this experimental approach is that PFGE in combination with MMS treatment generates fragmented chromosomes (Lundin *et al.*, 2005). Therefore, in the PFGE experiments presented in this thesis a recovery period was allowed before pulse field treatment, which overcame the problem of heat labile DNA sites.

Optimisation of PFGE experiments revealed the extent of chromosome degradation caused by the MMS concentrations used, with gels showing fragmented DNA as higher mobility smears. From these experiments, the lowest MMS concentration of 0.05% was selected for a recovery assay, in which samples were taken before (0 hours), during (2 hours) and after treatment (6 hours). After the six hour recovery period, the only strain to recover its three chromosomes was wild-type, which also resumed a normal growth pattern. In contrast the *rad21-45* mutant cells and the *rad21P:rec8*<sup>+</sup> *nmt1P:rad21*<sup>+</sup> repressible strain, both in the presence and absence of thiamine, failed to recover their three chromosomes. This latter strain was more sensitive when only SpRec8p was expressed, with cell numbers decreasing constantly over the 6 hour period.

Microscopic analysis of all three strains after the recovery period demonstrated that the wild-type MMS treated cells restored a wild-type phenotype, whilst the other two displayed an elongated phenotype characteristic of cell cycle arrest and G2 checkpoint activation. These data coincide with the observation that although *rad21-45* activates a checkpoint and arrests cells in G2, progression into the next stage of the cell cycle continues without the repair of DNA (Birkenbihl & Subramani, 1995).

Therefore, the data presented in Chapters 4 and 5 suggest that the DNA repair specific role of SpRad21p is not conserved in the meiotic cohesin SpRec8p. Despite the presence of *rec8*<sup>+</sup> mRNA at physiological levels in these cells this meiotic cohesin was not used for repairing damaged DNA.

The overall conclusion drawn from the experiments presented in this thesis are that the function of DNA repair is specific to the mitotic cohesin SpRad21p. This study has described a fundamental and significant difference between SpRad21p and SpRec8p. In the next Section further approaches are described to characterise this observation.

## 6.5 Future directions

The experiments described in Chapters 4 and 5 revealed a decrease in cell survival using the three damaging agents, UV-C, MMS and phleomycin. Interesting results were observed in strains where *rec8*<sup>+</sup> was expressed in mitosis, with *rad21*<sup>+</sup> under *nmt1*<sup>+</sup> control repressed in the presence of thiamine in the medium. An important experiment to confirm this analysis of the mitotic cohesin would be to repeat all these experiments with *rad21Δ* strain. Using this strain would not only confirm the absence of *rad21*<sup>+</sup> mRNA and protein cells expressing *rec8*<sup>+</sup>, but also overcome the potential problem arising from over-expression of SpRad21p.

Equally intriguing and important would be to repeat northern blot and PFGE experiments with phleomycin, since this glycopeptide is known to mimic the effects of  $\gamma$ -radiation, causing DSBs. The use of phleomycin would examine more effectively the direct involvement of the two cohesins in recombinational repair.

### **6.5.1 Induction of cohesin subunits as a result of DNA damage**

The *S. pombe* SpRad21p cohesin subunit was initially identified as a protein involved in the recombination DNA repair pathway (Birkenbihl & Subramani, 1992). Subsequent work in budding yeast revealed that *rad21*<sup>+</sup> had sequence homology to budding yeast MCD1/SCC1 and thus a cohesive function was attributed to SpRad21p (Guacci *et al.*, 1997; Michaelis *et al.*, 1997). The other *S. pombe* cohesin subunits were identified by Tomonaga and co-workers (2000).

Recent work in budding yeast has assigned a DNA repair role to the cohesin complex, perhaps by holding the sister chromatids together, and thus aiding the process of recombination repair. Of particular relevance to this work is that mutants of the SCC1 subunit in budding yeast, chicken and humans are all sensitive to ionizing radiation (Section 1.16.8). Ünal *et al.* (2007) demonstrated that *SCC1* transcription is induced alongside the induction of DSBs. Transcription profile data in this thesis have suggested that this may also be occurring in *S. pombe* (Figures 5.2 and 5.4). The fact that cells expressing *rec8*<sup>+</sup> instead of *rad21*<sup>+</sup> show reduced viability after DNA damage, and cannot repair their chromosomes, suggests that this function is specific to SpRad21p.

Data from budding yeast have also shown that the ScEco1p, which is necessary for establishing cohesion during S phase, is found to increase in response to DNA damage (Ström *et al.*, 2004; Ünal *et al.*, 2007). Further work within this context could examine if fission yeast SpEso1p behaves in a similar manner. In addition, SpRad21p co-localises with SpMis4p to chromosomes, and indeed the presence of SpMis4p is necessary for the association of the cohesin complex with chromosomes (Furuya *et al.*, 1998). Thus it would be interesting to see whether *mis4*<sup>+</sup> is also induced when DNA is damaged. Relating this knowledge back to this study FOCI experiments might show if ectopically expressed SpRec8p is co-localising with SpMis4p, and whether one reason why SpRec8p cannot repair DNA in mitotic cells is because it cannot interact with the same factors as SpRad21p. Experimentally this might be examined by using protein binding assays such as co-immunoprecipitation and pull down methods along with MALDI-TOF technologies. These methods might reveal if SpRec8p is able to bind to the same proteins as SpRad21p in mitotic protein extracts.

