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An investigation into cis and trans acting factors that may influence genomic rearrangements in the fission yeast, Schizosaccharomyces pombe

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An Investigation into *cis* and *trans* Acting Factors That may Influence Genomic Rearrangements in the Fission Yeast, *Schizosaccharomyces pombe*

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A thesis submitted for the degree of Doctor of Philosophy

• PRIFYSGOL CYMRU •
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2008

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ABSTRACT

Evolution is driven in part, by genetic events which result in global changes to genomic structure. Some genomic changes can be detrimental for cells / organisms. Genetic instability is a transient or a persistent state resulting from chromosomal breakage and rearrangements that causes a series of mutational events leading to gross genetic alterations, which can cause cancers and other genetic diseases. These alterations results from external and/or internal factors. External environmental factors include radiation or chemicals, which act by damaging DNA. Internal events such as oxidation and perturbation of the progression of the DNA replication by intrinsic factors such as DNA binding proteins, replication slow zones and replication fork barriers can also lead to genetic instability.

In this study, I used the fission yeast, *Schizosaccharomyces pombe* as a model organism to study aspects of the regulation of eukaryote genome stability. Firstly, I set out to determine whether Translin protein, which binds to chromosome breakpoint junctions in lymphoid malignancies and sarcomas, and its partner protein Trax, have any role in causation or suppression of genetic instability. I studied the effect of removal and over expression of Trax and Translin on *S. pombe* with regards to growth rate, DNA damage repair and repeat instability. I find that deletion and over expression of the *trax* and *translin* genes did not affect cell proliferation, morphology or DNA damage recovery. I found that Trax levels are post transcriptionally regulated by Translin, a function conserved in higher eukaryotes.

Secondly, I used two different genetic elements, *RTS1* and a tRNA gene cassette (*sup3-e*) which generate replication fork barriers (RFBs) to study the possible role of *RTS1* in the causation of genetic rearrangement. We find that the recombination potential of RFBs is not universal. We also demonstrate that intra-genomic ectopic recombination, initiated by a disruption to DNA replication, is regulated in a region-specific manner. Lastly, we demonstrate that the *trans* factor Swi1, a yeast TIMELESS homologue, differently regulate the recombination potential of replicative barriers, serving both to stimulate and suppress recombination in a barrier-specific fashion.

ACKNOWLEDGEMENTS

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ABBREVIATIONS

ATP	adenosine triphosphate
BND Cellulose	Benzoylated Naphthoylated Deae Cellulose
bp	base pairs
CDK	Cylin-dependent kinase
CML	chronic myelogenous leukemia
DAPI	4'-6-Diamidino-2-phenylindole
DSBR	Double-strand break repair
DMSO	Dimethyl sulfoxide
DNA	deoxyribonucleic acid
DNA PK	DNA-dependent protein kinase
DSBs	Double-strand breaks
dsDNA	Double-stranded DNA
EDTA	Ethylenenediaminetetra-acetic acid
EtOH	Ethanol
GCR	Gross chromosomal rearrangements
HJ	Holliday junction
HR	Homologous recombination
HU	Hydroxyurea
LB	Luria Bertani
LTR	Long terminal repeats
M	Molar
ml	Milliliter
mM	Millimolar
MM media	Mitchen's Minimal media
MMR	Mismatch repair
MMS	Methyl methane sulphonate
MOPS	Morpholine-5' propane sulphonic acid
NB media	Nitrogen base media
NER	Nucleotide excision repair

NHEJ	Non-homologous DNA end joining
nmt	No message in thiamine
nM	Nanometer
ORC	Origin Recognition Complex
ORF	Open reading frame
PCNA	Proliferating cell nuclear antigen
PCR	Polymerase Chain Reaction
Pol α	Polymerase α
r DNA	Ribosomal DNA
RIs	Recombination Intermediates
r.p.m.	Revolutions per minute
RFB	Replication Fork Barriers
RFP	Replication fork pause
RFs	Replication forks
RNA	Ribonucleic acid
RSZ	Replication slow zones
RTS1	Replication termination sequence 1
SDS	Sodium dodecyl sulphate
SDS-PAGE	Sodium dodecyl sulphate -polyacrylamide gel electrophoresis
ssDNA	Single-stranded DNA
tRNA	Transfer RNA
TRAX	Translin-associated Protein-X
UTRs	Untranslated regions
UV	Ultra violet
WCEs	Whole cell extracts
YE	Yeast extract
YEA	Yeast extract agar
2D gels	Two dimensional agarose gel

CONTENTS

Chapter 2 : Material and Methods

Publications:

1. Jaendling, A., Ramayah, S., Pryce, W. D. & McFarlane, J. R. (2008) Functional characterisation of the *Schizosaccharomyces pombe* homologue of the leukaemia-associated translocation breakpoint binding protein translin and its binding partner, TRAX. *Biochim. Biophys. Acta.*, 1783, 203-213.

2. Pryce, W. D., Ramayah, S., A. Jaendling, McFarlane, J. R. Recombination at DNA replication fork barriers is not universal and is differentially regulated by Swi1 (2009) PNAS 106 (12) 4770-4775

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CHAPTER 1

1. INTRODUCTION

1.1 Cancer

Cancers are complex diseases with many genes implicated in their development, progression, invasion and metastasis. They are characterised by uncontrolled cell proliferation. Cancers are one of the major causes of death in the developed world and there are more than 100 different cancer types (Jefford and Irminger-Finger, 2006). Most tumours occur from clonal selection and waves of expansion of a somatic cell that has acquired a genetic alteration in genes controlling pathways regulating cell death or cell proliferation.

Cancer causing genetic changes usually occur in somatic cells, but certain genetic changes can be inherited and cause a predisposition to cancer. The physiological changes that are acquired by cells during malignant tumour development are divided into six main groups; self sufficiency in growth signals, insensitivity to growth inhibitory signals (anti-growth signals), evasion of programmed cell death (apoptosis), limitless replicative potential, sustained angiogenesis, and lastly tissue invasion by metastasis (Hannah and Weinberg, 2000).

It is widely accepted that for a single cell to become cancerous, it must undergo a series of genetic changes (for review, see Jefford and Irminger-Finger, 2006) and various genotoxic agents (both internal and environmental) can provide an oncogenic stress capable of altering the genome. However, recent work on a range of tumour types (bladder, breast, lung, colon) has suggested that failures in DNA replication under normal physiological conditions are likely to contribute strongly to the generation of cancer causing genetic changes (Bartkova *et al.*, 2005; Bilousova *et al.*, 2005). It has been demonstrated that replication pauses can stimulate genetic recombination (Ahn *et al.*, 2005) and aberrant recombination can result in a range of genetic rearrangements, including chromosomal translocations, which are implicated in oncogenic transformation.

DNA double-strand breaks (DSBs) can be repaired by any one of several alternative and competing mechanisms. The repaired sequences often differ from the original depending on

which mechanism was used so that pathway choice can have profound influence on the genetic consequences (Preson *et al.*, 2006).

Therefore, understanding the molecular events which culminate in a cell becoming cancerous provides an insight into factors which can be used to identify possible predispositions to cancer formation and factors which can be used as early diagnostic markers. One of the successful examples of this is the discovery that mutations in the BRCA1 and BRCA2 genes result in predisposition to both breast and ovarian cancers (Frank, 1999). Individuals with BRCA1 and BRCA2 mutations are monitored closely and in some cases offered pre-emptive mastectomy, resulting in reduced mortalities due to breast cancers.

1.2 Chromosomal Rearrangements

Genetic instability is a transient or a persistent state that causes a series of mutational events leading to gross genetic alterations (Jefford and Irminger-Finger, 2006; Venkatesan *et al.*, 2006).

High fidelity DNA replication is essential to prevent eukaryotic genomes acquiring mutations. DNA replication is constantly challenged by intrinsic conditions in cells such as damaged DNA templates, protein complexes bound to DNA and inadequate supplies of deoxyribonucleotide triphosphates (dNTPs) (Noguchi *et al.*, 2004). As well as intra cellular events contributing to DNA damage levels, the alteration of a normal cell to a cancerous cell is also caused by changes that occur due to external or environmental factors, for example UV light, and gamma radiation (Venkatesan *et al.*, 2006). Cells have mechanisms to detect and repair DNA damage. However, inaccurate repair can result in genomic instability that could manifest as gross chromosomal rearrangements (GCRs) (Banerjee *et al.*, 2007).

GCRs include the structural changes that may occur either at the chromosome level, leading to loss or gain of large parts of chromosomes such as, translocations, deletions, inversions, amplifications, chromosome end to end fusions and ploidy changes or at the nucleotide level affecting gene structures or expression; for example mutations, deletions, gene amplifications, microsattellite amplification and gene silencing by epigenetic effects (Kolodner *et al.*, 2002; Aplan, 2006; Banerjee *et al.*, 2007)

GCRs are highly induced by initial mutations affecting DNA replication (Venkatesan *et al.*, 2006; Mirkin and Mirkin, 2007). During DNA replication, DNA replication forks can stall at damaged DNA, at naturally occurring sequences such as replication fork barriers or when collision occurs with other proteins associated with DNA metabolism such as RNA polymerase II (Prado and Aguilera, 2005). In mammals, cancer is frequently associated with large genome rearrangements arising from inaccurate repair of DSBs (Hasty *et al.*, 2003; Aplan, 2006).

1.3 Translocations

Translocations are rearrangements of chromosomes in which a chromosome arm is transplaced from one chromosome to another. Defects in chromosome replication can lead to translocations (Mirkin and Mirkin, 2007). The process of chromosomal translocation brings two previously unlinked segments of the genome together, which, in some cases, can result in the disruption of genes or cause the juxtapositioning of elements that disturbs the normal expression of the gene adjacent to the breakpoint. This phenomenon is particularly important when the breakpoint of the translocation results in inappropriate expression of an oncogene or synthesis of a new oncogenic fusion protein (Agarwal *et al.*, 2006). Such chromosome changes and mutations can activate cellular oncogenes in a dominant fashion.

Chromosomal translocations are frequently associated with a variety of cancers, particularly haematologic malignancies and childhood sarcomas (Aplan, 2006). In fact, the first specific translocation identified in a human neoplasia was t(9;22)(q34;q11), resulting in the Philadelphia Chromosome (Novell and Hungerford, 1960). The symbols t(9;22) (q34;q11) signify a translocation between chromosomes 9 and 22 with breakpoints in bands 9q34 and 22q11, respectively (Mitelman *et al.*, 2007). In addition to their diagnostic utility, chromosomal translocations are increasingly being used in the clinic to guide therapeutic decisions (Agarwal *et al.*, 2006; Aplan, 2006; Felix *et al.*, 2006; Fojo, 2007; Emerenciano *et al.*, 2007). However, the mechanisms that cause these translocations remain poorly understood (Aplan, 2006).

1.4 Mechanisms and Clinical Importance of Translocations

Translocations are divided into reciprocal and non-reciprocal translocations (Figure 1.1). Reciprocal translocations are usually an exchange of material between nonhomologous chromosomes. Reciprocal translocations can be balanced (no substantial net gain or loss of nuclear DNA and potentially fully functional) or unbalanced (where the exchange of chromosome material is unequal resulting in duplicated or deleted chromosomal regions). Reciprocal translocations are frequently balanced. Reciprocal translocations between nonhomologous chromosomes have been observed in a variety of malignancies including leukemias, lymphomas and sarcomas, and they have been implicated in the etiology of the diseases (Gollin, 2007).

Non-reciprocal translocations (Robertsonian translocation) are a one-way transfer of a chromosomal segment to another chromosome. Non-reciprocal translocations involve two acrocentric chromosomes that fuse near the centromere region with loss of the short arms. It is a special kind of translocation in that the acrocentric chromosomes (most often chromosomes 14 and 21 or 22) cause satellite-bearing short arm and a centric fusion t (14q21q or 14q22q) of the two remainder chromosomes, i.e., the long arms of the two pieces, results. The carrier of this trait has a larger probability of having offspring with trisomy / monosomy and this is independent of age.

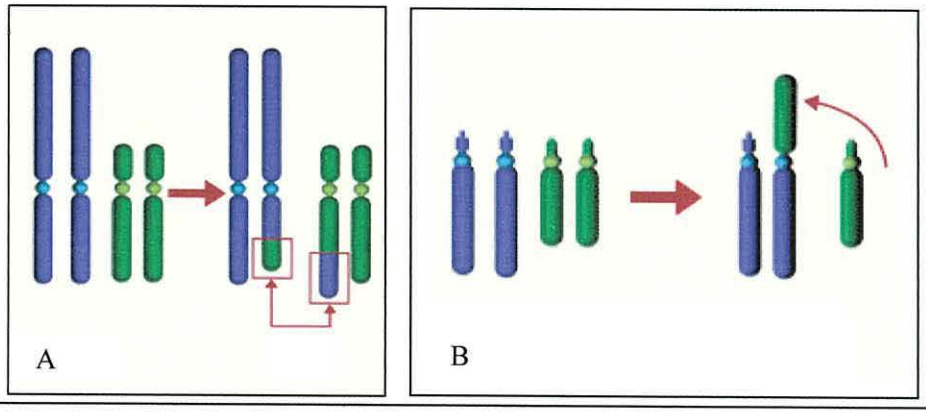


Figure 1.1 Reciprocal and nonreciprocal translocation

A. Reciprocal translocation
Two broken chromosome pieces from two non-homologous chromosome joining, creating a new hybrid chromosome.

B. Non- reciprocal Translocation
In a non-reciprocal translocation two chromosomes lose their short arms. Afterwards, the remaining partial pieces (q- arms) fuse to one another leading to centric fusion of two non-homologous chromosomes.

Beginning with the discovery of the Philadelphia chromosome in leukaemic cells of patients with Chronic Myelogenous Leukemia (CML), it soon became evident that leukaemias and lymphomas are often associated with nonrandom chromosomal abnormalities including deletions, inversions and translocations (Rabbits, 1994). Specific chromosomal translocations are often (but not invariably) associated with specific sub-types of leukaemia or lymphoma (Aplan, 2006).

Cytogenetic analysis of some malignancies, particularly haematologic malignancies, reveal a sole abnormality, such as a single balanced translocation, whereas epithelial tumours will typically display a much more complex pattern of numerical and structural chromosomal aberrations (Aplan, 2006). Figure 1.2 shows a comparison of karyotype analysis of malignant cells from a patient with CML versus a solid tumour (bladder carcinoma). Visible changes to chromosome structure and morphology have played a very important part as indicators of genetic damage in both clinical and cancer studies.

a b

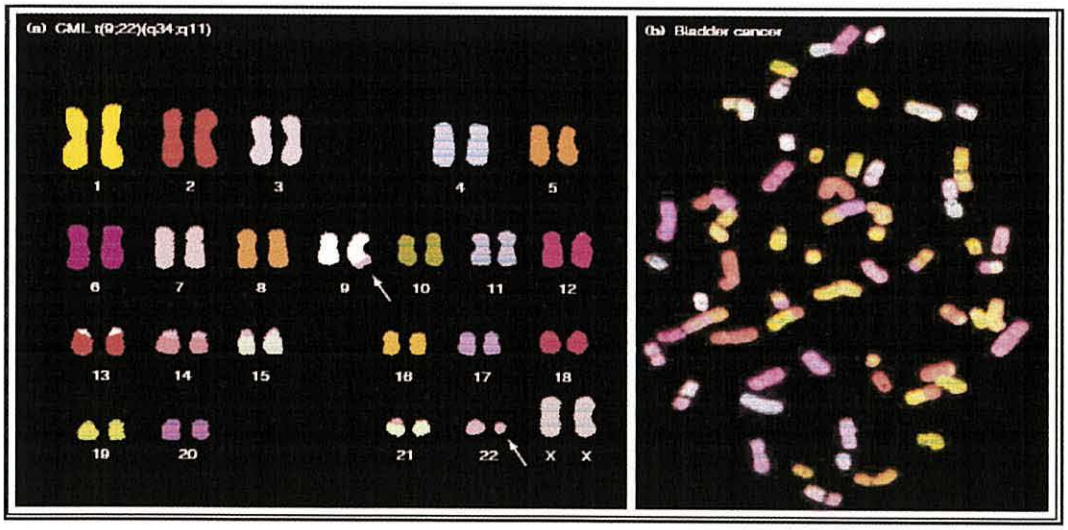


Figure 1. 2 Spectral karyotype analyses of malignant cells.

(a) A spectral karyotype from a patient with chronic myelocytic leukemia (CML), showing a pseudodiploid karyotype with a t (9: 22) (indicated by the white arrows) translocation as the only detectable gross anomaly.
(b) A spectral karyotype of a bladder carcinoma, showing numerous numerical and structural chromosomal anomalies.

Adapted from (Aplan, 2006)

A well studied example of a cancer generating translocation is seen in Burkitt's lymphoma. In most cases of this B cell tumour, a translocation is seen involving chromosome 8 and one of three other chromosomes [(2, 14 or 22; t(8;14)(q24;q32), t(2;8)(p11;q24) or t(8;22)(q24;q11)] (Mitelman *et al.*, 2007). In these cases, a fusion protein is not produced, but rather, the *c-myc* proto-oncogene on chromosome 8 is brought under transcriptional control of an immunoglobulin gene promoter. In B cells, immunoglobulin promoters are transcriptionally quite active and fusion to these proteins results in over expression of *c-myc*, which is known to have oncogenic properties. Hence, this translocation results in aberrant high expression of an oncogenic protein, which is almost certainly the cause of the Burkitt's tumour.

A second type of translocation arises with the breakpoint within the coding region of two genes, creating a fusion gene that encodes a chimeric protein. Such translocations were shown to involve reciprocal fusion of rather small pieces from the long arms of chromosome 9 and 22. The altered chromosome 22 is known as the Philadelphia chromosome (abbreviated as Ph1). When the breakpoint of the Ph1 chromosome was sequenced, it was found that this translocation creates a fusion gene by bringing together sequences from the *c-abl* proto-oncogene and another gene called *bcr* (for breakpoint cluster region) (Mitelman *et al.*, 2007) . The *bcr-abl* gene fusion encodes a phosphoprotein (p210) that functions as a disregulated protein tyrosine kinase and predisposes the cell to becoming neoplastic.

Other examples of translocation breakpoints associated with human cancer include: 14:18 translocation in follicular B cell lymphomas (*bcl-2* and immunoglobulin genes) 15:17 translocation in acute promyelocytic leukemia (pml and retinoic acid receptor genes) 1:19 translocation in acute pre-B cell leukemia (PBX-1 and E2A genes).

Although initially surprising, the identification of some of these oncogenic chromosomal translocations in peripheral blood from healthy individuals strongly suggests that the translocation alone is not sufficient to induce malignant transformation and that complementary mutations are required to produce a malignancy (Aplan, 2006). From analysis of the function(s) of the genes located at the translocation breakpoints, it emerges that these genes often encode transcription factors that are known to be important for

haematopoietic differentiation, or tyrosine kinases that are important regulators of cell proliferation. These observations have led to the hypothesis that at least two complementary events, one leading to a block in differentiation and a second leading to hyperproliferation are required for leukaemic transformation (Emerenciano *et al.*, 2007; Gilliland and Tallman, 2002).

1.5 DNA Replication

Cells duplicate by cellular division, which requires the duplication of the genetic material, DNA replication (Karp, 2005; Branzei and Foiani, 2007). DNA replication is the process of duplicating double-stranded DNA. Defects in chromosome replication can lead to translocation that arise as a result of recombination event following stalled replication forks and DNA replication pause sites can be hotspots for recombination (Labib and Hodgson, 2007).

DNA replication is semi-conservative as each daughter duplex contains one strand from the parent molecule. The events involved in the initiation of chromosomal replication are similar in Eubacteria, eukaryotes, and Archae: whereby replication starts with binding of specific initiator protein(s) to DNA sites, termed origins, and results in the localized unwinding of the DNA duplex followed by the establishment of replication forks (Zakrzewska-Czerwinska *et al.*, 2007).

1.5.1 Replication initiation.

Replication of the genome every time a cell divides is a highly coordinated process that ensures accurate and efficient inheritance of the genetic information. The molecular mechanism controlling replication origins in eukaryotes guarantee that many origins of replication fire only once per cell-cycle (Kawasaki *et al.*, 2006). The origin recognition complex (ORC) marks the position of replication origins in the genome and serves as the 'landing pad' for the assembly of a multiprotein, pre-replicative complex (pre-RC) at the origins. In the model organism *Schizosaccharomyces pombe,* the pre-RC consist of ORC, Cdc6, and Cdc10-dependent transcript (Cdt1) and mini-chromosome maintenance (MCM) proteins. The MCM proteins serve as key participants in the mechanism that limit eukaryotic DNA replication to once-per cell-cycle.

In the prokaryote, *Escherichia coli*, bidirectional replication starts from origin, *oriC* (Figure 1.3A) and the two replication forks travel in opposite direction until they meet at the terminus (Mirkin and Mirkin, 2007). The proper termination site in *E. coli* is determined by *Ter* sequences which function as polar replication terminators when bound by the Tus protein (Hill *et al.*, 1998; Heyer and Kanaar, 2004). The *E. coli* replication origin is approximately 250 base pairs (bp) in length and contain multiple 9-bp repeat elements, called DnaA boxes to which the DnaA initiator protein binds (Mott and Berger, 2007).

To ensure the eukaryotic genome is precisely duplicated during the limited period of S phase in every cell cycle, DNA replication initiates at a number of replication origins on eukaryotic chromosomes (Bell and Dutta, 2002; Gilbert, 2001) (Figure 1.3B). *Saccharomyces cerevisiae* is reported to have about 400 origins of replication, distributed about every 40-150 kb, which are responsible for the replication of the 14 Mb genome (Branzei and Foiani, 2007). The mechanisms of origin activation at the chromosome level are yet to be clarified in detail, although we have a growing understanding of protein factors involved in initiation and elongation of replication (Diffley, 2004).

Origins of DNA replication in *S. pombe* lack a specific consensus sequence analogous to the *S. cerevisiae* autonomously replicating sequence (*ARS*) consensus (Clyne and Kelly, 1995). Instead, the origins of DNA replication in *S. pombe* are larger (>500 bp), AT rich and mostly located in intergenic regions (Dai *et al.*, 2005; Hayashi *et al.*, 2007).

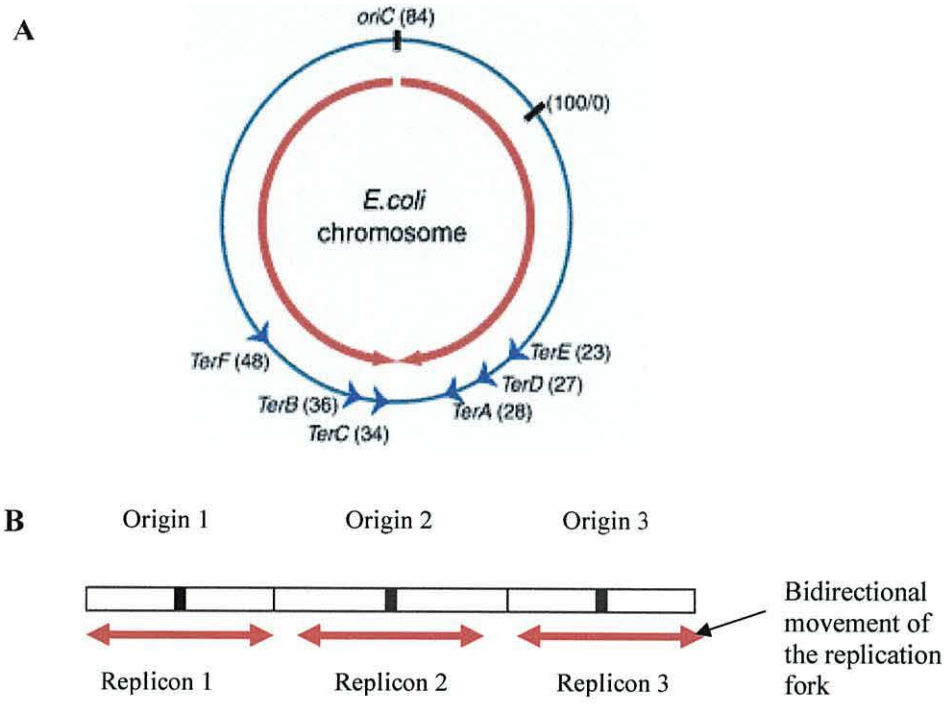


Figure 1.3: Origin of replication and termination in prokaryotes and eukaryotes

A. Circular bacterial chromosome (*E. coli*) replicates from one origin bidirectionally. The *E. coli* chromosome, the position of *oriC* and the six *Ter* sites are shown. The red arrows represent the direction of DNA replication initiated from *oriC*. The two forks approach the terminus region at approximately the same time having passed unimpeded through the *Ter* sites.

B. Linear eukaryotic chromosome replicates as many individual replicons moving bidirectionally.

Adapted from (Rothstein *et al.*, 2000)

1.5.2 Dynamics at the Replication Fork

The replication fork is the point where new DNA daughter strands are synthesized from the parental strands in a template-directed fashion. Due to its Y shaped structure; this reactive region is called a replication fork (Figure 1.4). Origin firing is followed by replication fork establishment, and factors required for DNA replication are loaded soon after the origins are licensed (Branzei and Foiani, 2007). At a replication fork, the DNA of new daughter strands is synthesized by a multi-enzyme complex that contains DNA polymerases. The replication process requires different activities and factors/proteins for the stages of initiation, elongation and termination.

In eukaryotes, just before the beginning of the S-phase of the cell cycle, protein kinases are activated, leading to the initiation of replication. The unwinding of the DNA duplex and separation of the strands require the aid of two types of proteins that bind to the DNA. Helicase acts to separate the duplex DNA and primase produces short RNA primers, which are required to initiate DNA synthesis. DNA helicases unwind the DNA duplex in a reaction that uses energy from ATP hydrolysis to break the hydrogen bonds that hold the strands together, exposing the single-stranded DNA templates. Concomitant with this multiple copies of the heterotrimeric single-stranded binding protein A (replication protein A; RPA), maintain the DNA in a single-strand state. The DNA polymerases ε and δ are involved in replicating both leading and lagging strands, together with their accessory proteins, such as replicating factor C (RFC) and proliferating cell nuclear antigen (PCNA), together these proteins form a large complex called the 'replisome' (Figure 1.4) (Waga and Stillman, 1998; Baker and Bell, 1998; Johnson and O'Donnell, 2005).

The formation of the replication fork involves many additional factors (Mcm10, Cdc45, Dpb11, Sld2, Sld3 and the GINS complex in *S. cerevisiae*) and activation of S-phase cyclin-depedent kinases (CDKs) and Cdc7- Dbf4 kinases (DDK), which both phosphorylate proteins of the repliosome (e.g. Mcm proteins, Sld2, Sld3) and others (Moldovan *et al.*, 2007). This reaction serves to assemble the replicative helicase, which is comprised of the Mcm2-7 complex together with associated factors, and to recruit the DNA polymerases and other factors required for DNA synthesis (Figure 1.4).

The two DNA strands are synthesized by different mechanisms. The leading strand can be replicated continuously through the 5'- to - 3' polymerase activity of the DNA polymerase δ (Pol δ) (Figure1.4). The lagging strand, meanwhile, is replicated in a discontinuous fashion, each Okazaki fragment being smaller than the stretch unwound in the replication fork structure. The initial RNA primer for DNA synthesis is made by the primase enzyme, followed by a short stretch of DNA synthesized by polymerase α (Pol α). Both enzymatic activities reside within a single primase-Pol α protein complex. Pol δ or Pol ε (bound to the sliding clamp PCNA, loaded by RFC), then take over from Pol α.

In lagging strand synthesis, when the replicative polymerase reaches an end of a previous Okazaki fragment, it partially displaces this fragment by ongoing DNA synthesis, and a flap structure is generated. Flap structure-specific endonuclease-1 (FEN-1, Rad27 in *S. cerevisiae*) cuts out this structure and the resulting nick is sealed by DNA ligase1 (Cdc9 in *S. cerevisiae*). Since the coordination between the FEN1 and Pol δ is more efficient than coordination between the FEN1 and Pol ε, the Pol δ is proposed to act on the lagging strand (Moldovan *et al.*, 2007).

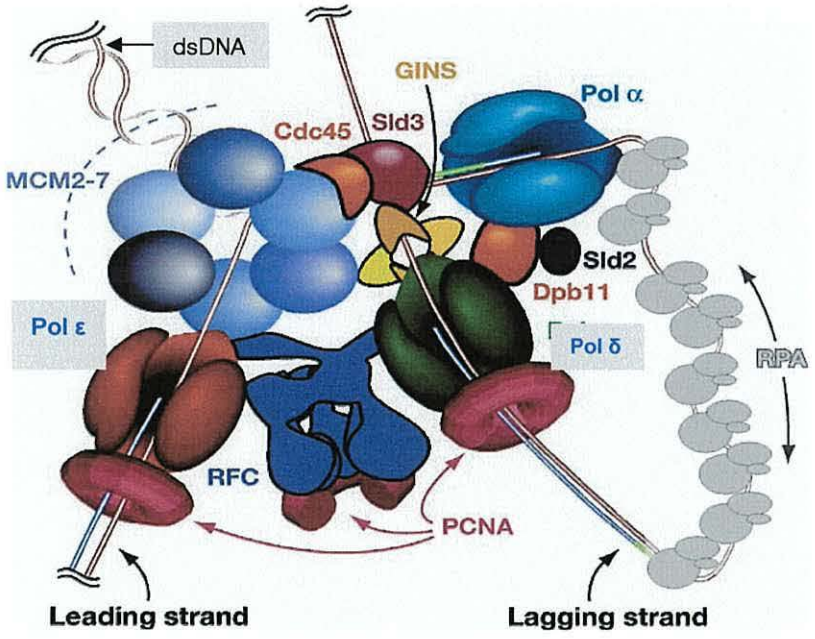


Figure 1.4 Model of *S. cerevisiae* DNA replication fork.

Hypothetical arrangement of proteins at the *S. cerevisiae* replication fork. The hexameric MCM complex encircles the leading strand, unwinding the double-stranded DNA. In this model, Pol ε is placed on the leading strand and Pol δ on the lagging (they are believed to work on both strands), with RFC bridging the two polymerases and helicase. Pol α / primase action places it on the lagging strand along with RPA bound to the looping single-stranded DNA. Other factors involved in replication and known to bind certain proteins at the replication fork include Cdc45, Sld2, Sld3, Dpb11, and the heterotetrameric GINS complex.

Modified from (Johnson and O'Donnell, 2005)

The catenation and positive super coiling ahead of the replication fork are counteracted by topoisomerases I and II. Cyclin-dependent kinase (Cdk) activity remains high from S phase throughout mitosis and prevents re-formation of the pre-replicative complex (Lambert and

Carr, 2005a). Cessation of Cdk activity permits the assembly of pre-RC for the next cell cycle (reviewed in Johnson and O'Donnell, 2005).

1.6 Replication fork progression and transcription

In *E. coli,* DNA replication proceeds throughout the whole life cycle. Furthermore, the speed of the replication fork movement is an order of magnitude faster than that of RNA polymerase. The combination of these two factors makes collisions between replication and transcription inevitable (Brewer, 1988; Rudolph *et al.*, 2007). The polymerase complexes charged with these tasks share the same template and so there is a potential for conflict between the two (Rudolph *et al.*, 2007).

Studies, both *in vivo* and *in vitro*, indicate that RNA polymerase complexes can indeed arrest the progress of replication forks and that cell viability is endangered as a result. The data on the organization of bacterial genomes revealed that there is a bias towards co-directional alignment of transcription units with replication, pointing to selection against head-on collisions (Blattner *et al.*, 1997). Studies in *E. coli* by Mirkin and Mirkin (2005) showed that collision between replication and transcription are avoidable when both the processes proceed in the co-directional orientation. By contrast head on collision lead to severe inhibition of the replication fork progression. This is basically collision between the RNA polymerase (e.g.RNAPIII transcribing tRNA genes) and DNA polymerase (e.g. Pol δ and ε involved in DNA replication).

Replication fork stalling that requires fork restart and / or activation of checkpoint mechanisms in the absence of any external damage is a common event in both bacteria (Cox *et al.*, 2000) and eukaryotes (Cha and Kleckner, 2002). Replication forks could restart from either endogenous DNA damage (e.g., oxidation) or replication barriers. Modification of damaged replication forks is emerging as a crucial factor for efficient chromosomal duplication and the avoidance of genetic instability (Calzada *et al.*, 2005).

1.6.1 Natural pause sites in E. coli

Orderly replication of DNA is a prerequisite to the faithful segregation of the chromosomes during cell division. This process can be perturbed by natural pausing at specific pause sites

or due to damage in the DNA or DNA binding proteins (Rothstein *et al.*, 2000). Terminators of DNA replication can play special biological roles in ensuring a proper genomic replicative program (Rothstein *et al.*, 2000), such as termination of *E. coli* replication by *Ter* sites (Hill, 1992). In fact, studies in *E. coli* have given us our first detailed insight into the importance of a replication termination signal in controlling genome duplication (Neylon *et al.*, 2005). As previously mentioned, *E. coli* has circular chromosome and the bidirectional replication of the chromosome starts at one origin and ends at diametrically opposed termination sites (Figure 1.3).

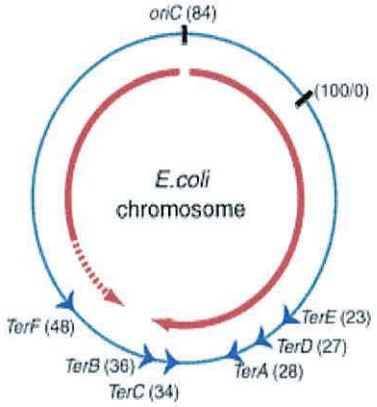


Figure 1.5: Origin of replication and termination in *E. coli*

In the *E. coli* circular chromosome, the position of *oriC* and the six *Ter* sites are shown. The red arrows represent the direction of DNA replication initiated from *oriC*. The two forks approach the terminus region at approximately the same time having passed unimpeded through the *Ter* sites; the counter clockwise replication fork encountered a replication block and restarted replication (broken red line). As a result, the clockwise replication fork enters into the terminator region and, in this example, pauses briefly at *TerC*, continues to *TerB*, and again pauses to await the arrival of the counterclockwise fork. Refer to text.

Adapted from; (Rothstein et al., 2000)

The terminus region is flanked by specific nucleotide sequences, the *Ter* sites, which are bound by the protein Tus (for reviews see Hill, 1992 and 1998). The *Ter*-Tus complex blocks the progression of replication forks in a polar manner by inhibiting the unwinding action of replicative helicase, DnaB (Sahoo *et al.*, 1995).

Ter sites have a role in the maintenance of genome integrity by limiting the encounter of two replication forks to a defined region of the chromosome. Nevertheless, arrest of the

replication fork leads to the formation of a Y structure, with a single-stranded gapped region and DNA ends from the newly replicated strands at the fork junctions, any of which can be substrates for DNA recombination enzymes (Michel, 2000). Consequently, *Ter* sites have the potential to stimulate homologous recombination and thus compromise genome integrity, possibly via multimerization (Rothstein *et al.*, 2000). The first link between replication arrest at *Ter* and homologous recombination was described by Horiuchi and co workers (Horiuchi *et al.*, 1994). The *dif* locus (deletion-induced filamentation) of *E. coli* is a resolvase site capable of driving de-multimerization, located close to the *ter* region of the chromosome, which reduces chromosome multimers to monomers. The *xerC* and *xerD* genes encode the resolvase proteins which function at *dif* site (Tecklenbug *et al.*, 1995). In strains in which this site has been deleted, a fraction of the cells are filamentous, have abnormal nucleotide structure, and exhibit elevated levels of the SOS repair system.

Eukaryotic replication termination is thought to occur randomly in the intervening regions between replication origins (Zhu *et al.*, 1992). However, at several genetic loci, replication termination is site-specific. Some homologous and illegitimate recombination events have already been correlated with replication pauses or arrest sites when the stalled forks are not stabilized leading to genetic instability (Bierne and Michel, 1994; Krings and Bastia, 2004; Cha and Kleckner, 2002).

1.7 Regulation of the repliosome at a paused eukaryotic DNA replication fork.

Replication through obstacles can make the repliosome pause or stall. The S-phase checkpoint responds to replication fork (RF) stalling and to intra-S-phase damage (mainly ssDNA gaps and DNA DSBs), preventing the firing of late replication origins and entry into mitosis. In this way the checkpoint contributes to the maintenance of functional forks by preventing their collapse. Several factors associate with the RF to prevent stalling or collapse. In *S. cerevisiae*, these include the Rrm3 helicase (Ivessa *et al.*, 2003), required for RF progression through natural impediments, Mrc1, which forms a complex with Tof1 and Csm3 and functions in RF maintenance together with the Sgs1 helicase, and the Asf1 chromatin assembly factor (Katao *et al.*, 2003; Calzada *et al.*, 2005; Aguilera and Gomez-Gonzales, 2008) (Figure 1.6).

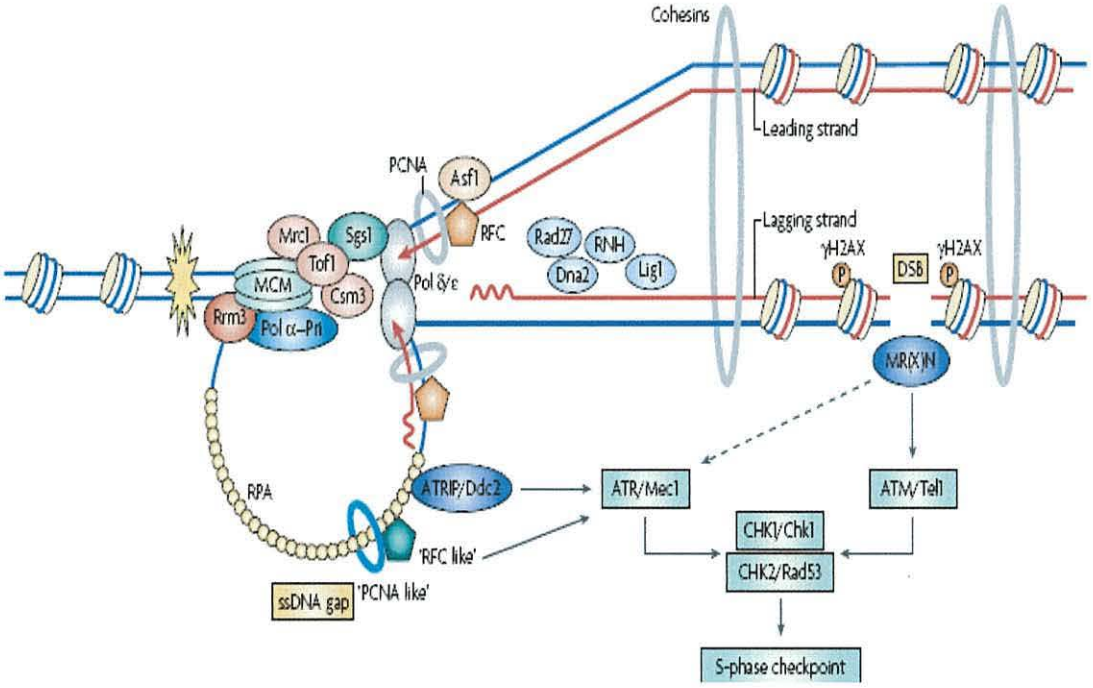


Figure 1.6 Replication fork progression and stalling.

Apart from protein complexes/factors required for DNA replication additional factors are recruited in the RF complex encountering an obstacle.

Encountering an obstacle can cause RF stalling, leading to ssDNA gaps and double-stranded breaks (DSBs). Several factors associate with the RF prevent its collapse, including the *S. cerevisiae* Rrm3 helicase, the Mrc1 checkpoint mediator in association with Tof1 and Csm3, or the nucleosome assembly factor Asf1.

ssDNA gaps and DSBs are sensed by the S-phase checkpoint which is activated through Tel1 (ATM in humans) and Mec1 (ATR in humans). In the case of a DSB, the checkpoint signaling spreads around the DSB site by histone H2AX phosphorylation (γH2AX) in humans (H2A in yeast). ATRIP/Ddc2, ATR/Mec1 interacting protein; CHK1/Chk1 and CHK2/Rad53, serine/threonine-protein kinases; MCM, replicative helicase; MR(X)N, a nuclease complex; RPA, replication protein A; Sgs1, ATP-dependant helicase. (see text)

Adapted from (Aguilera and Gomez-Gonzales, 2008).