### **6.5.2 Fluorescence activated cell sorting (FACs) analysis**

*S. pombe* G2 comprises the greatest portion of the mitotic cell cycle and it is possible that this phase is prolonged or shortened with increasing concentrations of DNA damaging agents. Similarly, the time that cells spend in G1 and S phase may be affected. It is even possible that differences might be detectable in cell cycle progression following treatment with different damaging agents. A method to detect changes in the time cells spend in the various stages of the cell cycle is fluorescence activated cell sorting (FACS) which measures the DNA content of individual cells: cells in G1 have a 1 C content, while those in G2 have a 2 C content. Therefore FACS could be used to examine cell cycle progression in the three strains after DNA damage.

### **6.5.3 Induction of damage in synchronous samples**

In this study, all experiments were carried out on asynchronous samples. G2 phase is known to be the period during which recombinational repair has the opportunity to take place. Thus it would be interesting to see the effects of transcript levels, protein levels and in turn strain viability, if DNA damage with each of the respective agents occurred at specific points of the cell cycle using synchronous cell populations for each of the three strains used. This could be achieved by either using fission yeast temperature sensitive *cdc* mutants arrested at various stages of the cell cycle before treatment with DNA damaging agent, or in wild-type cells synchronised for division by centrifugal elutriation.

### **6.5.4 Mutation analysis of promoters and ORFs**

The generation of truncated proteins and point mutations of SpRad21p and SpRec8p, may disclose the domains specific to cohesin function, and if any sites overlap, between the two proteins within their respective conserved regions. This work could possibly provide functional insights of the non-conserved regions, and whether these are the regions either conferring the exclusive properties, or if they are involved in the associations of SpRad21p and SpRec8p with other function specific proteins. A number of proteins have been identified that interact with either cohesin and each of these could be tested with this modified form of the cohesins.

#### 6.5.4.1 Promoter analysis

The consensus DNA sequence motif 5' –ACGCGT– 3' or MCB element is present in the promoter region of all MCB-regulated genes, which undergo periodic transcription at the G1-S boundary, either in mitosis or meiosis. The differences in position and arrangement of MCB elements in the promoters may reflect cell cycle specific transcriptional regulation. (Figure 3.1). MCB motifs within the promoters fall into three main categories (Cunliffe *et al.*, 2004). Type I/Type II confer meiotic specific gene expression. Type I contain a single 5' –ACGCGT– 3' motif within 1 kb of the ATG gene and type II contain several copies of this single motif within their promoters. The *rec8*<sup>+</sup> gene has the type II MCB promoter (Figure 3.1B). Type III MCB motifs include both mitotic and meiotic genes. This motif group contains one double MCB motif and may also have a single MCB element at a distance from the double motif. The *rad21*<sup>+</sup> gene falls into this category (Figure 3.1A). Type IV MCB motifs contain complicated arrangements of several MCB motifs. At least one double and one single motif can be found within the genes promoters.

Of particular relevance to this study are the type I/II and type III elements which are found in the promoters of *rec8*<sup>+</sup> and *rad21*<sup>+</sup>, respectively. With the knowledge that the two cohesin promoters contain different MCB type elements, an attractive experiment to undertake would be to identify the exact region within the *rad21*<sup>+</sup> promoter region necessary for mitotic DNA damage induction, and whether this is missing from the *rec8*<sup>+</sup> promoter. This could be done by cloning small fragments from the *rad21*<sup>+</sup> promoter into a heterologous reporter construct to determine whether they can confer transcription after DNA damage. Once this sequence was identified, to confirm its mitotic specificity it could be introduced into the promoter of *rec8*<sup>+</sup> to determine if this would confer mitotic gene expression to this gene following DNA damage.

Work in this thesis has shown that *rad21*<sup>+</sup> expression is specifically and rapidly induced after DNA damage. This implies that a novel DNA damage responsive transcription machinery operates to make this occur. Therefore, another interesting avenue of research following the observation that *rad21*<sup>+</sup> mRNA levels increased in response to MMS damage would be to identify the transcription factors involved, and whether levels of these are induced in response to damage. This could be done using methods such as electrophoretic mobility shift assays. Here, the promoter fragment identified by reporter assays to confer DNA damage responsive transcription would be radioactively labelled and mixed with fission yeast protein extracts from cells after DNA damage. Proteins that bind

to the promoter fragment could be detected by shifts in electrophoretic mobility. Identification of such proteins might also permit their characterisation through biochemical purification and MALDI-TOF technologies.

#### **6.5.4.2 Analysis of ORF**

Analysing the *rad21*<sup>+</sup> and *rec8*<sup>+</sup> ORFs in more detail could suggest specific amino acid sequences or domains which could be investigated further to determine if they are required for DNA repair. Site directed mutagenesis could be used to verify the amino acids necessary for this function in SpRad21p. If these could be identified, a chimeric protein could be constructed by inserting these domains from *rad21*<sup>+</sup> into *rec8*<sup>+</sup> to see if they could confer SpRad21p functions to SpRec8p.

#### **6.5.5 SpRec8p protein and phosphorylation status**

An important area to further study would be the analysis of SpRec8p protein levels and status. The most immediate way to do this would be to raise antibodies against the SpRec8p protein, which would allow a number of experimental approaches, including western blotting. Western blotting allows the quantification of protein levels and should be utilised to show that the SpRec8p protein in the *rad21P:rec8*<sup>+</sup> *nmt1P:rad21*<sup>+</sup> strain is produced at an equivalent level to native SpRad21p during the mitotic cycle. This would confirm that at physiological levels the two cohesins are functionally interchangeable, at least under normal growth conditions.

Since both SpRad21p and SpRec8p are phosphoproteins, western blotting could determine whether there is any protein modification occurring as a consequence of the damaging agent. Any changes in phosphorylation status suggested by western blot analysis could be confirmed by MALDI-TOF analysis to determine the phosphorylated sites in each protein, which would permit their study through, for example, mutation.