The mammalian transducer kinase ataxia telangiectasia mutated (ATM) and ataxia telangiectasia and RAD3 related (ATR) are the key players in triggering the S-phase checkpoint response. ATR acts in response to stalled RFs and other types of damage that lead to the accumulation of ssDNA, such as UV-induced damage or resected DSBs, whereas ATM responds directly to DSBs to which it is recruited through the MRN complex

(MRE11–RAD50–NBS1). ATR is recruited by its cofactor ATRIP, which recognizes RPA-coated ssDNA, but requires further activation by the RAD9–RAD1–HUS1 (9–1–1) replication processivity clamp (PCNA)-like complex, which is loaded onto stalled forks by the RAD17 'RFC-like' complex. ATR and ATM kinases phosphorylate the effector kinases CHK1 and CHK2 to trigger the checkpoint response. In this sequence of events, MCM is phosphorylated, which contributes to its association with active forks; in *S. cerevisiae*, the ATR orthologue Mec1 phosphorylates Sgs1 and Mrc1 to prevent replisome disassembly and collapse (Katao *et al.*, 2003) (Refer to Figure 1.6).

Restarting of the RF is mediated by the S-phase checkpoint to prevent unscheduled recombination. This was deduced from the observation that the budding yeast S-phase checkpoint mutations (such as *rad53*) and mutations in checkpoint target genes (such as *sgs1*) cause fork collapse in the presence of replication inhibitors, and accumulation of Holliday junction structures (Lambert *et al.*, 2005b). If DSBs are generated, histone H2AX is phosphorylated (γH2AX) at its C-terminal tail as one of the earlier events occurring at the break site. γH2AX spreads around the break, thus amplifying the initial damage signal and resulting in large, megabase-long chromatin domains that are responsible for the stable accumulation of damage-response and cohesion factors that favour repair by sister-chromatid exchange (Strom *et al.*, 2004). If this entire process is disrupted by replication stress or S-phase checkpoint inactivation, breaks accumulate that could trigger genomic instability.

Sgs1 (in *S. cerevisiae*) or Rqh1 (in *S. pombe*) belong to the RecQ family of DNA helicases, which is highly conserved in evolution from bacteria to mammals (Bachrati and Hickson, 2006). The family name was derived from its *E. coli* member, RecQ. This helicase is important in unwinding the DNA ahead of the replication fork and it is known to be part of a complex with topoisomerase (Karow *et al.*, 2000). There are five human RecQ family members (RECQ1, BLM, WRN, RECQ4 and RECQ5) and defects in three of them give rise to inherited human disorders such as Bloom's syndrome (BLM), Werner's syndrome (WRN) and Rothmund-Thomson syndrome and RAPADILINO (RECQ4).

BLM is mutated in Bloom syndrome, which is characterized by growth retardation, sunlight sensitivity, immunodeficiency, genomic instability, and a high incidence of cancer. WRN is

mutated in Werner syndrome, which is characterized by premature aging, genomic instability, and a high incidence of cancer. RECQL4 is mutated in Rothmund-Thomson syndrome, which is characterized by skin rash, small stature, skeletal dysplasias, chromosomal instability, premature aging, and a high incidence of cancer. All three of these syndromes involve genome instability and a predisposition to cancer (Sharma *et al.*, 2007).

1.8 Replication fork pauses / barriers and recombination

Replication arrests are associated with genome rearrangements, which result in either homologous or non-homologous recombination. Some proteins involved in homologous recombination are also able to convert an arrested replication fork into a recombination intermediate, which promotes replication restart and thus presumably prevents genome rearrangements (Michel, 2000). Eukaryotic cells regulate the progression and integrity of DNA replication forks to maintain genomic stability and couple DNA synthesis to other processes.

Eukaryotes chromosomes do not have *ter*-like elements and to date little progress has been made to determine the nature of general genome DNA replication termination (Rothstein *et al.*, 2000). In eukaryotes, sequence-specific termination seems to be the exception rather than the rule. However, replication fork barriers (RFBs) and replication fork pauses (RFPs) have been located at different genomic regions. RFP sites are defined as transiently arresting replication fork movement and RFBs are defined as factors that cause irreversible 'blocks' during replication of genome (Hyrien, 2000).

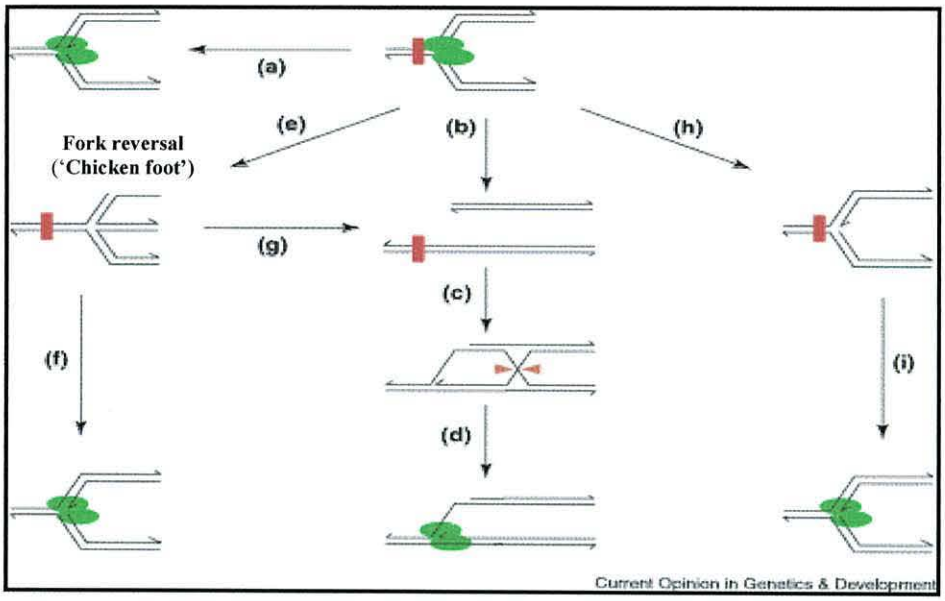
Natural RFBs occur within the rDNA and centromeric regions of many organisms and at other genomic loci such as tRNA genes (Dashpande and Newlon,1996) and the *RTS1* (Replication Termination Sequence 1) at the *mat1* locus in *S. pombe* (Vengrova *et al.*, 2002). In eukaryotes RFB sites have been identified in rRNA gene repeats (rDNA) from yeast to human (Rothstein *et al.*, 2000). A RFB has been located in the 3' end of the rRNA genes in *S. cerevisiae*. This RFB requires the *FOB1* gene (for fork blocking) for its activity. The biological roles of the RFB is thought prevent collision between the replication and transcription machineries (Kobayashi, 2003).

1.8.1 Replication restart from pauses

Chromosome replication is not a uniform and continuous process. The repliosome deals with replication barriers in different ways dependent on the nature of the block (McGlynn, 2004; Michel *et al.*, 2001). Firstly, the repliosome might remain associated with the fork and simply pause, restarting once the block has been removed (Figure 1.7a). Alternatively the block might result in the collapse of the RF resulting in the fork being exposed to other processing molecules, such as recombinases. The block might be dealt with following repliosome collapse and then the repliosome is re-established at the RF and replication proceeds (Figure 1.7A i). It is possible that the fork might break, possibly due to a nick in one of the template strands, resulting in the formation of a DSB (Figure 1.7A b). This would require processing by recombination proteins resulting in the re-establishment of the replication fork (Figure 1.7A c and d). Another alternative is that there is replication fork progression following dissociation of the repliosome; this will generate a structure known as the 'chicken foot' (fork reversal) which resembles a Holliday junction (Figure1.7 e). Such a structure could be processed in a number of ways, including resolution to a DSB (Figure 1.7A g), which would require a subsequent recombination processing to restore the replication fork, or re-conversion directly to a functional replication fork once the blockage is removed (Figure 1.7A f).

Another alternative is that the replication machinery can simply by pass the block, depending on the nature and the strand on which it is located (Figure 1.7B). (Replication bypass of DNA damage-induced lesions will not be discussed here). Studies in *S. cerevisiae* demonstrated that elimination of *MEC1* (ATR homolog) leads to fork stalling and chromosome breakage at genetically encoded replication slow zone (RSZ) (Cha and Kleckner, 2002), similar to that of mammalian fragile sites, which are triggered by delayed progression through normally late-replicating regions (Laird *et al.*, 1993). These resemble fragile sites in humans which become more fragile in the absence of ATR. These regions in the chromosomes are prone to formation of secondary structures which could provide obstacles for the replication fork, resulting in increased chromosomal rearrangements (Mirkin and Mirkin, 2007).

A



B

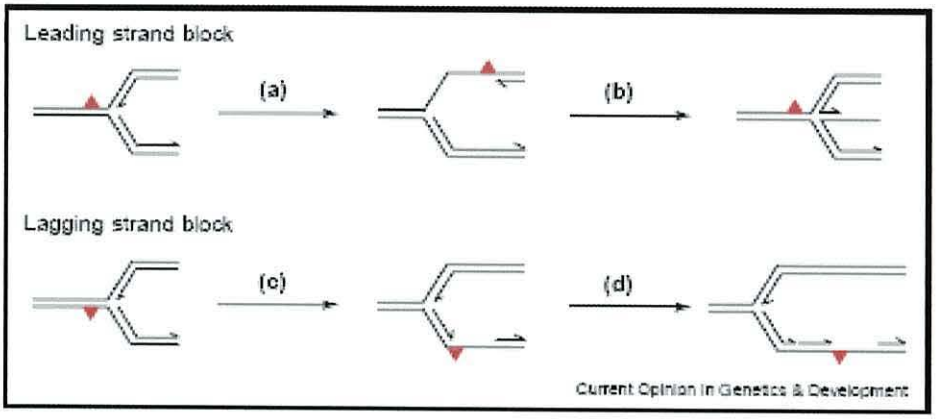


Figure 1.7 Replication restart from pauses / blocked replication forks

A. Possible pathways taken when replication blockage occurs in a chromosome. Refer to text for details.
B. By passing of replication block. The way in which the repliosome deals with a block can be dependent upon whether or not there is strand specificity to the block. a & b leading strand block; this might result in fork regression and the generation of are recombinogenic structure.
c & d lagging strand block ; might result in block or lesion by pass, resulting in gap which is repaired post replication.

Adapted from; (McGlynn, 2004)

1.9 Replication stalling in *RTS1* in *S. pombe*

RTS1 is a well characterised, polar eukaryote RFB. *RTS1* is a site-specific replication terminator. It is located proximal to the mating type locus *mat1* in *S. pombe*, which regulates the direction of replication at *mat1*, optimizing mating-type switching that occurs as a replication-coupled recombination event (Codlin and Dalgaard, 2003).

S. pombe exhibit two different mating types, called plus (P) and minus (M). During starvation, haploid cells of opposite mating types mate and the resulting zygote then undergoes meiosis and sporulation to produce four haploid spores. Homothallic haploid cells are able to switch between the mating types during mitotic growth in a distinct asymmetrical pattern (Figure 1.8). Mating type switching is determined by the *mat1* locus located on chromosome II (Dalgaard and Klar, 2000).

1.9.1 Mechanism of mating type switching

The *S. pombe* mating type region consists of three gene cassettes, *mat1*, *mat2P* and *mat3M*, each flanked by the homology boxes *H1* and *H2* (Figure 1.9A). The donor loci *mat2P* and *mat3M*, containing P (plus) and M (minus) information respectively are transcriptionally silenced (Kelly *et al.*, 1988). *mat1*, which contains either P or M information, is transcriptionally active and determines the mating type of the cell. Switching between mating types occurs via a recombination event between *mat1M* and *mat2P* or *mat1P* and *mat3M* cassettes, which replaces the information at *mat1* with the information of the opposite mating type (for review see Dalgaard and Klar 2001). A remarkable feature of this system is that switching occurs in a highly regulated fashion (Figure 1.8). The cell that is incompetent to switch (Pu) produces two daughter cells that are of same mating type but having switchable and unswitchable traits in them. The subsequent replication cycle would produce four daughter cells of which one would have a switched mating type. These specific characteristics are determined by imprint in the *mat1* locus. *RTS1* plays a major role in imprinting process thus contributes to mating type switching in *S. pombe*.

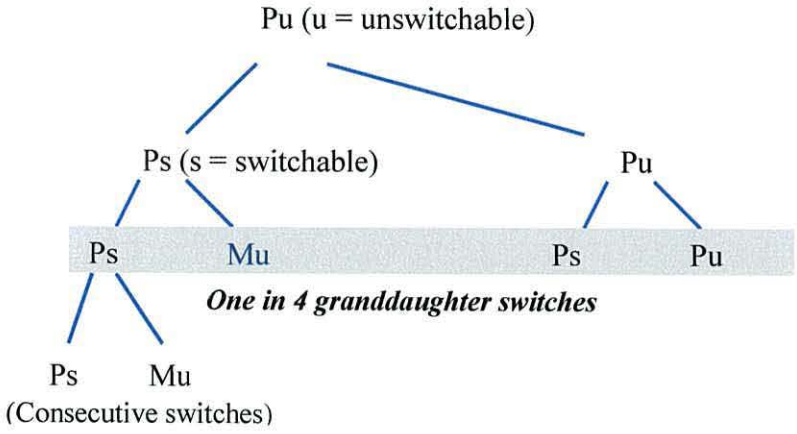


Figure 1.8 The pattern of *mat1* switching in cell pedigrees of haploid *S. pombe* cells

In a first replication cycle, a single mating type P (unswitchable cell) produces two daughter cells of one is switchable and another unswitchable of the same mating type (P). The second cycle of replication produces one mating type switched cell and 3 unswitched cells). In the third replication cycle the swichable cells will produce two daughter cells of different mating types. Refer to Text

Adapted from (Klar, 2007)

The programmed recombination at *mat1* requires a replication pause to occur adjacent to the H1 site, at the right hand side of the expressed *mat1* gene (Figure 1.9A). This pause is thought to result in an imprint (Imp in Figure 1.9B) which is proposed, generates a recombination initiating lesion (Vengrova and Dalgaard, 2004). To ensure that the imprint (Imp) is set correctly at the Imp site, DNA replication must come from a specific direction, right to left in Figure 1.9. To ensure that this occurs, a polar replication barrier, *RTS1*, is located at the distal side of *mat1* relative to Imp site.

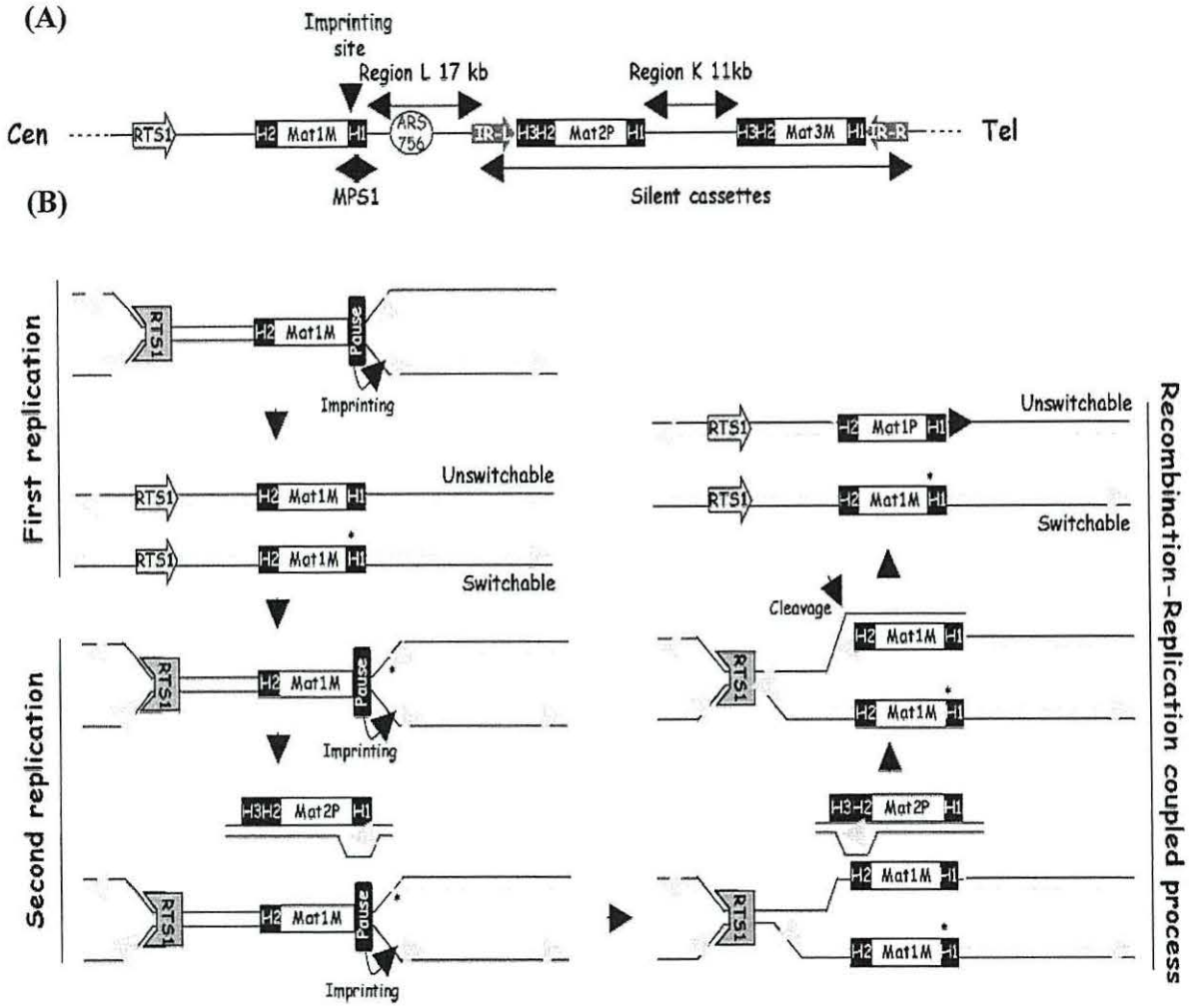


Figure 1.9 Outline of the mating-type region and the mating type switching of *S. pombe*.

A. The *mat1* cassette is expressed; *mat2P* and *mat3M* are unexpressed transcriptional silent cassettes. H1 and H2 are homologous regions present on each cassette. H3 is specific to *mat2* and *mat3*. Site specific recombination between H regions drives mating type switching. A DNA strand-specific imprint at *mat1* initiates a gene conversion event leading to transfer of a copy of *mat2* or *mat3* genetic information to *mat1*.The reaction is non reciprocal gene conversion reaction and so the information at *mat2P* and *mat3M* is maintained. IR-L and IR-R are inverted repeats regions. The *RTS1* is the replication termination site. *MPS1*: mating type pause site (Refer to Text).

B. Mating type switching in *S. pombe* cells. The fork traveling in the opposite direction pauses at the *MPS1* site, allowing imprint in the lagging strand (the imprint is marked *) each time the locus is replicated. Thus one cell inherits an unswitchable chromatid whereas the other one inherits switchable one (Refer to Text).

Adapted from (Lambert *et al.*, 2007).

RTS1 is thought to function to prevent, or limit, DNA replication coming from a direction where the *mat* imprint will not be established, as the pause generating signal at Imp is polar (Dalgaard and Klar, 2001). The imprint was identified as a RNase-sensitive modification and consists of one or two RNA residues incorporated into the *mat1* DNA (Vengrova and Dalgaard, 2004; Vengrova and Dalgaard (2006). The RNase-sensitive modification in the DNA is required for the initiation of the replication-coupled recombination event that leads to mating type switching event in *S. pombe*.

During the first replication, any fork traveling from the centromere to telomere is stalled by *RTS1*. The fork traveling in the opposite direction pauses at *MPS1* site in *mat1*, allowing introduction of imprint in the lagging strand (marked * in Figure 1.9B) each time the locus is replicated. Thus one cell inherits an unswitchable chromatid whereas the other one inherits a switchable chromatid.

During the second round of DNA replication, the leading strand is synthesized until it reaches the imprint (Figure 1.9B). This results in a polar DSB with a 3' end which can invade the H1 box of *mat2* or *mat3* to initiate a gene conversion event. Then the leading strand's template is cleaved and re-synthesized using the newly synthesized leading strand template. Therefore, the two strands of the switched *mat1* cassette are synthesized *de novo*. Thus, one cell inherits a switched but unswitchable chromatid and the other one inherits a switchable chromatid.

1.9.2 The structure and function of RTS1

RTS1 consists of an 859 bp region which has four 55 bp repeat elements (marked B Figure 1. 10) and a purine-rich region of approximately 60 bp (marked A Figure 1.10). *RTS1* RFB function requires the actions of at least four *trans* activators, Swi1, Swi3, Rtf1 and Rtf2 and is thought to have similarities to human rDNA RFBs (Codlin and Dalgaard, 2003). Rtf1 binds to repeat region B (Figure 1.10) and Rtf2 is proposed to bind to the purine-rich region A (Figure 1.10). Swi1 and Swi3 are proposed to function as a link between the Rtf1-Rtf2-*RTS1* complex and the repliosome to stall the progression of DNA replication. Another associated *trans* factor, Swi7, is DNA polymerase α which is primarily involved in lagging-strand synthesis (Singh and Klar, 1993). The '*swi*tching' genes (*swi*) have been known to be

important in the mating type switching. *swi1* is conserved among eukaryotes, and homologues include *TOF1* (*S. cerevisiae*) and *Tim* (*Timeless*) family of genes in higher eukaryotes (Dalgaard and Klar, 2000). In the *swi1, swi3, swi7* mutants, reduction in the *mat* DSB have been observed (Codlin and Dalgaard, 2003). This observation supports the idea that the *mat* DSB is the rate-limiting step for switching (Egel *et al.*, 1984). The Swi1 and Swi3 proteins promote imprinting both by pausing replication at *mat1* and by terminating replication at *RTS1* (Dalgaard and Klar, 2000).

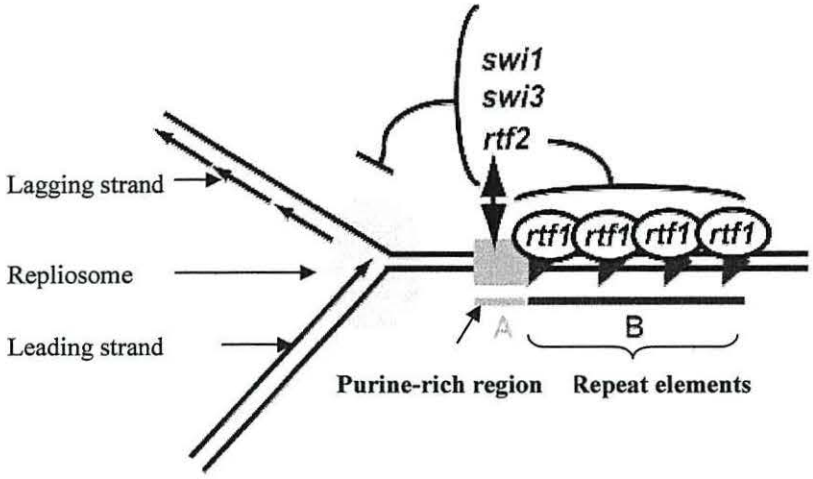


Figure 1.10 Model for the termination of DNA replication at *RTS1* RFB by *trans* and *cis* factors.

RTS1 RFB function requires a number of *cis* factors; *RTS1* has purine-rich domain (A) and outer repeat elements (B). A line drawing of a replication fork stalled at *RTS1* is showing putative binding of Rtf1 to the repeated motifs. Barrier activity also depends on the *trans*-acting factors Swi1 and Swi3, which might act at the replication fork along with Rtf2 at the purine-rich domain.

Adapted from (Codlin and Dalgaard, 2003)

The Swi1-Swi3 complex is also required for the survival of fork arrest and for activation of the replication checks point kinase Cds1, thus the complex is called a fork protection complex and it is proposed to coordinate leading and lagging strand synthesis and stabilizes stalled replication forks (Noguchi *et al.*, 2004; Noguchi *et al.*, 2003). In agreement with this, the frequency of ade+ recombinants in an intra chromatid *ade6* recombination reporter system was reported to be significantly higher in a *swi1Δ* strain than in the equivalent wild-type strains, possibly due to collapse of the replication fork leading to increased

recombination (Ahn *et al.*, 2005; Sommariva *et al.*, 2005). Other studies suggest that Swi1 and Swi3 act at several different protein-DNA complexes in the rDNA spacer regions to arrest replication but not all fork barriers required their activity to arrest forks (Krings and Bastia, 2004).

1.10 Transfer RNA (tRNA) genes and recombination

Transfer RNA (tRNA), is a small RNA chain (73-93 nucleotides) that transfers a specific amino acid to a growing polypeptide chain in the ribosome during translation. It has a 3' terminal site for amino acid attachment. This covalent linkage is catalyzed by an aminoacyl tRNA synthetase. It also contains a three base region called the anticodon that can base pair to the corresponding three base codon region on mRNA. Each type of tRNA molecule can be attached to only one type of amino acid, but because the genetic code contains multiple codons that specify the same amino acid, tRNA molecules bearing different anticodons may also carry the same amino acid.

Most tRNA genes distributed throughout eukaryotic genomes, are frequently found in multicopy families (Pratt-Hyatt *et al.*, 2006) and are said to have close association with retroelements / retrotransposons (Ty elements) in *S. cerevisiae* (Hani and Feldmann, 1998). tRNA genes have been found in clusters in centromere regions in *S. pombe* (Takahashi *et al.*, 1991; Kuhn *et al.*, 1991). This high-density distribution of tRNA genes in the centromere regions is surprising, as the fission yeast centromeres were thought to form transcriptionally inactive structures (Takahashi *et al.*, 1991). Recombination between dispersed repetitive DNA is a common source of genetic variation (Virgin and Bailey, 1998). Therefore the presence of tRNA genes at certain part of the genome could act as a programmed pause site, since RFPs activity has been found to be a general property of tRNA genes (Deshpande and Newlon, 1996).

1.10.1 tRNA gene transcription and RFP activity

RNA polymerase III is required for the transcription of eukaryotic tRNA genes. Transcription complex assembly on tRNA genes occurs in three steps; first TFIIIC binds to the intragenic promoter element, boxA and box B. TFIIIC then promotes binding of TFIIIB to a region upstream of the transcription start site (Deshpande and Newlon, 1996). tRNA

genes in *S. cerevisiae* were shown to behave like RFP sites by transiently arresting replication fork movement. These RFP sites stall replication forks only when they oppose the direction of tRNA transcription (Deshpande and Newlon, 1996). Likewise, tRNA transcription occurring in the absence of replication does not affect either recombination or replication fork progression.

In *S. cerevisiae*, tRNA gene transcription by RNA polymerase III (polIII) suppresses nearby transcription by RNA polymerase II (polII) (Pratt-Hyatt *et al.*, 2006). Transcription of genes by RNA polymerase II is also reported to increases their susceptibility to recombination (Prado and Aguilera, 2005), but little is known about susceptibility to recombination for RNA pol III transcription units (Saxe *et al.*, 2000). Pratt-Hyatt *et al.*, (2006), demonstrated that recombination was elevated between two transcriptionally active tRNAs genes. In this respect, RNA pol III transcription appears to behave similarly to RNA pol II transcription in that transcription stimulates recombination (Mirkin *et al.*, 2006; Aguilera, 2002). These phenomena are termed transcription-associated recombination (TAR) (Prado and Aguilera, 2005). However, using specifically designed plasmid-borne constructs Prado and Aguilera (2005) demonstrated that transcription by itself is not sufficient to induce recombination and TAR requires replication fork progression opposite to transcription and it is associated with the appearance of a RFP.

Most tRNA genes from eukaroytes are organized as monomeric transcription units, unlike genes from *E. coli* and *Bacillus subtilis* which are generally clustered and give rise to multimeric transcripts. In many cases the multiple genes coding for one specific tRNA are dispersed throughout the genome, with the members of such a gene family maintaining a common nucleotide sequence during evolution (Amstutz *et al.*, 1985). Five cases of dimeric tRNA genes have been found in yeasts, including three $tRNA^{Ser}$-$tRNA^{Met}$ dimers from *S. pombe* (Hottinger-Werlen *et al.*, 1985). The dimeric arrangement might be desirable for cells to co-regulate the appearance of the products of the two genes or it may be that the dimeric structure is required to permit proper function of one or the other of the genes; for example, if the $tRNA^{Met}$ gene were by itself a poor promoter, coupling it to the $tRNA^{Ser}$ could increase its rate of transcription (Hottinger-Werlen *et al.*, 1985).

The codons UAA, UAG and UGA are chain terminators for polypeptide synthesis. Suppressor tRNAs recognizing the different termination codons have been characterized at the nucleotide sequence level in prokaryotes. These nonsense suppressor tRNAs differ from their corresponding wild type tRNAs by base change in the anticodon. Nucleotide sequence of the opal suppressor $tRNA^{SER}$ was isolated and sequenced from *S. pombe* strain *sup3-e* (Rafalski *et al.*, 1979). The $tRNA^{SER}$ would insert serine at the UGA codon, thus suppressing the termination codon.

The role of tRNA genes as possible RFB that induce genetic instability is actively studied. Admire and co-workers (2006) attributed chromosome translocation in *S. cerevisiae* partially to tRNA genes. They explored a genetic system to identify naturally occurring sites which served as fragile sites for the generation of genomic changes such as translocations (Admire *et al.*, 2006). Low levels of dNTPs, defects in a replication helicase (Rrm3) and defects in replication checkpoint controls were factors associated with increase of chromosomal instability (Cha and Kleckner, 2002; Prado and Aguilera, 2005; Admire *et al.*, 2006). In addition , Admire and coworkers (2006) observed that instability frequently involved a site that contains multiple tRNA genes and when this site was deleted the frequency of unstable chromosomes were reduced, implicating tRNA genes in driving genomic instability.

tRNA gene position effect can influence the expression of some chromosomal genes, and the Ty retrotransposons gene in *S. cerevisiae*. Extrapolation to the entire genome indicates that as many as 12 genes in the yeast genome were down-regulated by tRNA genes position effects by 3.5- fold or more, and there may be many more affected to a lesser extent (Bolton and Boeke, 2003). Retroelement insertion can also alter the expression of nearby genes. The five Ty retrotransposons found in the budding yeast *S. cerevisiae* apparently target their integration to nonessential regions or "safe havens." The *S. cerevisiae* retrotransposons Ty1–Ty4 are transcribed by RNA pol II and target their integration upstream of genes transcribed by RNA pol III, mainly tRNA genes. Since tRNA genes can repress nearby RNA pol II-transcribed genes, it is hypothesized that transcriptional interference may exist between Ty1 insertions and RNA pol III-transcribed genes, the preferred targets for Ty1 integration (Bolton and Boeke, 2003). This implies that tRNA genes could possibly function to promote gene silencing thus indirectly help to maintain stability of the genome.

1.11 Genome maintenance and DNA repair

The DNA physiochemical constitution alone does not guarantee life-long stability or proper function. Faulty genome maintenance not only leads to cancer but also to premature aging (Thoms *et al.*, 2007). Exposure to environmental genotoxic agents such as ultraviolet light (UV), ionizing radiation and numerous genotoxic chemicals including thousands in cigarette smoke can cause alteration to the DNA structure. By products of normal cellular metabolism also constitute a problem to DNA integrity from within. These by products include reactive oxygen species (superoxide anions, hydroxyl radicals and hydrogen peroxide) derived from oxidative respiration and products of lipid peroxidation. Some chemical bonds in DNA tend to spontaneously disintegrate under physiological conditions; this may be due to hydrolysis of nucleotide residues leaving non-instructive abasic sites, spontaneous or induced deamination of cytosine, adenine, guanine or 5-methylcytosine which converts these bases to the miscoding uracil, hypoxanthine, xantine and thymine respectively. The out come of DNA damage, if left unrepaired may lead to mutations triggering cell-cycle arrest or death and enhance cancer risk (Hoeijmakers, 2001).

Many of these lesions block transcription and trigger the DNA replication checkpoint. This is a complex signal transduction pathway, present in all eukaryotic cells, that functions to maintain genomic integrity and cell viability when DNA replication is perturbed. In view of the diversity in types of DNA lesions that are possible, there is no single repair process that can cope with all kinds of damage. Many repair pathways are essential and therefore are highly conserved in evolution across the prokaryotic and eukaryotic world. There are at least five main, partly overlapping damage repair pathways operating in mammals (Hoeijmakers, 2001), namely nucleotide-excision repair (NER), base-excision repair (BER), mismatch repair, homologous recombination (HR) and non-homologous DNA end joining (NHEJ).

Briefly, NER deals with the wide class of helix-distorting lesions that interfere with base pairing and obstruct transcription and normal replication. Small chemical alterations of bases are targeted by BER. These lesions may or may not be impede transcription and replication, although they frequently miscode, therefore they are particularly relevant for preventing mutagenesis. Most NER lesions arise from exogenous sources whereas BER is mostly

concerned with damage of endogenous origin. In this chapter we will concentrate on the repair pathways for DNA DSBs which are HR and NHEJ.

1.11.1 DNA double-strand breaks and their repair

DSBs can be caused by external factors such as ionizing radiation, chemicals or endogenous factors such as free radicals, single-stranded breaks encountered by DNA replication, and collapsed replication forks (van Gent *et al.*, 2001). Extensive DNA damage due to DSBs can result in cell death, which can occur due to cells entering a state of irreversible growth arrest (replicative death) or they can trigger apoptosis. DSBs can also be beneficial when they occur in a controlled manner in the context of specialized events that demand that genome sequences be rearranged, such as during development of the immune system [(V(D)J recombination)] and homologue conjoining in meiosis (Wyman and Kanaar, 2006).

HR repair (HRR) comprises a series of interrelated pathways that function in the repair of DNA DSBs, and provides support for DNA replication in recovery of stalled or broken replication forks, contributing to tolerance of DNA damage (Li and Heyer, 2008). Following DSB formation, a complex cascade of events is initiated to slow down the cell cycle and recruit DNA repair enzymes. After DNA replication, cells utilize HRR (Figure 1.11) because of the existence of a second identical sister chromatid. HR occurs between sister chromatids during late S and G2 of the cell cycle and this is a high fidelity repair process (Jackson, 2002). In contrast NHEJ (Figure 1.12) is error prone (as it can generate deletions) and is more prevalent in G1 prior to DNA replication (Jackson, 2002). Malfunction of either of the repair pathway results in enhanced cellular genomic instability (Khanna and Jackson, 2001).

Diploids cells in G1 have only the homologous chromosome for HR. NHEJ simply links ends of a DSB together, without any template, using the end-binding KU70/KU80 complex and DNA-PK_{CS}, followed by ligation by XRCC4-ligase IV (Figure 1.13). The KU70/KU80 complex might be involved in end protection and approximating the ends, in addition to a signaling function by DNA-PK_{CS}. NHEJ may be further facilitated when the ends are still held together within the nucleosome or other structures. The main disadvantage of this repair method is that it can contribute to the loss or gain of a few nucleotides therefore it is considered error prone (Li and Heyer, 2008; Hoeijmakers, 2001; Weterings and David, 2008).

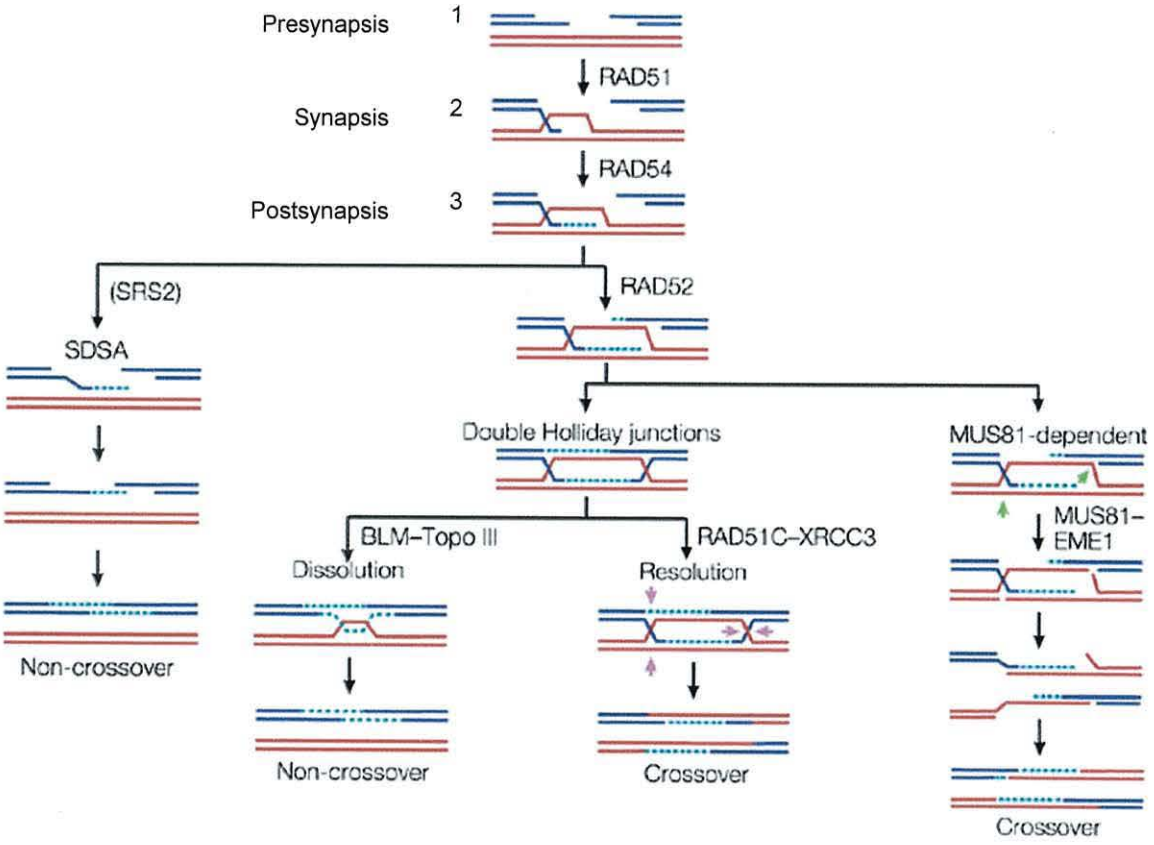


Figure 1.11 Double strand break repair through homologous recombination.

HR can be conceptually divided into three stages: presynapsis, synapsis and post synapsis. During presynapsis, DSB ends are recognized and processed to a 3′-OH ending single-stranded tail (steps 1-2). In synapsis, DNA strand invasion by the Rad51-ssDNA filament generates a D-loop (step 3). At least three different pathways are proposed after the D-loop intermediate.

The assembly of a RAD51 nucleoprotein filament leads to interactions with homologous duplex DNA and strand invasion. This process is known as single-end invasion (SEI) and the intermediate structures might be stabilized by the RAD54 protein.
In some pathways for recombination (centre), SEI is followed by capture of the second DNA end in reactions that are likely to involve RAD52. This intermediate can proceed to form double Holliday junctions, and any remaining gaps might be filled by new DNA synthesis. The resulting Holliday junctions might then serve as the substrate for a classic Holliday-junction-resolution reaction, involving RAD51C, XRCC3 and other as-yet-unidentified factors, or be dissociated by the combined actions of BLM (Bloom's syndrome protein) and topoisomerase IIIα (Topo III). The BLM–Topo-III reaction primarily leads to the formation of non-crossover products, as mutations in BLM cause an increase in crossover formation. Recombinants can also form by a MUS81-dependent pathway that does not involve Holliday-junction formation (right).

Similarly, DSBs can be repaired by synthesis-dependent strand annealing (SDSA), a pathway that is dependent on the SRS2 helicase (left).