#### **6.5.6 Localisation and Chromatin immuno-precipitation (ChIP)**

In Chapter 5 experiments were presented that showed that although ectopically expressed *rec8*<sup>+</sup> was present in cells, repair did not occur (Figure 5.5). Further work using fusion proteins of GFP with the two cohesin subunits, might illustrate if the expressed proteins are

localising to the correct regions within the cell. Furthermore, ChIP assays could reveal the chromatin sites on which they are loaded in a damaged and non-damaged environment.

### **6.5.7 Meiotic experiments**

#### **6.5.7.1 DNA damage**

Expression of the mitotic cohesin in the alternative meiotic cycle would give further insight into the behaviour of the cohesins. DSBs are a natural occurrence in the meiotic cycle for the purpose of recombination to generate genetic diversity. It would be interesting to see if ectopic expression of SpRad21p in the meiotic cycle prevents meiotic specific recombination events from occurring, since SpRad21p is known to be involved in the process of repair of DSBs rather than utilising them.

#### **6.5.7.2 Chromosome segregation**

A specific function of the meiotic SpRec8p cohesin is its involvement in accurate segregation during meiosis. Riddell (2003) showed that SpRad21p was able to restore normal ascus development: using DAPI to measure nuclear DNA content it was shown there was an increase in spore viability when compared to a *rec8Δ* meiosis. Meiotic work undertaken in this study to further these initial observations involved labelling the centromere of chromosome 1 with a fluorescent tag to allow a detailed analysis of chromosome segregation to understand whether SpRad21p when expressed during meiosis allowed normal chromosome segregation to occur.

This approach involved exploiting the principles of the bacterial LacO-LacI system, whereby the LacI repressor protein binds to the LacO repeats (Nabeshima *et al.*, 1995; 1998). The original parental strain (GG 484) a gift from Dr Pascal Bernard, (University of Bordeaux, France), contained a series of LacO repeats integrated into the *lysI*<sup>+</sup> site, located close to the centromere of chromosome 1 (*cen1*). A fusion gene encoding GFP-LacI tagged with a nuclear localisation signal (NLS) placed under the control of the *disI*<sup>+</sup> promoter was integrated at the *hisI*<sup>+</sup> site. When the expressed GFP-LacI-NLS protein enters the nucleus to bind specifically to the LacO repeats this enables visualisation of the movements of *cen1* in *S. pombe* cells. The strains used for the meiotic experiments, were created via a series of matings with GG 484 and are listed in Appendix II.

This method could reveal specific segregation defects occurring when SpRec8p was replaced with SpRad21p during meiosis. Work in vegetative cells showed the system to be working, however, when meiotic work was carried out to assess spore viability, following tetrad analysis even the wild-type tagged strain had a lower viability when compared to *rec8Δ* and cells in which SpRad21p was expressed (Appendix I).

To determine if the observed decrease in viability was due to the treatment of spores during tetrad manipulation or if it was the presence of the GFP protein causing an affect, untagged wild-type strains were mated. Spore viability after tetrad analysis for the untagged strains was 100%. This observation suggests that it might be the presence of the GFP itself affecting viability and not a consequence of tetrad manipulation. Therefore this method was not used further.

An alternative experimental approach to determine accurate segregation could be to use fluorescent in situ hybridisation (FISH) or chromosome painting techniques.

### **6.5.8 General Summary**

Previous work in the laboratory manipulating SpRec8p so it was expressed artificially during the mitotic cycle formed the basis for this study (Riddell, 2003). These results showed that:

- Ectopically expressed SpRec8p at physiological levels and at the appropriate time during the mitotic cell cycle was able to complement the essential function of SpRad21p.
- Replacing SpRad21p with SpRec8p neither caused an increase in cell division time, nor caused a delay in S phase.

The work described in this thesis took these initial observations further by looking at the specific role of the mitotic cohesin SpRad21 and confirmed that while SpRec8p can complement SpRad21p during mitosis in unperturbed cells under normal growth conditions, the specific role of DNA repair is exclusive to SpRad21p. The observations that allow this conclusion are as follows:



- Opening experiments showed that *rad21*<sup>+</sup> is expressed exclusively during the mitotic cell cycle and *rec8*<sup>+</sup> solely during meiotic development.
- Three strains: wild-type, *rad21-45* and *rad21P:rec8*<sup>+</sup> *nmt1P:rad21*<sup>+</sup> were treated with UV-C, MMS and phleomycin, and data showed a gradual decline in cell viability in all strains following treatment with increasing concentrations, although *rad21P:rec8*<sup>+</sup> *nmt1P:rad21*<sup>+</sup> was the most sensitive to DNA damaging agents.
- Further work with MMS and transcriptional profiling of *rad21*<sup>+</sup> and *rec8*<sup>+</sup> mRNA levels in wild-type and *rad21-45* cells revealed an induction of *rad21*<sup>+</sup> expression in response to damage, but not *rec8*<sup>+</sup>. This induction was rapid and not dependent on the cell cycle stage of the cells implying that fission yeast has a specific transcription control mechanism that regulates *rad21*<sup>+</sup> expression under DNA damage conditions.
- However, the repressible strain in the presence of thiamine *nmt1P* “off” showed constant levels of *rec8*<sup>+</sup>. Thus, despite the presence of SpRec8p it is not utilised for repair.
- PFGE experiments studying the recovery of three *S. pombe* chromosomes following DNA damage showed recovery in the wild-type strain only, and not in strains where *rad21*<sup>+</sup> was either mutated or where *rec8*<sup>+</sup> was expressed instead.
- Samples in the same chromosome recovery experiments taken hourly to monitor growth rates showed the wild type strain recovering a normal pattern of growth over the course of the experiment. In comparison the other two strains failed to recover.
- Microscopic analysis of cells taken at equivalent times during the recovery experiment demonstrated wild-type cells having a normal phenotype after the 6 hours, which was not seen in the *rad21-45* nor *rad21P:rec8*<sup>+</sup> *nmt1P:rad21*<sup>+</sup> strains.
- Thus, the chromosome recovery experiments show that SpRec8p when expressed during mitosis does not replace the DNA damage function of SpRad21p, showing a significant functional difference between the two fission yeast cohesins.