Adapted from (Liu and West, 2004; Li and Heyer, 2008)

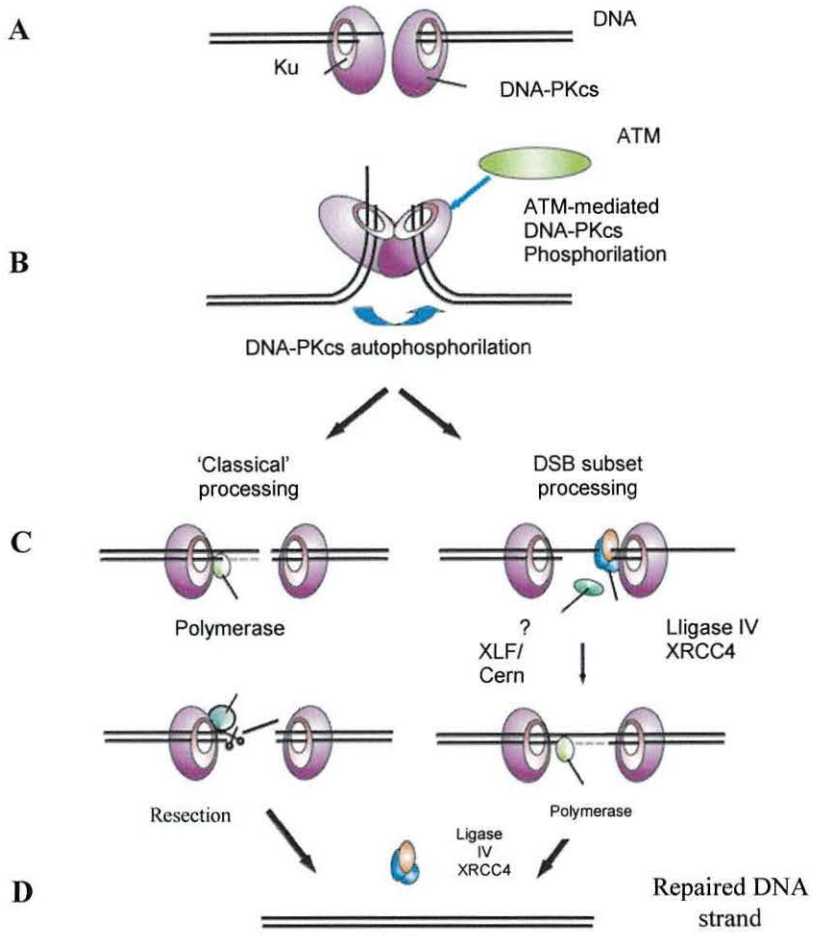


Figure 1.12 A model for double-strand break repair by NHEJ.

(A) The Ku70/80 heterodimer associates with the two ends of the broken DNA molecule. This DNA-Ku scaffold attracts DNA-PK_{CS}, which protects the DNA termini against degradation and premature ligation.
(B) The DNA-PKCS molecules on both DNA ends form a synaptic complex which tethers the DNA ends. Trans DNA-PK_{CS} autophosphorylation then introduces a conformational change that makes the DNA termini accessible for other NHEJ enzymes. In addition to autophosphorylation, ATM-mediated DNA-PKCS phosphorylation may play a role in this conformational change.
(C) Non compatible DNA termini need to be processed before ligation can proceed. This can be done in the 'classical' way, by either filling (polymerases) or resection (Artemis) of single-strand overhangs. The NBS1-MRE11-RAD50 complex might be involved in unwinding and /or nucleolytic processing of the ends.
(D) Ligation of the blunted ends by DNA ligase IV/XRCC4. Alternatively, in a subset of DSBs with partially complementary overhangs, ligase IV/XRCC4 and XLF/Cernunnos mediate the joining of one single-strand overhang with the opposite DNA end, followed by filling of the gap by polymerases.

Modified from (Weterings and David, 2008)

1.12 Possible Role of Translin and the Translin associated factor X (Trax) in oncogenic chromosomal rearrangements and cancer formation

Translin is a highly conserved protein which associates with breakpoint junctions of chromosomal translocations linked with development of some human cancers (Aoki *et al.*, 1995). Translin was originally identified as a protein that binds to a DNA consensus sequence, ATGCAG and GCCC (A/T)(G/C)(G/C)(A/T) (Aoki *et al.*, 1995), found at break points of many chromosomal translocations in cases of lymphoid neoplasms and solid tumours, implicating this protein in oncogenic translocation formation (Kasai *et al.*, 1994, Aoki *et al.*, 1995; Chalk *et al.*, 1997). Subsequently, Translin binding sequences have been identified in other cancer-associated translocation breakpoints (Wei *et al.*, 2003a; Kanoe *et al.*, 1999; Hosaka *et al.*, 2000; Atlas *et al.*, 1998; Abeysinghe *et al.*, 2003).

Chromosomal translocations are associated with carcinogenesis (Weinstock *et al.*, 2006; Aplan, 2006), thus it was hypothesized that Translin might be involved in mediating chromosomal rearrangements including translocations (Gajecka *et al.*, 2006a; Gajecka *et al.*, 2006b) and potentially playing a role in the recognition of staggered DNA ends. Interaction between Hepatitis C virus core protein and Translin protein has been reported in hepato cellular carcinoma and lymphoma caused by Hepatitis C virus, suggesting a possible role of Translin protein in the disease pathogenesis (Li *et al.*, 2003).

The Translin Associated Protein X (Trax), was identified by yeast two hybrid assays (Aoki *et al.*, 1997b) and by immunoprecipitation (Wu *et al.*, 1999a), as a protein that interacts with Translin (Aoki *et al.*, 1997b; Taira *et al.*, 1998). Trax contains a bipartite nuclear targeting motif and has a heptad repeat of hydrophorbic amino acids which may be a leucine zipper (LZ) domain. This latter domain may be important for formation of Trax homodimers or heterodimers with Translin. Both Trax and Translin are co-expressed in many tissues and form a complex with the GS1-DNA complex. GS1 is a protein that binds in a sequence-specific manner to single-stranded DNA and is highly enriched in the brain (Taira *et al.*, 1998).

Mouse Translin was independently identified as the testis brain RNA-binding protein (TB RBP) (Wu *et al.*, 1997) and has been implicated in mRNA metabolism, particularly in

neurones and in the testis (Wu *et al.*, 1997; Gu *et al.*, 1998; Muramatsu *et al.*, 1998; Wu and Hecht, 2000; Chennathukuzhi *et al.*, 2003b; Li and Baraban, 2004; Cho *et al.*, 2005; Yang *et al.*, 2003). In support of this mice and fruit flies lacking translin exhibit a range of neurological behavioural problems (Chennathukuzhi *et al.*, 2003a; Suseendranathan *et al.*, 2007).

1.12.1 Trax and Translin: structure of the genes and protein

The *S. pombe trax* gene (SPCC736.09c) is a non-essential gene, (Decottignies *et al.*, 2003; Laufman *et al.*, 2005; Jaendling *et al.*, 2008). Its open reading frame is 696 bp and codes for a 231 amino acid predicted protein of 26.7 kD (Refer to Figure 3.1). Human and mouse Translin consist of 228 amino acids, of which only three amino acids differ between them (Laufman *et al.*, 2005). The *trax* gene is present as a single copy in the human genome located at 1q42 (Devon *et al.*, 2000).

Both human and mouse Trax proteins, which share 90% amino acid identities, contain identical dipartite nuclear targeting sequence near their N terminus (Refer to Figure 3.1). The *S. pombe* Translin consists of 236 amino acids and *S. pombe* and human Translin share 36% identity and 54% similarity, with higher degree of identity and similarity in the N-terminal half of the proteins (Laufman *et al.*, 2005) (Figure.1.13). Human Translin consists of eight subunits having a highly helical secondary structure that assemble into a ring (Figure 1.15). DNA and RNA are proposed to bind inside the ring (Kaluzhny *et al.*, 2005). In human Translin the C-terminal region contains a putative nuclear export signal and putative GTP binding site. These sites are also highly conserved in the *S. pombe* Translin (Figure.1.13). The DNA binding region required for binding of single-stranded DNA and RNA (ssDNA and ssRNA) in human and mouse Translin is only partially conserved in *S. pombe* Translin.

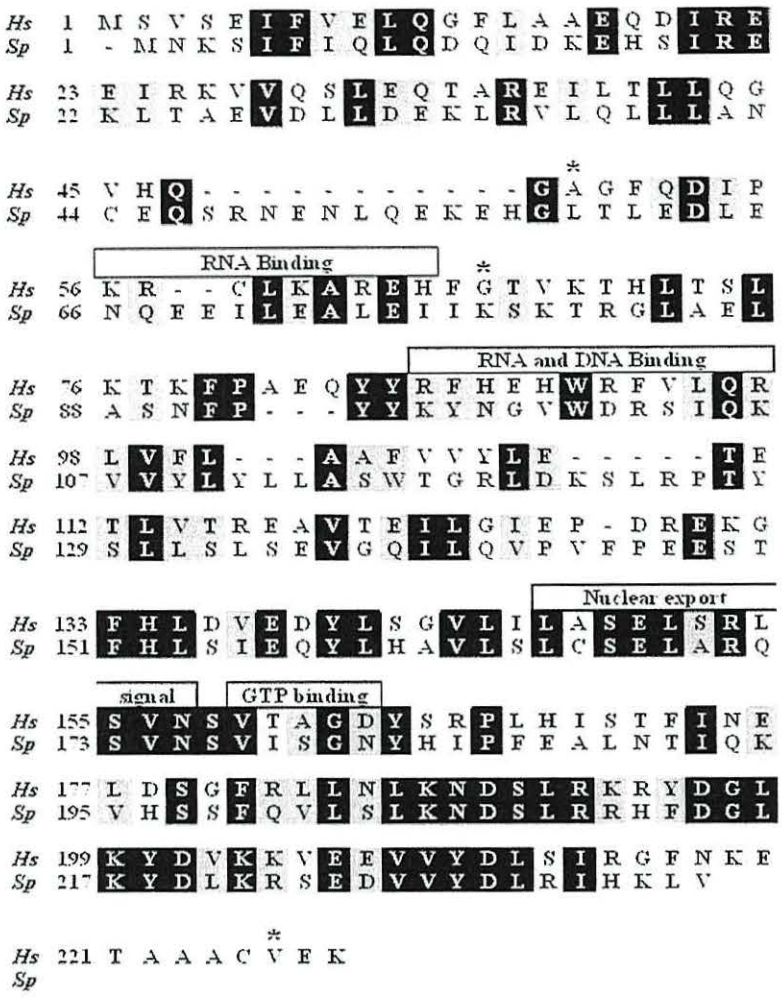


Figure.1.13 Amino acid sequence alignment of the human and the *S. pombe* Translin.

The sequences of the human Translin (*Hs*; *Homo sapiens*) and its *S. pombe homologue* (*Sp*; *S. pombe*) were aligned using BLAST. Identical residues are highlighted in black. Similar residues are highlighted in grey. Previously identified motifs in the human Translin (also shared by the mouse Translin) are marked with boxes and are described in the text. The asterisk indicates the amino acids in the human Translin that have been substituted with other amino acids in the mouse Translin.
Adapted from (Laufman *et al.*, 2005)

Comparison between the human Trax and Translin revealed that they not only share high homology in amino acid sequences of their encoded proteins but also a high degree of structural similarity at genomic level. Each of them consist of six exons and five introns,

encompassing approximately 27 kb in genomic DNA (Meng *et al.*, 2000). There are Trax - like proteins in all eukaryotes that also contain a Translin ortholog (Meng *et al.*, 2000).

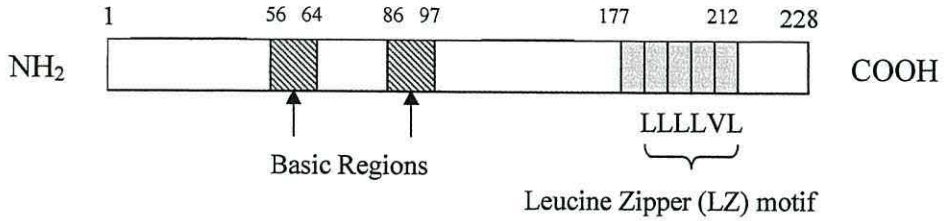


Figure 1.14 Schematic representation of the Translin protein.

The predicted amino acid sequence contains two relatively basic regions (amino acids 56-64 and 86-97) indicated by the hatched regions. The COOH-terminal region of the molecule (amino acids 177-212), indicated by the shaded region, contains the hypothetical structure referred to as the leucine zipper (LZ).

Adapted from (Kasai *et al.*, 1997)

The human Trax protein has a heptad repeat of hydrophobic amino acids consisting of leucine, alanine, leucine, leucine, isoleucine and leucine. This putative leucine zipper (LZ) motif of Trax required for multimer formation, was shown to be encoded by exons 3 and 4, while that of Translin was encoded by exon 6. The translation, initiation and termination sites were localized to exons 1 and 6 in both human *trax* and *translin*. Like in the promoter region of *translin*, several promoter-like domains were found in the upstream region of human *trax* gene, and this region was highly GC rich.

The native form of Translin was established to be a ring-shaped structure by electron microscopy and crystallographic studies (Figure 1.15). This ring shaped structure is believed to bind to the consensus sequences that exist at the chromosomal breakpoints in malignant cells. It was also determined that this multimeric Translin formed by the subunits is responsible for its binding to target sequences situated only at single-stranded DNA ends. The results support the hypothesis that staggered breaks occur at recombination hot spots and Translin has a pivotal function in recognition of the generated single-stranded DNA ends. (Kasai *et al.*, 1997; Aoki *et al.*, 1995). However these hypotheses are not proven.

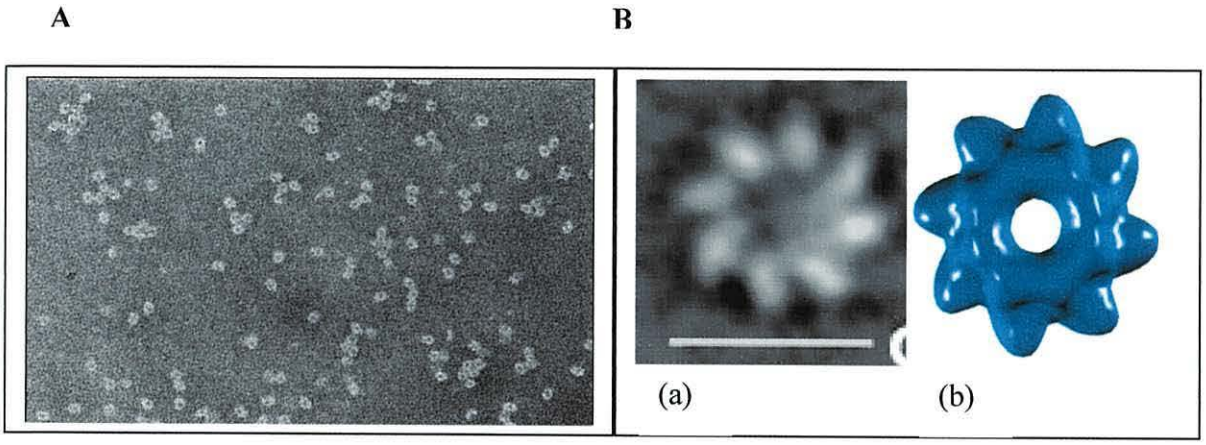


Figure 1.15 The Structure of the human Translin protein

A. Visualization of native Translin under the electron microscope (EM).
The native form of Translin is a ring-shaped octamer. (The recombinant Translin was prepared on a thin carbon film supported by a mesh copper grid and negatively stained with potassium phosphotungstate) (Kasai *et al.*, 1997).

B. Visualization of octameric Translin; (a), magnified-scale bar is 1000Å and (b), Electron Microscope structure reconstructions of Translin

Adapted from (VanLoock *et al.*, 2001)

1.12.2 trax /translin *expression profile*

The analysis of *trax* expression in humans showed that it is expressed in various human tissues including the spleen, thymus, small intestine, colon and peripheral blood lymphocytes. The expression pattern was similar to that of *translin* (Meng *et al.*, 2000). Expression of *trax* mRNA in mouse tissues, obtained from different developmental stages through to adult, tested with Northern blots, showed that *trax* mRNA was present in the heart, brain, spleen, lung, liver, skeletal muscle, kidney and testis tissues, but higher levels of *trax* mRNA seem to be found in the heart and liver tissues compared to lower levels found in lung and skeletal muscles (Devon *et al.*, 2000). This wide-ranging expression profile suggests that mouse *trax*

is expressed ubiquitously throughout development of the embryo and continues to be expressed in adult tissues, implying that it has a function at all stages of development.

In the mouse brain, both TB-RBP and *trax* are expressed in neurons, especially in cerebella Purkinje cells and in the hippocampus (Wu *et al.*, 1999b; Kobayashi *et al.*, 1998). Trax forms a complex with TB-RBP in neuronal dendrites and has been proposed to be involved in dendritic RNA processing (Finkenstadt *et al.*, 2000; Erdemir *et al.*, 2002a)

1.12.3 Trax and Translin binding / affinity to RNA and DNA

The Trax protein contains bipartite nuclear targeting sequences, suggesting a possible role in the selective nuclear transport of Translin which lacks any nuclear targeting motifs (Meng *et al.*, 2000). Translin binds to both RNA and DNA (Kasai *et al.*, 1997; Aoki *et al.*, 1999; Wu *et al.*, 1998). Unlike Translin, Trax does not bind to DNA or RNA directly, but might be part of the RNA or DNA-binding complex, thereby modulating the nucleic acid-binding affinity of Translin (Finkenstadt *et al.*, 2002; Sengupta and Rao, 2002; Gupta *et al.*, 2005; Chennathukuzhi *et al.*, 2001). Translin forms an octomeric toroidal structure (Kasai *et al.*, 1997; VanLoock *et al.*, 2001), which has similarities to the structures of other protein complexes associated with DNA replication, repair and recombination (Hingorani and O'Donnell, 2000).

In addition to binding to DNA, translin is also capable of binding to highly conserved sequences in the 3' untranslated regions (UTRs) of certain RNAs, thereby suppressing their *in vitro* translation (Devon *et al.*, 2000). Translin has a high affinity for single-stranded microsatellite GT repeats, $d(GT)_n$, and G-strand telomeric repeats, $d(TTAGGG)_n$, but not to corresponding double-stranded DNAs (Aharoni *et al.*, 1993; Jacob *et al.*, 2004). This suggests a role for translin in microsatellite repeat / telomere regulation.

Telomeres are specific protein–DNA complexes that provide a protective cap at the ends of linear eukaryotic chromosomes. Their main function is to prevent chromosome ends from being recognized and processed as DNA breaks. Loss of capping function results in genetic instability and loss of cellular viability. The emerging evidence indicates that maintenance of an appropriate telomere structure is essential for their proper function. Telomeric DNA

consists of tandemly repeated sequence motifs that typically contain clusters of three or four guanines (e.g., TTGGGG in *Tetrahymena* and TTAGGG in humans). The terminal regions of telomeric DNA is not thought to be packaged into nucleosomes, but instead provide binding sites for sequence-specific DNA binding proteins (Smogorzewska and de Lange, 2004). These DNA-binding proteins in turn recruit other partners to the telomere, forming a multiprotein telomeric complex.

Telomerase appears to play a role in the formation and maintenance of telomeres. Many human cells progressively lose terminal TTAGGG sequences from their chromosomes during the process of cell division, a loss that correlates with the apparent absence of the telomerase enzyme in these cells. There has been great interest in the possible relationship between human telomeres in the one hand and cellular senescence (aging) and cellular immortality on the other. This interest includes the question of a role for telomerase in the malignant process and the question of the use of agents that inhibit telomerase as anti-tumor agents. (Reviewed in Smogorzewska and de Lange, 2004)

However, fission yeast recombinant translin protein possesses higher affinities for RNA $(GU)_n$ and $(GUU)_n$ repeats, implying a role in regulation of RNA metabolism, rather than DNA metabolism, in this simple eukaryote (Laufman *et al.*, 2005). This is consistent with a role for TB-RBP in mRNA metabolism. Trax has been shown to enhance the DNA binding capacity of TB-RBP, while decreasing its RNA binding ability (Chennathukuzhi *et al.*, 2001).

1.12.4 Role of Trax / Translin in Translocation, DSB Repair and double-strand DNA damage response.

Chromosomal translocations are associated with carcinogenesis (for reviews, see Aplan, 2006; Weinstock *et al.*, 2006) but the mechanism of formation of translocations is not fully understood. Other than the association with breakpoint sequences, several lines of evidence implicate Translin and Trax functions in maintaining genome stability, particularly in response to DNA damage. Murine Translin (TB-RBP) interacts with GADD34, a protein implicated in the DNA damage response (Hasegawa and Isobe, 1999). Translin is primarily cytoplasmic in many cell lineages but was found in the nucleus in haemopoietic cells lines. However, treatment of HeLa cells with Mitomycin C or etopside agents that induce DNA

DSBs, was reported to cause translocation of Translin into the nucleus, indicating a functional link to DNA repair (Kasai *et al.*, 1997). Translin recognition sites have been also reported at the breakpoints with deletion of the distal band of the short arm of human chromosome 1 (monosomy 1p36, which is a most common terminal deletion syndrome) that has been rearranged (Gajecka *et al.*, 2006a). This further supports the idea that Translin is involved in chromosome rearrangements.