In conclusion, in *S. pombe* the mitotic *rad21*<sup>+</sup> and meiotic *rec8*<sup>+</sup> cohesin subunits form a pair of homologous genes which are expressed in alternative cycles. Work presented here has shown that when SpRec8p is expressed during vegetative growth at physiological levels normal unperturbed conditions growth is maintained, and thus a significant overlap in function between the two cohesins exists. However, this study then examined the mitotic specific role of SpRad21p in DNA repair and showed that this is a specific function of this mitotic cohesin, and not the meiotic cohesin subunit *rec8*<sup>+</sup>. Thus significant differences between the two fission yeast cohesins also exist.

Thus this study has formed a fundamental basis for future work with these two cohesin subunits to investigate further why organisms have two homologous cohesin genes with similar but clearly distinct functions.

## Appendices

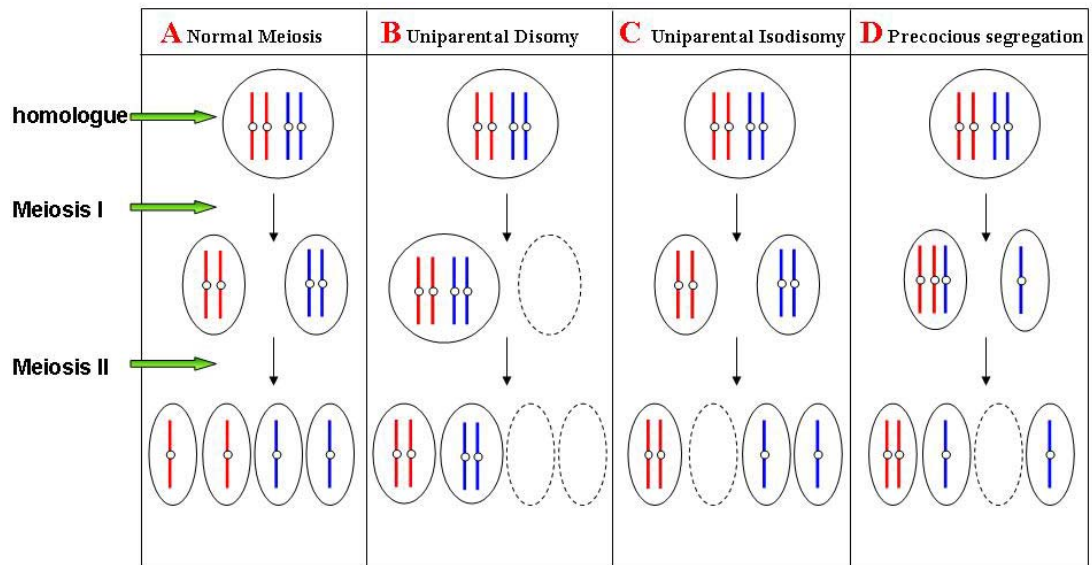
### Appendix I – Meiotic segregation experiments

#### **Analysis of *rec8*<sup>+</sup> to study chromosome segregation during the meiotic cycle. Can SpRad21p replace the function of SpRec8p?**

##### **Introduction**

Cohesins hold sister chromatids together after replication at the metaphase plate, both in mitosis and meiosis. The links are then destroyed during the metaphase to anaphase transition, when sister chromatids separate to the opposite poles (Uhlmann, 2001).

SpRec8p is expressed specifically in the meiotic cycle with peak transcripts at the G1-S interval (Mata *et al.*, 2002; Chapter 3). A cohesin complex containing SpRec8p binds sister chromatids together at centromeres and along chromosome arms. In meiosis I homologous chromosomes pair, and at anaphase I SpRec8p along the chromosome arms is cleaved allowing the pairs to disengage and move to opposite poles. In the second meiotic division, at the onset of anaphase II, SpRec8p at the centromeres is cleaved resulting in the separation of sister chromatids (Figure 1.10; Section 1.16.4). The types of segregation defects seen in both mitotic and meiotic cells are illustrated in Figures A I-1



**Figure AI-1: Meiotic chromosome segregation events.**

**A**, Normal *S. pombe* meiosis: homologous chromosomes separate during the first meiotic division (MI); reductional division and sister chromatids remain associated. During meiosis II (MII), sister chromatids separate in an equational pattern of division, generating four haploid spores. **B**, Meiosis I non-disjunction, also called uniparental disomy, occurs when homologues fail to segregate during MI. **C**, Meiosis II non-disjunction or uniparental isodisomy: sister chromatids fail to segregate during MII. **D**, Precocious separation of sister chromatids results in individual chromatids segregating randomly during MI and MII.

## Analysis of the importance of SpRec8p in meiotic chromosome segregation by testing whether meiotic expression of SpRad21p can compensate for loss of SpRec8p.

The premise that SpRec8p is involved in chromosome cohesion is based on the observation that cells which lack SpRec8p randomly segregate chromosomes during meiosis (Watanabe & Nurse, 1999). During meiosis SpRec8p is present along the chromosome arms and at the centromeric region, with its cleavage resulting in separation of both homologous chromosomes and sister chromatids. In contrast, during the mitotic cycle, SpRad21p cleavage occurs only once resulting in the separation of sister chromatids.