The study using haematopoietic stem cells exposed to sub-lethal levels of ionizing radiation demonstrated that cells from Translin deficient mice (Translin $^{-/-}$) had a severe delay in recovery from radiation compared to the wild type (Fukuda *et al.*, 2008), suggesting a possible role of Translin in protection from radiation induced injury. In this study wild type (Translin $^{+/+}$) and Translin deficient mice were exposed to 4-Gy dose of ionizing irradiation and examined after 1, 2 and 4 weeks. Histological features of extramedullary hemotopoiesis in the spleen of both the wild type and Translin deficient mice were assessed by hematoxylin and eosin staining. The hematopoietic colony formation in the spleen of wild mice started 1 week after irradiation and peaked at 2 weeks. However, the same hematopoietic colony formation in the spleens of Translin deficient mice was delayed more than 2 weeks compared to wild type mice.

Apart from this, none of the other studies carried out showed that either Trax or Translin had any effect on the repair of damaged DNA or irradiation. Mice deficient of TB-RBP showed normal development of B and T cells (Chennathukuzhi *et al.*, 2003a). Mouse Embryonic Fibroblasts (MEFs) from TB-RBP deficient mice did not exhibit increased sensitivity to DNA damaging agents or irradiation (Yang *et al.*, 2004a). *Drosophila* with single or double mutants of Trax and Translin did not show any increased sensitivity to DNA DSBs (Clauβen *et al.*, 2006).

C1D, a gamma-irradiation inducible nuclear matrix protein, can interact with Trax and prevent association with Translin (Erdemir *et al.*, 2002a). Using *in vitro* competition experiments, Erdemir *et al.* (2002a) demonstrated that binding of C1D and Translin or Trax is mutually exclusive. If Trax binds to Translin first, it is no longer available for C1D. Interactions of C1D and Trax occurs in mammalian cells following γ irradiation, raising the

possibility of involvement of Trax in DSB repair and providing evidence for biological functions of the nuclear matrix protein C1D and Trax in DNA damage recovery (Erdemir *et al.*, 2002a).

1.12.5 Regulation and interaction between Trax / Translin

Trax levels are reported to be regulated by Translin levels in both yeast and metazoan models (Jaendling *et al.*, 2008; Yang *et al.*, 2004a; Chennathukuzhi *et al.*, 2003a). It has been suggested that the regulation of Trax levels by Translin is by directed Translin-Trax interactions controlling the levels of ubiquitin-mediated Trax proteolysis (Jaendling *et al.*; 2008; Clauβen *et al.*, 2006; Yang *et al.*, 2004a), indicating that Translin functions to mediate stable levels of intracellular Trax. It seems the regulatory interaction is highly conserved, suggesting conservation of a biological process important for both lower eukaryotes and metazoans.

C1D is an activator of the DNA-dependent protein kinase (DNA-PK), which is essential for the repair DSBs and V(D)J recombination (Erdemir *et al.*, 2002b). The C1D protein may play a role in regulation of Trax/Translin complex formation (Erdemir *et al.*, 2002a). The putative LZ region of Trax is important for its interaction with Translin (Aoki *et al.*, 1997b) and C1D. The N-terminal region of Trax that contains an intact LZ region is sufficient for its interaction with C1D, whereas Translin requires full length Trax with an intact LZ region for interaction/ binding. When increased amounts of C1D are expressed (as in situation when cells are exposed to γ irradiation), more C1D binds to Trax, masking the Translin docking site on Trax, thereby preventing the Trax / Translin complex formation and binding to DNA. Therefore, CID may act as a regulator for Trax / Translin complex formation, rather than regulate the association of any preformed Trax / Translin complex (Erdemir *et al.*, 2002a). However, it was later demonstrated that Trax does not inhibit the Translin RNA binding activity, instead it forms part of the integral component of the active RNA binding complex *in vivo*, along with Translin (Finkenstadt *et al.*, 2000).

1.12.6 Role in cell proliferation

Both Trax and Translin have been implicated in the regulation of cell proliferation. MEFs obtained from TB-RBP depleted mice have a reduced cell proliferation rate, compared to

MEFs from heterozygous littermates (Yang *et al.*, 2004a). However, Trax protein was depleted in these cells, despite having normal *trax* mRNA. So the effect of loss of Translin might be indirect (Chennathukuzhi *et al.*, 2003a; Yang *et al.*, 2004a). Reduction in Trax levels in HeLa cells resulted in reduced levels of proliferation adding credence to the possibility that the reduced levels of proliferation in Translin-deficient cells is due to reduced levels of Trax protein (Yang and Hecht, 2004b).

Similarly reduction of Translin or Trax by RNA interferences slows cell growth rates of NIH3T3 murine fibroblast cells and progression through G2/M phases (Yang *et al.*, 2004a; Yang *et al.*, 2003; Yang and Hecht, 2004b). It was also shown that over expression of Translin leads to an acceleration of cell proliferation (Ishida *et al.*, 2002). Mean while, it was reported that both Trax and Translin are essential for normal cell proliferation (Yang *et al.*, 2004a). However, the most recent study in *Drosphila* showed that growth and development of *Drosophila* was not affected by absence of the *trax* and / or *translin* genes (Clauβen *et al.*, 2006).

1.12.7 mRNA regulations by Trax / Translin

Complexes of TB-RBP in association with specific mRNAs have been found in the nuclei, cytoplasm and intracellular bridges connecting male germ cells, suggesting roles in both intracellular and intercellular mRNA transport (Morales *et al.*, 2002). In dendrites the inhibition of TB-RBP causes disruption of mRNA sorting for TB-RBP associated mRNAs (Servert *et al.*, 1999).

TB-RBP/ Translin has also been identified as an RNA-binding protein that binds to a variety of brain and testes RNAs and is known to suppress the translation of stored mRNA by binding to H and Y elements in the 3' untranslated region (UTR) of a number of testicular and brain mRNAs (Kwon and Hecht, 1991; Kwon and Hecht 1993). However, further studies proved that the RNA binding complex that suppresses selected mRNA transcripts during spermotocyte maturation had both Translin and Trax components (Finkenstadt *et al.*, 2002). The synthesis and storage of mRNAs prior to their translation is necessary during spermatogenesis as global transcription ceases several days prior to the completion of spermatid differentiation. Translational repression is essential for spermatid differentiation as

premature translation can lead to an arrest in spermatid differentiation and cause dominant male sterility (Braun, 1998).

Immunohistochemical studies have localized Translin to neuronal dendrites (Finkenstadt *et al.*, 2000). Accordingly, it has been hypothesized that the Translin/ Trax complex may also regulate translation of dendritic transcripts (Li and Baraban, 2004). Collectively these observations indicate that Translin may function in more than one important biological pathway. It is possible, that due to these characteristics, Trax and Translin are found in high concentration in brain and testis.

The Trax/Translin complex binds with high affinity to an RNA oligo that contains Y and H elements. It was generally assumed that sequences that match or closely resemble these *cis* elements are required for binding to this complex. It was proven that recognizable Y and X elements are not required; instead the presence of G residues embedded within these sequences play a key role in mediating affinity binding complex (Li and Baraban, 2004). A minimum length of oligo appears to be critical. Basically, the search for the binding site of the Trax/ Translin complex should not be restricted to the Y and H elements alone but extended to sites that closely match them (Li and Baraban, 2004).

1.12.8 Other Reported Trax / Translin activity

It has been reported that Trax and Translin associate *in vivo* as components of the NS1 strand specific DNA binding complex enriched in brain (Taira *et al.*, 1998). Apart from that, Trax has also been found to bind to Mea2, a golgin family protein which is highly expressed in pachytene spermatocytes and is indispensable for mouse spermatogenesis.This suggests, that interaction between Mea2 and Trax could be important for augmentation of reproductive potency through an unknown mechanisms (Matsuda *et al.*, 2004).

Studies of TB-RBP-null mice by Chennathukuzhi and co-workers (2003) showed that the TB-RBP-null mice were 10-30% smaller than their wild-type littermates at birth and remained so to about 6 to 9 months of age and accumulated visceral fat. The TB-RBP- null males were fertile and sired offspring but had abnormal seminiferous tubules and reduced sperm counts. They also reported that the TB-RBP- null female mice were subfertile and had

reduced litter sizes (Chennathukuzhi *et al.*, 2003a). These findings suggest that TB-RBP may play an important role in mouse fertility.

The products from the *Tsnaxip1, Golga3, Sun1* and *Akap9* genes are found to interact with Trax but not TB-RBP in mouse male germ cells (Bray *et al.*, 2002), implying that the Trax protein could have roles other than acting as a binding partner of Translin. Interestingly, the *trax* gene has been associated with schizophrenia in humans. Two of the major candidate genes, DISC1 and DISC2 are disrupted by a translocation that results in major psychiatric illness. Several of the DISC1 transcripts are reported to contain *trax* sequence at the 5' end, resulting from intergenic splicing (Millar *et al.*, 2000). The relevance to the role of the gene in unicellular eukaryotes is not clear.

1.13 *S. pombe* model system

S. pombe can be genetically manipulated, and serves as excellent model organism for research into genetic diseases such as cancer. Fifty of the *S. pombe* genes were identified to be related to human diseases. The largest group of human disease-related genes are those implicated in cancer. Twenty three of this family of genes are involved in DNA damage and repair, checkpoint controls, and the cell cycle, all processes involved in maintaining genomic stability. Its genome has been completely sequenced (Wood *et al.*, 2002). It is hoped that the use of simple eukaryote model system like *S. pombe,* in which there may be less redundant molecular pathways, may provide insight into the function of *trax* and *translin* genes as well as tRNA genes in relation to their role as RFB in the genome.

1.14 The Aim of the research project

I aim to gain insight into factors regulating genome rearrangements, which may be the underlying causes of cancer.I focus on two aspects of genome stability. Firstly, I used the facile fission yeast to disrupt the conserved *trax* gene to functionally characterize the gene and the protein it encodes. Secondly, I studied the role of different RFBs in driving genome instability.

I investigated the effect genetic elements have on recombination in the *S. pombe* genome, namely *RTS1* and tRNA genes (*sup3-e*). The role of some of the *trans* and *cis* factors associated with these elements, (namely *swi1* and *rqh1*) in maintenance of genomic stability was also analyzed.

CHAPTER 2
MATERIALS AND METHODS

2.1 PCR based deletion of *trax* gene in *S. pombe*

The plasmid, pARC782 (Bähler *et al.*, 1998), containing the *kanMX6* marker gene was isolated from *Escherichia coli* (Laboratory Strain BE9). pARC782, was used a template for PCR to generate a fragment containing *kanMX6* gene with the outer 80 base pairs (bp) being homologous to the region flanking the *S. pombe trax* gene. The *kanMX6* DNA was amplified using PCR primers with 80 bp flanking sequence homologous to the *trax* gene in the *S. pombe* genome. These primer sequences were obtained from the Fission Yeast Functional Genomic Software of the Wellcome Trust Sanger Institute;
http://www.sanger.ac.uk/cgibin/PostGenomics/S_pombe/PPPP/pppp_deletion.pl.
The primer sequences are as shown in Table 2.1

Table 2.1. PCR primers used to obtain the transformation module for deletion of *trax*

Primers	Sequence
Trax :: kan forward	5'- TATAGACTTATACATTTATACCTTCCACACGGCTTTGCTGAATTG AGGATATTATAAAACTTTAACCGAATTTGCCAAAT – cggatccccgggttaattaa -3'
Trax :: kan reverse	5'- TTATGATTTTCAAAAGCTGCAAAACAGAAAAACTTTTAATAAACT AGTAAGGTGTCTGTCGAGAGCTGTCGATCATATAT- gaattcgagctcgtttaaac -3'

Note:
Upper case indicates sequence homology to ends of the *trax* gene and lower case indicates sequence homology to the DNA flanking of the selectable marker *kanMX6*

The 50 µl PCR reactions were mixed in a 0.5 ml PCR tube consisting of, 1 µl of Expand High Fidelity Polymerase enzyme (Finnzyme), 1 µl of the template DNA (0.02 µg or 20 ng of plasmid DNA), 1 µl of 10 x dNTPs, 1 µl of 20 ng/ µl each of forward and reverse primers, 4 µl of Expand buffer, 3.5 µl of 25 mM $MgCl_2$, and 36.5 µl sterile distilled water. The PCR cycles were as follows; denaturing, start at 94°C for 2 minutes, 25 cycles; 94°C for 1 minutes ; annealing, 55°C for 1 minutes; extension at 72°C for 2 minutes; finishing at 72°C for 4 minutes. 10 reaction mixtures were pooled in a 1.5 ml Eppendorf tube. 5-10 µl of the product was run on a 1% agarose gel to visualise product size. 250 µl of phenol and 250 µl of chloroform were added and gently vortexed. The tubes were then centrifuged at 3,000g for 5

minutes (Eppendorf centrifuge 5415D). The clear top layer was aspirated and transferred into a fresh Eppendorf tube. Twice the volume of ice cold ethanol was added and 0.01 M (final concentration) of NaCl were added, mixed and the tube incubated at - 20°C overnight or at - 80°C for one hour. The tubes were centrifuged at 13,000 r.p.m. for 15 minutes at 4°C. The supernatant was carefully aspirated, leaving the DNA pellet behind. 1 ml of 70% ethanol was gently added in and drained out. The tubes were left open to air dry. The pellet was resuspended in 20 µl of TE. 1 µl of the DNA concentrate was run on a 1% agarose gel. The concentration of DNA was determined using a spectrophotometer at a wavelength of 260 nM. 10 µg of this DNA was used for the transformation of *S. pombe* cells.

After the transformation process (refer 2.3 Materials and Methods) the cultures were plated onto YEA. Transformed cells have resistance to Geneticin. YEA plates were replica plated on selective media (YEA + Geneticin), covered in aluminium foil and incubated at 30-33°C for 2-3 days. Large colonies were re-streaked onto fresh YEA with Geneticin plates for further tests. PCR, Southern blot and Western blot tests were carried out on the isolates to determine/confirm if the *trax* gene was deleted.

2.2 Isolation of plasmid from *E. coli*

The plasmids were obtained using the method as described in the GenElude™ HP Plasmid Miniprep Kit. *E. coli* from the -70°C freezer stock was streaked on Luria Bertani (LB) media (see section 2.29) with the appropriate antibiotic and grown overnight at 37°C. A single colony from this culture was inoculated into a 5 ml LB broth with the appropriate antibiotic and incubated overnight at 37°C in an orbital incubator. Briefly, the cells were harvested by centrifugation at 3,000 g for 1 minute and resuspended in 200 µl of Resuspension Solution containing RNase A. Cells were then lysed with 200 µl of the lysis buffer. The cell debris was precipitated with 350 µl of Neutralization / Binding Buffer and separated by centrifugation at 3, 000 g for 1 minute. The washing buffer was added to the supernatant and centrifuged. Lastly, the plasmid was eluded out with 50 µl Elute solution using the column supplied with the kit.

2.3 Chemical Transformation of *S. pombe* using Lithium Acetate (LiAc)

Culture media, strain storage and *S. pombe* transformation were as described by Moreno and co-workers (Moreno *et al.*, 1991). A single colony of the *S. pombe* strain to be transformed was cultured to a concentration of 1 X 10^7 cells / ml in YEL. The cells were harvested by centrifugation at 3,000 r.p.m. for 5 minutes and then washed with an equal volume of water. The cell pellet was resuspended in 1 ml of sterile distilled water, and transferred to a 1.5 ml microcentrifuge tube, washed once with LiAc / TE prewarmed to 30°C. The cell pellet then resuspended in LiAc/TE at a concentration of 2 X 10^9 cells /ml. 100 µl of the concentrated yeast cells were mixed with 2 µl of 10 mg / ml Salmon sperm DNA and 10 µl of DNA solution to be used for transformation (Refer to 2.1).

After 10 minutes of incubation at room temperature, 260 µl of prewarmed (30°C) 40% PEG/ LiAc / TE pH 7.3 was added. The cell suspension was mixed gently and incubated for 60 minutes at 30°C. 43 µl DMSO was added and cells were heat shocked for 10 minutes at 42°C. The cells were allowed to cool for 10 minutes at room temperature. The cells were then washed with 1 ml of sterile distilled water and resuspended in 0.5 ml of sterile distilled water. 100 µl of the cell suspension was plated onto appropriate solid medium and incubated at 30-33°C.

Table 2.2 Primers used for PCR checking of *S. pombe* for deletion of *trax* gene.

Primer designation		Sequence
P1	TRAX Check F	5'- CAAATAGTCATCTTGATTTGC-3'
P2	TRAX Check R	5'- TCTAACATATAGAAAGCAGCG-3'
P3	TRAX_intF	5'- ATAAGAGGGAGAAAATTATTCG -3'
P4	TRAX_intR	5'- CTCCTCGGGAGGAGTTGC-3'
P5	TRAX_midF	5'- CTGATGGATTTCCTCTACCC-3'
P6	TRAX_midR	5'- GGAGAACAGCATTTCAAAAG-3'
P7	Screen Tag F	5'- GGATGTGATGTGAGAACTGT-3'
P8	Scrkan 1154F	5'- AGAAAACTCACCGAGGCAGT-3'

2.4 PCR of candidate *trax* deleted *S. pombe* strains

The PCR was undertaken using DNA obtained by the cell lysis procedure as described in 2.5. It was performed using 1 µl of the DNA extracted from the candidate *trax* deleted strains, 1 µl of Phusion polymerase enzyme, 1 µl 10 x dNTPs, 10 µl of 5 x Phusion buffer, combinations of 1 µl each of forward and reverse primers (Refer to Table 1.1 for sequence), and 35 µl of sterile distilled water or by using 1 µl of the prepared DNA with a PCR tube containing 47 µl of a commercial master mix (Megamix Blue, Helena Bioscience), added with 1 µl each of the combinations of forward and reverse primers. These were run in the thermocycler using the same program as for synthesizing the DNA fragments for transformation (see section 2.1). The PCR product was examined by agarose gel electrophoresis.

2.5 Preparation of chromosomal DNA for PCR

5 ml of overnight *S. pombe* culture was grown to saturation in YEL+ adenine (200 mg/l), in a shaker at 30-33°C. Cells were washed at 3,000 g for 1 minute. The pellet was resuspended in 1 ml of sterile distilled water and transferred to a 1.5 ml Eppendorf tube and re-washed. 200 µl lysis buffer [2 % Triton X-100, 1% SDS, 100 mM NaCl, 10 mM Tris-HCl (pH 8.0), 1 mM EDTA in water], 0.3 g of acid-washed glass beads, 100 µl phenol and 100 µl chloroform were added to the pellet. Tubes were vortexed vigorously for 3-4 minutes. 200 µl TE (pH 7.5) was added and centrifuged for 5 minutes at 3,000 g. The clear top layer was removed and added to 1 ml of cold 100% ethanol. This was mixed gently by inversion and centrifuged for 5 minutes at 3,000 g. The supernatant was removed, the pellet gently dried and resuspended in 50 µl of TE buffer pH 7.5-8.0. The DNA was then stored at 4°C.

2.6 PCR sequencing

1 µl volume of chromosomal DNA solution (prepared via method 2.5), was mixed with 46 µl of Megamix Blue® PCR master mix and 1.5 µl of appropriate forward and reverse primers of 10 pM primer / ddH_2O concentrations. The PCR program was set at one denaturing cycle of 1 minute at 95°C followed by 25 cycles of 94°C for 1 minute/ 55°C for 1 minute and 72°C for 1-2 minutes and for 4 minutes. 5-10 of aliquots were then removed from each reaction and checked for product size and approximate concentration on 0.8% analytical agarose gel. PCR products were then purified using Roche® PCR product purification kit. Appropriate

quantities of PCR product (amounts specified by MWG Biotech Germany), were then precipitated and sent to MWG Biotech (Germany) along with the appropriate primers for sequencing.

2.7 Purification of amplification products after PCR (Roche® Applied Sciences)

100 µl of PCR product was mixed with 500 µl of Binding Buffer. This was centrifuged in High Pure filter tube at maximum speed (3,000 g) for 30-60 seconds. The High Pure filter tube was washed twice with 500 µl and 200 µl of wash buffer. Following this the purified PCR fragments was obtained by centrifugation with 50-100 µl of Elution Buffer at 3,000 g for 1 minute.