Here, experiments are described that attempted to examine if the mitotic cohesin SpRad21p can compensate for the dual role of the meiotic cohesin SpRec8p during meiosis. Three types of strains were used: wild type with functional SpRec8p; a *rec8* deletion strain (*rec8Δ*) with no functional SpRec8p; and *rec8<sup>+</sup>P:rad21<sup>+</sup>ORF rec8Δ*. In this final strain the *rec8<sup>+</sup>* promoter is ligated to the *rad21<sup>+</sup>* open reading frame (Figure AI-2), and this DNA construct integrated into *rec8Δ* cells. When meiosis is triggered *rad21<sup>+</sup>*, now under the regulation of the *rec8<sup>+</sup>* promoter, is expressed during the meiotic cycle, replacing *rec8<sup>+</sup>*.



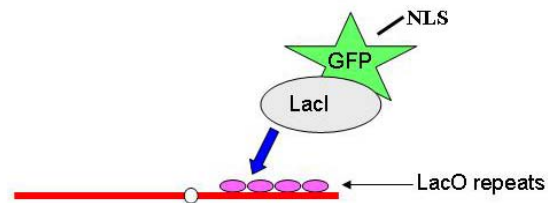
**Figure AI-2: *rad21<sup>+</sup>* under control of *rec8<sup>+</sup>* promoter.**

To allow the mitotic cohesin *rad21<sup>+</sup>* to be expressed in the meiotic cycle the *rec8<sup>+</sup>* promoter was ligated to the *rad21<sup>+</sup>* open reading frame (Appendix III). This construct was then integrated into the chromosome of a *rec8Δ* strain.

To enable visualisation of chromosomes, a GFP tagging protocol was adopted based on a method for observing *S. cerevisiae* centromeres, which utilises the *E. coli* Lac Operon system (Straight *et al.*, 1996) and adapted for fission yeast (Nabeshima *et al.*, 1998). This approach involves exploiting the binding properties of the LacI repressor protein and the LacO DNA repeats (Figure AI-3). A series of LacO repeats were integrated into the fission yeast *lys1<sup>+</sup>* site which is located 30 kb from the centromere of chromosome I. The

GFP-LacI fusion protein is tagged with a nuclear localisation signal (NLS) with this is under the control of the *dis1*<sup>+</sup> promoter. This fusion protein was integrated into the *his7*<sup>+</sup> site on chromosome II. The expressed GFP-LacI-NLS protein could thus enter the nucleus and specifically bind to the operator sequences linked to chromosome 1, allowing visualisation of the movements of cen1.

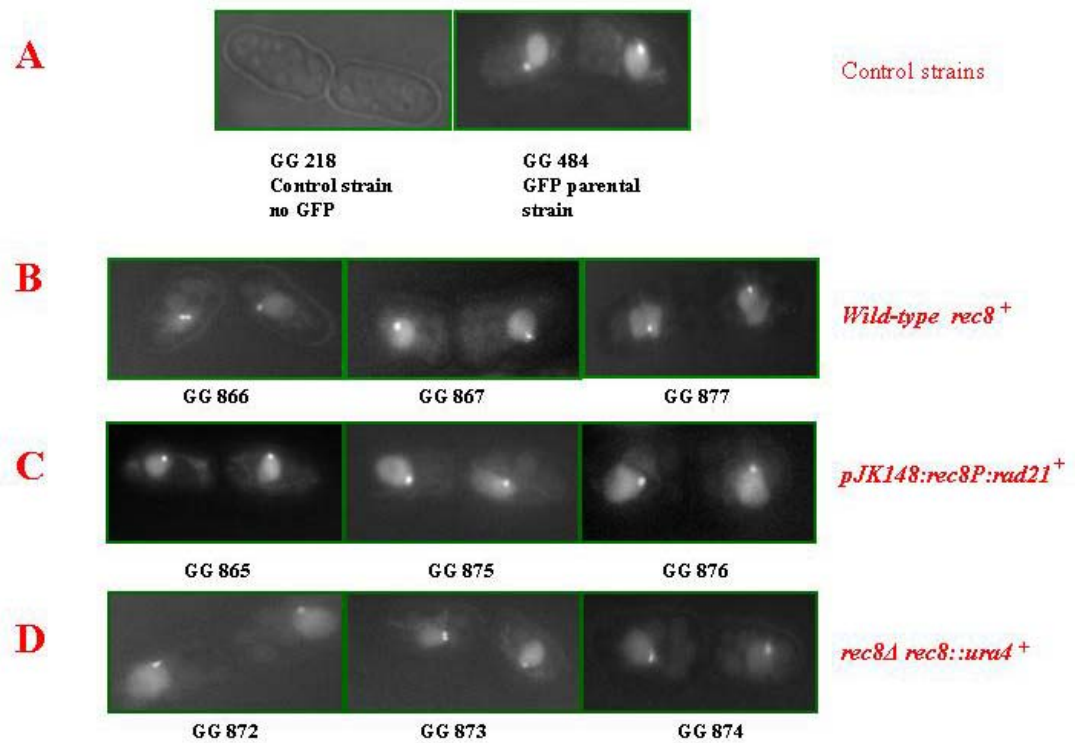
### LacO-LacI-GFP



**Figure AI-3: LacO-LacI-GFP system for chromosome visualisation in fission yeast.**

The GFP-LacI-NLS fusion protein binds to the LacO repeats (Nabeshima *et al.*, 1998). Fluorescent microscopic visualisation of GFP can be used to monitor movement throughout the phases of the cell cycle.