2.8 Purifying DNA fragments from agarose gel (Roche® Applied Sciences)

The required DNA band was cut out of the agarose gel after electrophoresis. To each 100 mg agarose gel cut out, 300 µl of binding buffer was added and the tube was heated at 56°C for 10 minutes, to ensure that the agarose gel had melted. 150 µl of isopropnol was added for every 100 mg of agarose and vortexed thoroughly. The entire amount (700 µl) was transferred into one High Pure filter tube and centrifuged at maximum speed of 3,000 g for 30-60 seconds. The supernatant discarded. The tube was similarly centrifuged twice with another 500 µl and 200 µl of wash buffer. Finally 50-100 µl Elution Buffer was used to obtain the purified PCR product.

2.9 Meiotic crosses and strain selection

Cells of the opposite mating type (h^+ and h^-) were grown in 5 ml yeast extract liquid (YEL) supplemented with adenine (200 mg/l) to a density of approximately 2 X 10^7 cells/ ml. Equal volume of each culture were then mixed (0.75 ml) in microfuge tubes, centrifuged at 1,000 g and the supernatant was aspirated off. Cell pellets were washed with 1 ml of sterile distilled water and resuspended in 20 µl distilled water. Suspensions were spotted onto fully supplemented synthetic sporulation media (SPA) and incubated at 30°C for a minimum of 3-5 days. After incubation, sporulating cells were scraped into a microfuge tube containing 1 ml of a 0.6% β- glucuronidase® (Sigma)/ distilled water solution and incubated for 16 hrs at 25°C. 0.5 ml ethanol was added and incubated at room temperature for not longer than 5 minutes. Suspensions were then centrifuged, aspirated dry and cell pellets were resuspended

in 1 ml distilled water. Spores were plated out onto YEA and colonies were replica plated onto appropriate selective media for strain selection.

2.10 Iodine staining of meiotic products

The SPA plates with mated colonies were placed on iodine beads for up to 5 minutes, so that colonies were exposed to the iodine vapour. Strains of opposite mating types would produce spores; these are stained by iodine, producing dark/ black coloured spots.

2.11 *S. pombe* transformation via electroporation

200 ml cell cultures of *S. pombe* were grown to a density of 1 x 10^7 cells per ml (OD_{600}= 0.5) in supplemented nitrogen base (NB) broth (See 2.25 Reagents and Media). 50 ml of the cultures were then chilled on ice for 20 minutes before being harvested by centrifugation at 3,000 g for 5 minutes at 4°C. Each sample was then washed three times in ice cold 1 M sorbitol before being resuspended in ice cold 1 M sorbitol to a density of 1 x 10^9 cells/ml. Samples were then mixed in pre-chilled Eppendorf tubes containing 1 µl DNA/ plasmid to be transformed in 3 µl of TE buffer (pH 7.6) then immediately electroporated in prechilled Equibio ECU-102® cuvettes using settings 2.25 kV, 201 Ω (Ohm), 25 µF. Immediately after pulsing, 1ml of ice cold 1 M sorbitol was added into the cuvettes and they were maintained on ice. 50 µl of the aliquots were spread onto EMM2 plates with or without required supplements for selection and incubated for up to 6 days at 30°C.

2.12 Preparation of Electro competent *E. coli* cells.

E. coli cells from frozen stocks were streaked onto LB agar and incubated at 37°C overnight. One colony was selected and inoculated into 5 ml LB broth and incubated overnight in an orbital shaker at 37°C. 1 ml of the culture was inoculated into two 1 litre flasks with 250 ml LB broth and incubated overnight at 37°C to obtain growth to an OD_{600} of 0.5-0.75. The cultures were cooled in ice for more than 15 minutes. From this stage cultures were constantly maintained on ice. Cells were harvested at 3,000 g at 4°C for 10 minutes. The pellet was resuspended in 40 ml ice cold sterile distilled water and transferred to sterile 50 ml falcon tubes, and spun at 1,500 g in ice cold water and incubated at 4°C for 15 minutes. All the supernatant was aspirated off and cells were resuspended in 50 ml ice cold sterile distilled water and the centrifugation repeated as above. All the supernatant was removed and cells

were resuspended in 1 ml ice cold sterile 10 % glycerol. Aliquots of 50 µl cells were stored in 1.5 ml Eppendoff tubes at -80°C.

2.13 Southern blot protocol

Genomic DNA from appropriate strains was extracted as follows; Cultures of *S. pombe* cells were grown in 100 ml YEL overnight with shaking at 30-33°C. Cultures were harvested at 3,000 g. for 5 minutes and resuspended in 5 ml of 50 mM citrate / phosphate pH 5.6 (7.1 g/L $Na_2 HPO_4$, 11.5 g/ L citric acid), 40 mM EDTA pH 8.0 and 1.2 M sorbitol. 15 mg of Zymolase - 20T was added and the suspension incubated at 37°C for 30 – 60 minutes. Digestion of cell walls was checked using a phase contrast microscope on a 10 µl sample to which 1 µl of 10 % SDS was added. The suspension was spun down at 5,000 g. for 5 minutes and resuspended in 15 ml of 5 x TE (50 mM Tris-HCl, 5 mM EDTA pH 7.5). 1.5 ml 10 % SDS was added, mixed well and rechecked for lysis (if necessary, cells can be further incubated for 5 minutes at 65°C). 5 ml of 5 M potassium acetate was added and kept on ice for 30 minutes, then centrifuged at 5,000 g. for 15 minutes. The supernatant was passed through sterile gauze and 20 ml of ice cold isopropanol was added and samples left for 5 minutes at –20°C.

The tubes were centrifuged at 5,000 g for 10 minutes, drained well and the pellet was air dried. The pellet was resuspended in 3 ml 5 x TE and RNase was added to a final concentration of 20 µg/ ml followed by incubation for 2 hours at 37°C. 3 ml of phenol/chloroform (1:1) was added, mixed well transferred to a 15 ml Corex tube. This was centrifuged at 5,000 g for 10 minutes. The upper aqueous phase was transferred to another Corex tube, 0.3 ml of 3 M sodium acetate and 7.5 ml of ethanol, was added and mixed followed by incubation on dry ice for 1 hour or at –20°C for 4-5 hours. The DNA was precipitated by centrifugation at 5,000 g for 10 minutes. Finally, the pellet was washed with 5 ml cold 70% ethanol and dried under a vacuum. The DNA was resuspended in 0.2 ml of TE. The concentration was checked in a spectrophotometer at 260nM wavelength.

Enzymes were selected for the digestion of the genomic DNA. A total volume of 25 µl digestion mixture was prepared using DNA, appropriate buffer, bovine serum albumin and water. The mixture was digested overnight at 37°C. The DNA digest was run in a 0.8%

agarose gel in 1 x TAE buffer at 60 V for 4-5 hours. Then the gel was photographed and used for transfer onto GeneScreen membrane. The salt transfer protocol by GeneScreen (NEN Life Science Products) was used for transfer and preparation of the membrane for probe hybridization. DNA for probes were generated by PCR using pFA6a-kanMX6 as template using primers Screen Tag-Forward; 5'-CAGTTCTCACATCACATCCG-3' and primer Scr1458 – Reverse 5'-TGGTCGCTATACTGCTGTCG-3'; producing a neucleotide of 1051 bp. This was labeled with P^{32} γ-CTP using the Megaprime DNA Labelling System (Amersham Biosciences) and used to probe the membrane. The membrane was probed using the protocol described in the GeneScreen Protocol (NEN Life Science Products). Following the probing the membrane was scanned on a phosphor imager (BIO-RAD Molecular Imager FX).

2.14 Whole cell protein extraction and Western blots

Whole-cell protein extracts (WCEs) were obtained following the protocol described by Ilyushik and co-workers (Ilyushik *et al.*, 2005) with the addition of a specific protease inhibitor set (Roche Molecular Biochemicals, Lewes, UK). Samples of at least 50 ml cultures of *S. pombe* grown in 100 ml YEL overnight were centrifuged at 3,000 g for 5 minutes and resuspended in 1.5 ml ice cold STOP buffer (150 mM NaCl; 10 mM EDTA, pH 8; 1 mM NaN_3). Cells were washed and resuspended in 50 µl lysis buffer (50 mM tris-HCl, pH 7.4; 200 mM KAc; 0.5% (v/v) Triton; 1 mM sodium ortho-vanadate; 1 mM PMSF; 10 µl Bestati; 10 µM E64; 2 µM Leupeptin; 60 µM Apoptenin), with 0.75 g cold acid washed glass beads. Cells were pulse centrifuged and then vortexed for 6 x 30 seconds, returning to ice between each vortex. 350 µl lysis buffer was added and lysed cells were centrifuged at 5,000 g at 4°C for 20 minutes. Lysates were collected and centrifuged as above for 10 minutes.

Approximately 30 µg of WCE was mixed with equal amounts of loading buffer and 2 µl of Dithiothreitol (DTT) and boiled for 5 minutes before loading into the wells of sodium dodecyl sulphate (SDS) - polyacrylamide gels. This was resolved on 10% SDS - polyacrylamide gels and then electro blotted onto PVDF transfer membrane (Amersham Biosciences, UK Limited, Little Chalfont, UK). The membranes were incubated with phosphate- buffered saline (PBS) containing 10% non- fat milk and 0.1% Tween (blocking buffer) overnight at 4°C. Membranes were rinsed in clean blocking buffer and then incubated

with primary anti Trax purified polyclonal antibody for 1.5 hours at room temperature (approximately 20°C), membranes were washed 3 times in 1 X PBS-Tween and incubated in blocking buffer with anti- guinea pig or anti-mouse horseradish peroxidase (HRP) secondary antibodies (Santa Cruz) for 1.5 hours at room temperature. The blot was probed using anti-Trax polyclonal antibodies and donkey anti-guinea pig IgG-HRP (Jackson Immuno Research). After washing 3 times with 1 X PBS, proteins were visualized using the ECL protocol of Amersham. The blots were also probed using monoclonal anti-tubulin antibody (Sigma-Adrich) and goat anti-mouse IgG-HRP secondary antibody (Santa Cruz Biotechnology).

2.15 Generation of anti-Trax polyclonal antibodies

Anti-Trax polyclonal antibodies were generated by Eurogentec, Liege, Belgium. They were raised in guinea pigs via inoculation with synthetic peptides SDGFPLPKDFDRTSI and VDTATPPEEKRLRST corresponding to amino acid residues 46-60 and 217-231 respectively of the predicted Trax protein (SPCC736.09c).

Trax polypeptides sequence;

MEEEFLSFKN FLQEDQDKRE KIIRLSREIT IQSKRMIFLL HQTSS**SDGFP LPKDFDRTSI**
FEKKIHKELE SLKRELAGLN ADKFSSACTH GLQEYVEAVT FKFWLQTGTL LSCKDSSFRI
SINFIDYVLG VCDMTGEIMR FLVTNGSKFS VQQLTQQVKF LRGLHKNCSE IEHLPSKVKS
ELQQKLSVME NSISKVEGIC YSKILREADK RYLNLE**VDTA TPPEEKRLRS T**

Both serum sets were affinity purified with the corresponding peptide.

2.16 Cloning of the *S. pombe trax* gene into plasmid with *nmt* promoter

S. pombe vector pREP plasmids (Maundrell, 1993) have different strength *nmt* (no message in thiamine) inducible transcriptional promoters (high pREP4, medium pREP42 and low pREP82). They were isolated from *E. coli* strains using the GenElude™ HP Plasmid Miniprep Kit. (Refer to 2.2). *trax*$^{+}$, open reading frame (ORF) was generated by PCR using primers with suitable *Bam*HI restriction sites (CGCGGA) inserted in forward and reverse primers as below; The *Bam*HI site is bold.

Primers *Bam*HI trsynF;

5'- **CGCGGA**TCCATGTGGACCGTAATCGTTTCTCCTCGGGAG-3' (39mer)

and *Bam*HI trsynR ;

5'- **CGCGGA**TCCTTATGGAAGAGGAATTCCTCTCATTTAAAAATTTTTTACAG-3' (50mer).

The PCR product was digested overnight with *Bam*HI at 37°C purified using phenol precipitation and cloned into pREP plasmids similarly digested with *Bam*HI and treated with Akaline Phosphatase. Ligation was carried out using 0.5 µl of *Bam*HI cut plasmid (high *pREP4*, medium *pREP42* and low *pREP82*) 2.5 µl (approximately 5 µg) of digested *trax*$^{+}$ ORF DNA, 0.5 µl of DNA T4 Ligase (Promega), 14.5 µl of water and 2 µl of ligase-buffer. The mixture was incubated at 4°C, overnight. 2 µl of ligation mixture was transformed into electro competent *E. coli* by electroporation and plated onto LB agar containing Ampicilin. The transformed *E. coli* were PCR checked by colony PCR; using internal *trax*+ primers P3 and P4 (refer to Table 2.2).

Plasmid from selected *E. coli* cells was reisolated using the GenElude^{TM} HP Plasmid Miniprep Kit (as in 2.2) and PCR checked for presence of the *trax* ORF using Trax internal primers. Part of the plasmid preparation was sent for sequencing to verify correct sequence of the *trax* ORF (MWG Biotech, Germany). After confirmation by sequencing, the plasmids with *trax* ORF were transformed into *S. pombe* by electroporation (See 2.11) and used for further studies.

2.17 *S. pombe* growth rate analysis

100 ml cells were grown in EMM2 media with the required amino acids in 250 ml flasks in a 33°C shaker incubator. A sample was collected every 1.5 hours of incubation over a 9 hour period. At each time point the cell count, viable cell count and the OD at 600 nm was taken. A portion of the cells (1 ml) were fixed by adding 1 ml of 70% ethanol to washed cells. These were used for observation of morphology and septation index counts. Cultures were plated onto solid YEA media to determine total viable count. Similarly, a growth curve was generated for cultures having the *trax* ORF cloned into the *nmt* promoter plasmid (over expressed *trax* gene). The viable cell count was determined by plating serial dilutions onto EMM2 media with appropriate supplements.

2.18 Calcofluor and DAPI staining

A sample of 100-200 µl of ethanol fixed cells was washed in 1 ml of water. The pellet was resuspended in 50 µl of water, and heat fixed at 70°C. 1 mg / ml calcufluor stock was made in 50 mM sodium citrate and 100 mM sodium phosphate with NaOH added until the calcufluor dissolved. Working concentration was made up with 50 mM Tris pH 9.5. 50 µl was then added to the slides and left for 5 minutes. Slides were then rinsed with 2 ml 50 mM Tris and air dried. 3-4 µl DAPI (1 µl/ml in Vecra Shield) was added and viewed under the fluorescent microscope.

2.19 RNA extraction and Northern blotting protocol

The protocol was carried out as described at the Wellcome Sanger Institute website (http://www.sanger.ac.uk/PostGenomics/S_pombe/docs/rnaextraction_website.pdf). Briefly, the liquid nitrogen snap frozen cultures of *S. pombe* from the -80°C freezer were thawed on ice. 1 ml pre-chilled DEPC water (0.1% Diethyl Pyrocarbonate autoclaved in distilled water) was used to resuspend the cells. The cells were transferred to 2 ml Eppendorf tubes, spun for 10 seconds at 3,000 g and the supernatant removed. 750 µl of TES (10 mM Tris pH 7.5; 10 mM EDTA pH 8.0; 0.5 SDS made with DEPC treated water) was added and the cells were re-suspended with a pipette. 750 µl of acidic phenol-chloroform (refrigerated, Sigma P-1944) was immediately added, vortexed and incubated at 65°C in a heat block (in a fume hood). This was repeated for each sample. All samples were incubated at 65°C for 1 hour; vortexed for 10 seconds every 10 minutes. Samples were then placed on ice for 1 minute, vortexed for 20 seconds and centrifuged for 15 minutes at 5,000 g at 4°C. 2 ml yellow phase-lock (heavy) Eppendorf tubes were pre-spun for 10 seconds and 700 µl of acidic phenol-chloroform added. The aqueous phase from the centrifuged sample was added to the phase-lock tube, thoroughly mixed by inverting and centrifuged for 5 minutes at 5,000 g at 4°C.

To another 2 ml pre-spun phase-lock tube, 700 µl of chloroform: isoamyl alcohol (24:1; Sigma® C-0549) was added in a fume hood. 700 µl of the water phase from the above step was transferred to this phase-lock tube (Eppendorf) and mixed thoroughly as above and centrifuged for 5 minutes at 5,000 g at 4°C. A 2 ml Eppendorf tube was prepared with 1.5 ml of cold 100% Ethanol (-20°C) and 50 µl of 3 M NaAc, pH 5.2. 500 µl of the aqueous phase

from the above centrifuged mixture was added into this tube and vortexed for 10 seconds. Samples were precipitated at -20°C overnight (or -80°C for 30 minutes).

The tubes were centrifuged at room temperature for 10 minutes at 5,000 g The supernatant was discarded and 500 µl of 70% Ethanol (4°C) (made with DEPC water) was added and spun for 1 minute. The majority of the supernatant was aspirated and spun again for 5 seconds and the rest of the liquid removed with pipette. The pellet was air dried for 5 minutes at room temperature. 100 µl of DEPC treated water was added and incubated for 1 minute at 65°C or 10 minutes at room temperature. The pellet was dissolved by pipetting it up and down (~30 times) until no particulates were left. The OD $_{260/280}$ was measured using 5 µl to 995 µl in DEPC water (1:200).

Salt blotting was carried using GeneScreen protocols. All stock solutions were sterilised/ autoclaved. Agarose gel (1.5%) was made of 0.66 M formaldehyde and 1x MOPS buffer, (10 x MOPS buffer was made of 0.4 M Morpholinopropanesulfonic acid, 0.1 M Sodium Acetate-$3H_2O$, 10 mM EDTA-Na-$2Na_2$-$2H_2O$, and pH adjusted to pH 7.2 with NaOH and the volume made up to 1 litre) was melted and poured. The set gel (after about 30 minutes) was used to load the prepared RNA.

The approximately 10-15 µg of total sample of RNA was dried by vacuum centrifugation. 20 µl of loading buffer was added to each of the dried RNA pellets and the RNA was denatured for 2 minutes at 95°C and then cooled on ice. The set gel (from above) was loaded with 10-15 µg RNA samples in freshly prepared loading buffer and one well with RNA ladder. Loading buffer was prepared with 720 µl formamide, 160 µl 10 x MOPS , 260 µl of formaldehyde, 180 µl of distilled water, 100 µl of 80% glycerol and 80 µl of saturated bromophenol solution. Using 1 x MOPS as a running buffer the samples were loaded and gel run until bromophenol blue migrates three fourths of the length of the gel.

The gel was soaked in 5 volumes of distilled water for 5 minutes to remove the formaldehyde from gel. This was repeated up to 4 times. The GeneScreen Plus membrane was cut to the exact size of the gel and then the membrane was placed in distilled water for few seconds until fully hydrated. The membrane was then soaked in 10 x SCC buffer for 15 minutes. The

capillary blot was set up using 10 x SCC as transfer solution. Transfer was done overnight. The filter paper was removed and the membrane carefully lifted with plastic forceps. The membrane was briefly rinsed with 2 x SCC to remove the residual agarose. The membrane was placed on a filter paper with the RNA side up to dry. The membrane was fixed by ultraviolet irradiation.

The membrane was prehybridized in a minimum volume of prehybridization solution at 42°C for 2-4 hours in a hybridization oven. The solution was preheated to 42°C before adding to the hybridization chamber holding the membrane. The prehybridization solution was made of 5 x SSPE, 50% (w/v) deionized formamide, 5 x Denhardt's solution 1% SDS 10 % dextran sulphate, NaCl and 100µg/ ml salmon sperm DNA. The prehybridization solution was removed and fresh hybridization solution was added. This solution is prepared similar to the prehybridization solution except the salmon sperm was omitted. The ^{32}P labelled probe was denatured by boiling and then added to the hybridization buffer and left in hybridizer at 42°C for 16-24 hours. The probe was prepared using PCR of *trax*$^{+}$ fraction from wild-type *S. pombe* genomic DNA (primers P3 and P4 were used, Table 2.1). 2 µl of this was labelled with ^{32}P γ CTP using the Megaprime DNA labelling system (Amersham Bioscience).