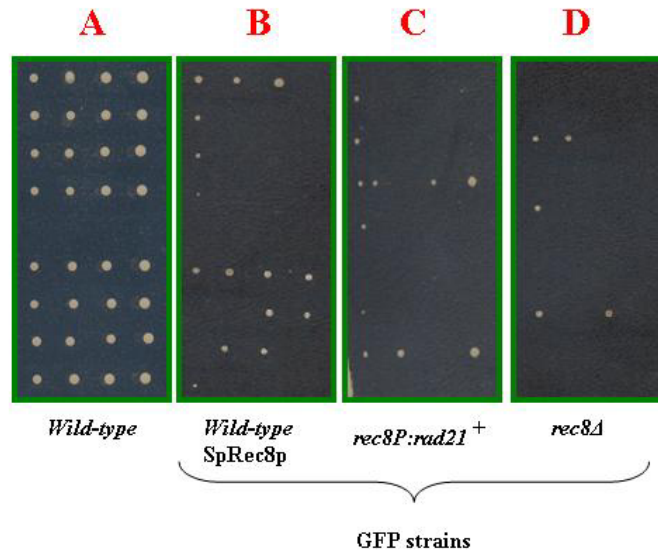
Strains in which chromosome 1 was tagged with GFP were created through a series of mating reactions with a parental strain GG 484, to introduce the GFP-LacI-NLS fusion protein and the LacO repeats. Three strains with manipulated cohesion expression were created: wild-type, *rec8*<sup>+</sup>*P:rad21*<sup>+</sup>ORF *rec8Δ* and *rec8Δ*. The three strains were then grown at 25°C in liquid minimal media to detect the presence of expressed GFP by fluorescent analysis using a Zeiss Axiovert microscope indicating that chromosome 1 had been successfully tagged, (Figure AI-4).



**Figure AI-4: GFP-tagged chromosome 1 in mitotic fission yeast cells.**

**A**, Control strains with no GFP (GG 218), and the parental strain with GFP (GG 484). Three isolates of each strain, **B**, *rec8<sup>+</sup>* (GG 866, 867, 877), **C**, *rec8Δ* (GG 872, 873, 874), **D**, *rec8P:rad21<sup>+</sup> rec8Δ* (GG 865, 875, 876). Presence of a fluorescent dot, indicates centromere 1 tagged with GFP.

Next, matings with fission yeast strains with and without the integrated LacO tag were completed. Using a micromanipulator (Singer Instruments Manual MSM), spores from individual asci were isolated and arranged in a grid pattern on a YE plate, and then incubated at 25°C for 3-7 days until colonies had grown. The resulting tetrads are shown in Figure AI-5:



**Figure AI-5: Tetrad analysis of GFP-tagged chromosome fission yeast strains.**

**A**, Control strain wild type  $h^+$  and  $h^-$  fission yeast without GFP in the strain background, demonstrating that spores had not been damaged by handling with the micro-manipulator. **B, C, D**, GFP strains  $rec8^+$  (WT SpRec8p),  $Rec8P:rad21^+$  and  $rec8Δ$ , left for the same length of time at the same temperature, asci coat failed to break down, and when they did break spore viability was decreased.

Unfortunately, mating reactions to integrate the LacO repeats and LacI-GFP-NLS fusion protein were not successful as the tetrad analysis of strains in which the fusion protein was present had significantly decreased spore viability compared with a wild type control. This suggested that the GFP tag was by itself affecting function, and so precluded its use for further experiments.

Future experiments might involve testing if it was either the LacO repeats or the fusion protein which was having an effect. An alternative approach to view chromosomes could be to use fluorescent in situ hybridisation (FISH).



## Appendix II – *S. pombe* strains used in this study

GLASGOW LAB NUMBER	ORIGIN	GENOTYPE
GG 217/905	Lab Stock	h <sup>-</sup> wild type
GG 218/904	Lab Stock	h <sup>+</sup> wild type
GG 300	Lab Stock	h <sup>+</sup> <i>rec8::ura4<sup>+</sup> pat1-114 ura4-D18 leu1-32 ade6-M216</i>
GG 301	Lab Stock	h <sup>-</sup> <i>rec8::ura4<sup>+</sup> pat1-114 ura4-D18 leu1-32 ade6-M210</i>
GG 347	Lab Stock	h <sup>-</sup> pJK148 <i>pat1-114 leu1-32 ade6-M216</i>
GG 349	Lab Stock	h <sup>-</sup> pJK148: <i>rec8<sup>+</sup>P:rad21<sup>+</sup> ORFrec8::ura4<sup>+</sup> pat1-114 ura4-D18 leu1-32 ade6-M210</i>
GG 358	Lab Stock	h <sup>-</sup> pJK148: <i>rad21<sup>+</sup>/rec8<sup>+</sup>ORF h- cdc25-22 leu1-32 ura4-D18 rad21::ura4<sup>+</sup> nmt<sup>+</sup>promoter:rad21<sup>+</sup></i>
GG 375	Lab Stock	h <sup>-</sup> /h <sup>-</sup> <i>pat1-114 /pat1-114 leu1-32 /leu1-32 ade6-M216 /ade6-M210 GG 339</i>
GG 434	Lab Stock	h <sup>+</sup> pJK148: <i>rec8<sup>+</sup>P:rad21<sup>+</sup> ORF rec8::ura4<sup>+</sup> pat1-114 ura4-D18 leu1-32 ade6-M216</i>
GG 435	Lab Stock	h <sup>+</sup> pJK148 <i>pat1-114 leu1-32 ade6-M210</i>
GG 482	Lab Stock	h <sup>+</sup> <i>ade6-210 leu1-32 lys1-131 his7-366 ura4-D18</i>
GG 484	Pascal Bernard (Nabeshima <i>et al.</i> , 1998)	h <sup>-</sup> LacO- <i>lys1<sup>+</sup> GFP-LacI-his7<sup>+</sup> ade6-210 leu1-32 ura4D-18</i>
GG 825	This Study	h <sup>+</sup> <i>rec8::ura4<sup>+</sup> pat1-114 lys1-131 his7-366 ade6-M210 leu1-32 ura4-D18</i>
GG 826	This Study	h <sup>+</sup> <i>rec8::ura4<sup>+</sup> pat1-114 lys1-131 his7-366 ade6-M210 leu1-32 ura4-D18</i>
GG 827	This Study	h <sup>+</sup> <i>rec8::ura4<sup>+</sup> pat1-114 lys1-131 his7-366 ade6-M210 leu1-32 ura4-D18</i>
GG 828	This Study	h <sup>+</sup> <i>rec8::ura4<sup>+</sup> pat1-114 lys1-131 his7-366 ade6-M210 leu1-32 ura4-D18</i>
GG 833	This Study	h <sup>+</sup> pJK148: <i>rec8P:rad21<sup>+</sup> ORF rec8::ura4 pat1-114 ura4-D18 leu1-32 ade-M210 his7-366</i>
GG 834	This Study	h <sup>-</sup> pJK148: <i>rec8P:rad21<sup>+</sup> ORF rec8::ura4 pat1-114 ura4-D18 leu1-32 ade-M210 lys1-131</i>
GG 850	This Study	h <sup>+</sup> pJK148: <i>rec8P:rad21<sup>+</sup> ORF rec8::ura4 pat1-114 ura4-D18 leu1-32 ade-M210 his7-366 lys1-131</i>
GG 851	This Study	h <sup>+</sup> pJK148: <i>rec8P:rad21<sup>+</sup> ORF rec8::ura4 pat1-114 ura4-D18 leu1-32 ade-M210 his7-366 lys1-131</i>
GG 852	This Study	h <sup>-</sup> pJK148 <i>pat1-114 leu1-32 lys1-131 ade6-210</i>
GG 853	This Study	h <sup>+</sup> pJK148 <i>pat1-114 leu1-32 his7-366 ade6-216</i>

## Appendix II – *S. pombe* strains used in this study - continued

GLASGOW LAB NUMBER	ORIGIN	GENOTYPE
GG 854	This Study	h <sup>+</sup> pJK148 <i>pat1-114 leu1-32 his7-366 lys1-131 ade6-210</i>
GG 865	This Study	h <sup>-</sup> pJK148: <i>rec8P:rad21</i> <sup>+</sup> ORF <i>rec8::ura4 pat1-114 LacO-lys1</i> <sup>+</sup> GFP-LacI- <i>his7</i> <sup>+</sup> <i>ade6-210 leu1-32 ura4D-18</i>
GG 866	This Study	h <sup>-</sup> pJK148 <i>pat1-114 LacO-lys1</i> <sup>+</sup> GFP-LacI- <i>his7</i> <sup>+</sup> <i>ade6-210 leu1-32</i>
GG 867	This Study	h <sup>-</sup> pJK148 <i>pat1-114 LacO-lys1</i> <sup>+</sup> GFP-LacI- <i>his7</i> <sup>+</sup> <i>ade6-210 leu1-32</i>
GG 868	This Study	h <sup>-</sup> pJK148: <i>rec8P:rad21</i> <sup>+</sup> ORF <i>rec8::ura4 LacO-lys1</i> <sup>+</sup> GFP-LacI- <i>his7</i> <sup>+</sup> <i>ade6-210 leu1-32 ura4D-18</i>
GG 869	This Study	h <sup>-</sup> pJK148: <i>rec8P:rad21</i> <sup>+</sup> ORF <i>rec8::ura4 LacO-lys1</i> <sup>+</sup> GFP-LacI- <i>his7</i> <sup>+</sup> <i>ade6-210 leu1-32 ura4D-18</i>
GG 870	This Study	h <sup>-</sup> pJK148 LacO- <i>lys1</i> <sup>+</sup> GFP-LacI- <i>his7</i> <sup>+</sup> <i>ade6-210 leu1-32</i>
GG 871	This Study	h <sup>-</sup> pJK148 LacO- <i>lys1</i> <sup>+</sup> GFP-LacI- <i>his7</i> <sup>+</sup> <i>ade6-210 leu1-32</i>
GG 872	This Study	h <sup>-</sup> <i>rec8::ura4</i> <sup>+</sup> <i>pat1-114 LacO-lys1</i> <sup>+</sup> GFP-LacI- <i>his7</i> <sup>+</sup> <i>ade6-M210 leu1-32 ura4-D18</i>
GG 873	This Study	h <sup>-</sup> <i>rec8::ura4</i> <sup>+</sup> <i>pat1-114 LacO-lys1</i> <sup>+</sup> GFP-LacI- <i>his7</i> <sup>+</sup> <i>ade6-M210 leu1-32 ura4-D18</i>
GG 874	This Study	h <sup>-</sup> <i>rec8::ura4</i> <sup>+</sup> <i>pat1-114 LacO-lys1</i> <sup>+</sup> GFP-LacI- <i>his7</i> <sup>+</sup> <i>ade6-M210 leu1-32 ura4-D18</i>
GG 875	This Study	h <sup>-</sup> pJK148: <i>rec8P:rad21</i> <sup>+</sup> ORF <i>rec8::ura4 pat1-114 LacO-lys1</i> <sup>+</sup> GFP-LacI- <i>his7</i> <sup>+</sup> <i>ade6-210 leu1-32 ura4D-18</i>
GG 876	This Study	h <sup>-</sup> pJK148: <i>rec8P:rad21</i> <sup>+</sup> ORF <i>rec8::ura4 pat1-114 LacO-lys1</i> <sup>+</sup> GFP-LacI- <i>his7</i> <sup>+</sup> <i>ade6-210 leu1-32 ura4D-18</i>
GG 877	This Study	h <sup>-</sup> pJK148 <i>pat1-114 LacO-lys1</i> <sup>+</sup> GFP-LacI- <i>his7</i> <sup>+</sup> <i>ade6-210 leu1-32</i>
GG 921	Suresh Subramani (Birkenbihl & Subramani, 1992)	h <sup>-</sup> <i>rad21-45</i>

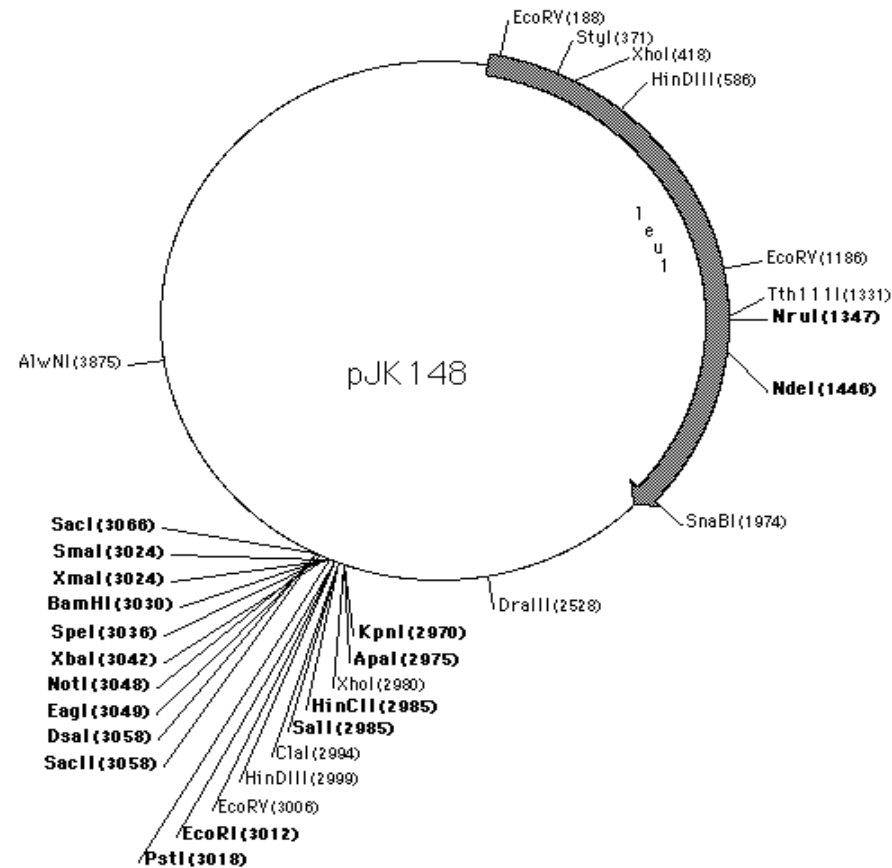
## Appendix III - Oligonucleotides

GLASGOW LAB NUMBER	PURPOSE	SEQUENCE
GO 118 GO 119	<i>rad21</i> <sup>+</sup> northern probe <i>rad21</i> <sup>+</sup> northern probe	GCGTTAGGGTCCTTTGGCCA CAAGCATAACGGTGAGGTG
GO 146 GO 147	<i>rad21</i> <sup>+</sup> promoter <i>rad21</i> <sup>+</sup> promoter	GTTACCCTCGAGTTCATCCTTCAATTCTCTTT CATTCCCCGGGAAGAAGCTTAATATGATTAG
GO 148 GO 149	<i>rad21</i> <sup>+</sup> ORF <i>rad21</i> <sup>+</sup> ORF	CATTCA <del>CCCCGGG</del> GATGTTCTATTCAGAGGCCAT GGTCAAG <del>GCGGCCGCT</del> CATAGTGATGAAAGTAGCA
GO 150 GO 151	<i>rec8</i> <sup>+</sup> promoter <i>rec8</i> <sup>+</sup> promoter	GTACCA <del>CTCGAG</del> GTAGTAATTTTGTACTTGTT TTAACG <del>CCCCGGG</del> GGTGAAAGACAGGAACGCGT
GO 152 GO 153	<i>rec8</i> <sup>+</sup> ORF <i>rec8</i> <sup>+</sup> ORF	TCAATT <del>CCCCGGG</del> GATGTTTTACAATCAAGATGT AATGGAG <del>GCGGCCGCT</del> CAAATGGCATCGGTGCTTT
GO 694 GO 695	<i>rec8</i> <sup>+</sup> northern probe <i>rec8</i> <sup>+</sup> northern probe	TGACATGAGTTCTTTCCTC CGTGGTTTGATTGTGATACT

Oligo pairs are grouped together, and restriction sites inserted for PCR cloning are in blue.

## Bacterial vector

GB 186 *leu1<sup>+</sup>* pJK148



**Figure AIII- 1: Plasmid map of the fission yeast intergration vector pJK148 (GB 186).**

The vector pJK148 (Keeney & Boeke, 1994) carries unique *Nru I* and *Nde I* sites within the *leu1<sup>+</sup>* gene. Upon linearisation with either of these two restriction enzymes this allows integration of the vector into the fission yeast genome at the *leu1<sup>+</sup>* locus. Unique restriction sites are shown on the circle map in **bold** (adapted from [www-rcf.usc.edu/~forsburg](http://www-rcf.usc.edu/~forsburg)).

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