After overnight incubation, the membrane was carefully washed in 200 ml of 2 x SSPE at room temperature for 15 minutes. The membrane then was removed from the hybridization oven, covered in plastic seal, and placed with the RNA side facing down in a cassette and with film overnight. The exposed film was read in a Phosphor imager (BIO-RAD Molecular Imager FX).

2.20 Drop Tests for genotoxic Agents

Fully supplemented YEA was prepared and appropriate chemical agents were added to the media to the required amount, plates were poured and dried. The plates were prepared freshly each time and used on the same day. The agents were prepared as appropriate stock solutions and added to make up to final concentrations as in Table.2.2.

Table 2.3 DNA damaging agents and final concentration used for tests.

Chemicals / Agents	Concentration Tested
Methyl Methanesulfonate (MMS) (Fluka Chemical)	0.0075%, 0.005 and 0.0025%
Hydroxyurea (Sigma)	10 mM, and 5 mM
Camptothecin (Sigma)	0.5 µg / ml
Phleomycin (from *Streptomyces verticillus*) (Sigma)	1, 2.5, 5 and 10 µg / ml
Mitomycin C (Sigma)	0.15 mM

S. pombe cultures to be tested were cultured in approximately 5 ml YEL overnight with rotation at 30°-33°C. Cell concentration was checked by microscopic cell count and approximately 1 x 10^7 cells per ml concentrations were obtained. From this serial dilutions [10^{-1}, 10^{-2}, 10^{-3}, 10^{-4}] were prepared for each strain to be tested. 10 µl of the serially diluted *S. pombe* cell suspension was gently dropped in a straight line, to make a circle on each of the plates containing different chemical agents and one plate without any agents (control). Each plate was inoculated with several strains in a straight line as in Figure: 2.1.

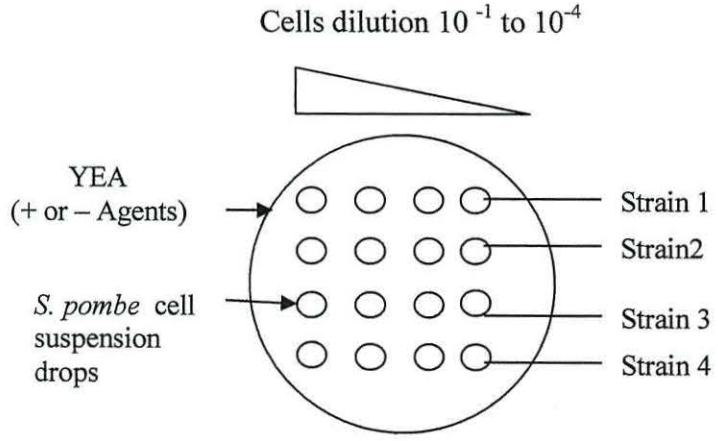


Figure 2.1 Organization of Petri dish for drop tests

Diagram showing organization of the drops of *S. pombe* cells (highest to lowest) in tests carried out to determine effect of DNA agents in a Petri dish. Each row represents a different strain.

Similar drop tests were also carried using cells that were grown in nitrogen free EMM2 media. The inoculated plates were incubated at 25°C, 30°C and 37°C for 2 to 5 days. The growth was compared with media without any added genotoxic agents.

2.21 UV Irradiation of *S. pombe*

The *S. pombe* strains were spotted as in Figure 2.1 on YEA and incubated for one hour in a 30°C incubator to dry. These were then exposed to an ultraviolet light source - 300nM (CL-1000 UV cross linker giving out 100 μJ/CM^2 energy) set at 2500 μJ/CM^2, 5000 μJ/CM^2, and 7500 μJ/CM^2. These plates were then incubated at 30°C. Growth was checked 2-4 days after incubation.

2.22 GT_8 repeats stability tests

Appropriate *traxΔ* and *trax*$^+$ *ade6-GT*$_8$ strains were constructed (see strain table). The presence of the GT_8 repeats was confirmed by PCR sequencing (ade6 C; 5'-TGGAGGTGGTCAATTGGGC-3' and ade6 B; 5'-GCAGCTTCAAGAGGGTTG-3'). The PCR cycle used was as in 2.24ii. Control strain (BP90), which was the parental strain used to obtain the *traxΔ* mutant strain, was similarly treated and selected for GT8 repeat integration. Fluctuation analysis was done (as in 2.23) for both the strains to determine proportion of adenine prototrophs obtained in wild type as compared to *trax*Δ strain.

2.23 Fluctuation Analysis

Recombination frequency was determined using fluctuation analysis. Single colonies of strains to be tested were inoculated into 5 ml NB or EMM2 liquid media, with appropriate supplements and grown to log phase. Serial dilutions were made from these cultures and dilutions of 10^{-1} to 10^{-5} were plated out onto appropriate medium and incubated at 30°C (2-3 days) until small colonies (~ 1 mm in diameter) grew. For each strain to be tested, 7 whole colonies were picked and inoculated into 7 different universal bottles and permitted to grow to saturation by incubation at 33°C.

1 ml of each independent culture was transferred into sterile a 1.5 ml micro-centrifuge tubes, centrifuged, and the supernatant was carefully discarded. 1 ml of sterile distilled water was added. This makes neat (undiluted 10^0) sample culture. 10 fold serial dilutions of up to 10^{-5}

was prepared and dilutions 10^0, 10^{-1}, 10^{-2}, 10^{-3} were plated onto selective media and spread with a glass spreader. Dilution 10^{-3}, 10^{-4} and 10^{-5} were plated onto non selective media to estimate the total viable cell count in the culture. After incubation at 33°C for 5-6 days, the number of colonies seen in each plate was counted and tabulated. The number of colonies that grew on selective media was determined. For each strain to be tested the recombination frequencies of seven independent cultures were measured and the mean values of the median values were generated. *P* values were generated by pair wise comparison using Student's *t*-test.

2.24 Construction of plasmid pSRS5 for plasmid by chromosome recombination assay (fluctuation analysis)

The plasmid based recombination assay, was carried out using a specially prepared plasmid, carrying mutated *ade6* gene. The a*de6* gene was mutated by deletion of one nucleotide 'G' at 1483 bp of the *ade6* gene (taking the A of the ATG start codon as position 1).

2.24.1 Preparation of the plasmid back bone for cloning

S. pombe vector, pREP42 with the *LEU2* marker was isolated from *E. coli* (Laboratory reference BE 79). The plasmid was prepared using GenElude™ HP Plasmid Miniprep Kit. (as in 2.2). The plasmid needed to be digested to expose the *Pst*1 and *Sac*1 multicloning site in order to clone the mutated *ade6* ORF insert into the site. 10 µl of the plasmid preparation was digested with 2 µl (10 u/µl) of *Sac*1 (Promega) and 2 µl (10 u/µl) of *Pst*1 (Promega) enzymes, 0.5 µl of BSA (bovine serum albumin), 5 µl of 10x NEB 1 buffer, topped up with 30.5 µl of sterile distilled water, at 37°C for 2 hours. This produced a back bone of the plasmid with the multicloning site at *Pst*1 and *Sac*1 site exposed for integration of the mutated *ade6* gene (see 5.2).

2.24.2 Preparation of the mutated ade6 *ORF insert*

DNA of the wild type strain (BP1) was used as a template for PCR to obtain the mutated *ade6* ORF for cloning into the digested plasmid pREP42 at the *Pst*1 and *Sac*1 cloning sites. 1 µl (10 nM) of each the primers ade6-clone-Pst1;

-5'- TATGCTGCAGCATTGAATTCTCCAATATATTTAGAATTAGC- 3'

and ade6-frameshift_f; -5'-CAGATGCCTCGAGGTGTCCCTGTCCCACTGTT-3' in 10 µl of 5x Finnzymes buffer, 1 µl of Phusion™ High Fidelity Polymerase enzyme (New England Biolabs), 1 µl of 10 x dNTPs and 1 µl of template DNA with 35 µl of sterile distilled water were used for synthesis of one end of the *ade6* gene. Whereas, 1 µl of primers ade6-clone-Sac1 -5'-ATACGAGCTCATACGCACATTGAAACATGGACG-3' and ade6-Frameshift-r -5'-AACAGTGGGACAGGGACACCTCGAGGCATCTG- 3' were used in similar conditions as above to synthesize the opposite end of the *ade6* gene. The PCR cycles used were 94°C + 2 minutes 25 cycles; 94°C for 1 minute, 55°C for 1 minute and 72°C for 1.5 minutes finishing at 72°C for 4 minutes.

Each of the PCR products was run on an agarose gel to confirm the presence of the expected band size from the respective PCR reaction. The remaining PCR product was purified using the Roche® PCR product purification kit (as in 2.2). 0.5 µl from each of the purified PCR products were mixed together and used as a template for synthesis of the mutated *ade6* gene using 1 µl each of primers ade6 clone -Pst1 and ade6 clone -Sac1, in 10 µl of 5 x Finnzymes buffer, 1 µl of Phusion™ High Fidelity Polymerase enzyme (New England Biolabs), 1 µl of 10 x dNTPs with 35 µl of sterile distilled water and PCR was carried out. The PCR product size was verified (mutated *ade6* ORF) using an agarose gel. 10 µl of the purified PCR product, was digested with 2 µl *Pst*1 and 2 µl *Sac*1 enzymes in 1 µl BSA, 5 µl of the 10 x NEB 1 buffer and 30 µl of sterile distilled water, then it was incubated at 37°C for 2 hours.

2.24.3 Cloning of the mutated ade6 *into plasmid vector*

The *Pst*1 and *Sac*1 enzymes digested plasmid vector pREP41 (back bone) (from 2.24 i) was used to clone the similarly digested PCR product above (2.24.2). 1 µl each of the digested plasmid and the 5 µl of mutated *ade6* ORF, 2 µl of T4 DNA Ligase (Promega), 2 µl of 10 x Ligase Buffer and 10 µl of sterile distilled water were mixed in a 1.5 ml Eppendorf tube and incubated at 4°C for 16 hours.

2.24.4 Chemical transformation into E. coli *using Promega pGEM®-T protocol*

10 µl of the above ligation mix was added into 50 µl of chemically competent *E. coli* cells, thawed on ice for 20 minutes, heat shocked at 42°C for 50 seconds, then reincubated on ice for a further 2 minutes. LB liquid media was added and the sample incubated for 1.5 hours at

37°C. The culture was plated out onto LB containing Ampicilin and incubated overnight at 37°C. Colonies growing on the plates were selected. These were grown in fresh 5 ml LB and the plasmids were isolated using GenElude™ HP Plasmid Miniprep Kit (as in 2.2). The plasmid DNA was sent for sequencing with appropriate primers as in 2.6. The plasmid containing the desired mutated *ade6* sequence was selected for use in all other plasmid-by-chromosome recombination assays (fluctuation tests).

2.25 Construction of *S. pombe* strains having *RTS1* and control (*his3'*$_{756}$) fragments.

Three PCR fragments were cloned into *ade6* gene in *S. pombe* for fluctuation analysis. They were the control fragment, *RTS1* orientation (1) and *RTS1* orientation (2) fragments.

2.25.1 Synthesis of* his3'$_{756}$ *fragment

A PCR fragment for integration into *S. pombe* as a spacer control insertion fragment was synthesized using 1 µl (10 nM) of each of the forward and reverse primers R1 and R2 (Refer Table 2.4), 1 µl DNA from of wild type *S. pombe* (BP1), 10 µl of 5 x Finnzymes buffer, 1 µl of Phusion™ High Fidelity Polymerase enzyme (New England Biolabs) with 36 µl of sterile distilled water. The cycles set in PCR were; denaturing, start at 98°C for 30 seconds; annealing, 55°C for 30 seconds; extension at 72°C for 2 minutes; finishing at 72°C for 30 seconds, for total 25 cycles. 5 reaction mixtures were pooled. 5 µl of the product was run on a 1% agarose gel to visualise product size. The PCR product was purified using the Roche® kit (refer 2.7) 10 µl of it was digested with 1 µl of *Bst*X1 enzyme, 5 µl of 10 x NEB Buffer 3, 34 µl of sterile distilled water at 37°C for 2 hours. This fragment was used for ligation into the pREP 42 plasmid back bone.

2.25.2 Synthesis of* RTS1 *orientation 1 and* RTS1 *orientation 2 fragments

RTS1 orientation 1 [*RTS1*(1)], PCR fragment for integration into *S. pombe* as was synthesized by PCR using 1 µl of each of forward and reverse primers R3 and R4 (Refer Table 2.4 for sequence) with 1 µl (~ 20 ng) DNA from of wild type *S. pombe*, 10 µl of 5 x Finnzymes buffer, 1 µl of Phusion™ High Fidelity Polymerase enzyme (New England Biolabs), 1 µl of nucleotides with 35 µl of sterile distilled water. The same was carried out for *RTS1* element orientation 2 [*RTS1* (2)], using primers R5 and R6 (Refer Table 2.4). The PCR cycles used were similar to that was used for *his3'* synthesis above (see 2.25.1). 5

reactions of each element were pooled. 5 µl of the product was run on a 1% agarose gel to visualise product size. The PCR product was purified using the Roche® kit (refer 2.8) and 10 µl of it was digested with 2 µl of *Bst*X1 enzyme, 5 µl of 10 x NEB Buffer 3, 33µl of sterile distilled water at 37°C for 2 hours. The digest was checked with an agarose gel and the required fragments purified with Roche® PCR purification kit (see to 2.7). These fragments were subsequently used for ligation into the pREP 42 plasmid back bone.

2.25.3 Plasmid preparation and transformation of the his3'$_{756}$, RTS1(1) *and* RTS1(2)

A plasmid with *ade6* ORF (pDP8) was isolated from *E. coli* (Laboratory reference number BE 98) using GenElude™ HP Plasmid Miniprep Kit (Refer to 2.2 for methods). 5 µl of the plasmid pDP8 was digested with 1 µl of *Bst*XI enzyme, 2 µl of 10 x NEB Buffer 3, and 42 µl of sterile distilled water at 37°C for 2 hours. The *Bst*XI digested *his3'$_{756}$*, *RTS1(1)* and *RTS1*(2) fragments were ligated to the plasmid pDP8 with Roche® ligation kit. 1 µl of digested plasmid preparation was mixed to 5 µl of the specific PCR fragment, 2 µl of 10 x buffers, 1 µl Ligase and 11 µl of sterile distilled water were added and incubated at 4°C for 18 hours The ligation mix was transformed into chemically competent *E. coli* using Promega pGEM®-T protocol (Refer to 2.24.4). The plasmid was reisolated from transformants and the sequencing was carried out to ensure the presence of the required fragment in the *ade6 Bst*XI cut site within the *ade6* gene.

Table: 2.4 Primers used in construction of *RTS1* and *his3'$_{756}$* (control) fragments for integration into the *ade6* genes in *S. pombe* strains

Primer designation		Primer sequence	Fragment synthesized
R1	RTS1his3 *Bst*X1-F	5'-CTGCAGAACCAAGAGTTTGGCAGCCTTA TGCTGTGCG-3'	*his3'$_{756}$* (control)
R2	RTS1his3 *Bst*X1-R	5'-TTCTGCAGCCAAACTCTTGGAATCAATA TAGGC-3'	
R3	RTS1 *Bst*X1-F	5'-CTGCAGAACCAAGAGTTTGGGAAAGGG TAAGGAGAGGACG-3'	*RTS1* (orientation 1)
R4	RTS1 *Bst*X1-R	5'- TTCTGCAGCCAAACTCTTGGACGTTTCA TTGTCAAGTTCG-3'	
R5	2RTS1 *Bst*X1-F	5'-CTGCAGAACCAAGAGTTTGGACGTTTCA TTGTCAAGTTCG-3'	*RTS1* (orientation 2)
R6	2RTS1 *Bst*X1-R	5'-TTCTGCAGCCAAACTCTTGGGAAAGGGT AAGGAGAGGACG-3'	

2.25.4 Integration of his3'$_{756}$, RTS1 *Orientation 1 and* RTS1 *Orientation 2 fragments into S. pombe*

The plasmid with the required PCR fragment was isolated from *E. coli* using the GenElude™ HP Plasmid Miniprep Kit (as in 2.2). The plasmid borne constructs were then co-transformed into *ade6*$^{+}$ *S. pombe* strains with a plasmid (pFY20) carrying a *ura4* marker gene. Transformants were plated onto selective minimal medium with limiting adenine (10 µg / ml), a concentration which permits growth of adenine auxotrophs with a red pigmentation in the colony. Strains which are *ade6*$^{-}$ produce colonies that are red in colour in YEA and minimal medium with limiting adenine. Red coloured colonies were selected and tested for integration of the appropriate construct within the genomic a*de6* gene. Correct homologous integrants were subjected to PCR and restriction analysis using specific PCR primers and sequenced; subsequently, the co-transformed plasmid was lost. Southern blot analysis was carried by another member of our team, using the *RTS1* sequence as a probe to ensure that only one integrant of the *RTS1* element had been integrated (Dr. Pryce -personal communication).

2.26 Construction of *S. pombe* strains having *sup3e* and *his3$_{283}$'* control

Three PCR fragments were constructed for cloning into *ade6* gene in *S. pombe*. They were the control *his3'$_{283}$* fragment, *sup3-e* orientation (1) and *sup3-e* orientation (2) fragments.

2.26.1 Synthesis of his3'$_{283}$ *fragment*

PCR fragment for integration into *S. pombe* as control was synthesized by PCR using 1 µl (10 nM) of each of forward and reverse primers S1 and S2 (Refer Table 2.4 for details of sequence), 1 µl DNA from of wild type *S. pombe* (BP1), 10 µl of 5 x Finnzymes buffer, 1 µl of Phusion™ High Fidelity Polymerase enzyme (New England Biolabs), 1 µl 10 x dNTPs with 35 µl of sterile distilled water. The cycles set in PCR were; denaturing, start at 98°C for 60 seconds.; annealing, 55°C for 30 seconds; extension at 72°C for 10 seconds; finishing at 72°C for 60 seconds for total 25 cycles. 5 reaction mixtures were pooled. 5 µl of the product was run on a 1% agarose gel to visualise product size. The PCR product was purified using the Roche® PCR product purification kit (refer 2.16) and 10 µl of it was digested with 1 µl of *Bst*X1 enzyme, 5 µl of 10 x NEB Buffer 3, 33 µl of sterile distilled water at 37°C for 2

hours. The digest was checked with an agarose gel and purified with a Roche® PCR purification kit. This fragment was used for ligation into the plasmid.

2.26.2 Synthesis of sup3e - *orientation 1 and* sup3e - *orientation 2 fragments*

sup3-e orientation 1 (*sup3-e*-1), PCR fragment for integration into *S. pombe* was synthesized by PCR using 1 µl (10 nM) of each of forward and reverse primers S3 and S4 (Refer Table 2.4 for sequence) with 1 µl DNA from *S. pombe* with *sup3-e* integrant (BP1092), 10 µl of 5x Finnzymes buffer, 1 µl of Phusion™ High Fidelity Polymerase enzyme (New England Biolabs), 1 µl 10 nM dNTPs with 35 µl of sterile distilled water. The same was carried out for the *sup3-e* orientation 2 element [(*sup3-e* (20)], using primers S5 and S6 (Refer Table 2.4 for primer sequence). The PCR cycles used were similar to those used for *his3'*$_{283}$ synthesis (see 2.26.1).

Five reactions of each element were pooled. 5 µl of the product was run on a 1% agarose gel to visualise product size. The PCR product was purified using the Roche® (refer 2.8) and 10 µl of it was digested with 2 µl of *Bst*X1 enzyme, 5 µl of 10 x NEB Buffer 3, 33 µl of sterile distilled water at 37°C overnight. These digested fragments were subsequently used for ligation into the plasmid pREP42 back bone.

2.26.3 Plasmid preparation and integration of the his3'$_{283}$, sup3-e(1) *and* sup3-e(2) *fragments.*

Plasmid preparation and integration of the *his3'*$_{283}$, *sup3-e(1)* and *sup3-e(2)* fragments into the *ade6* gene were carried out exactly similar to *his3'*$_{283}$, *RTS1(1)* and *RTS1(2)* (see 2.25.3, and 2.25.4). Primers used for synthesis of various fragments which were incorporated into the *ade6* gene of *S. pombe* are as in Table 2.5.

Table: 2.5 Primers used in construction of *sup3-e* and *his3'$_{283}$* (control) fragments for integration into the *ade6* gene in *S. pombe* strains

No	Primer names	Primer sequence	Fragment synthesized
S1	tRNA-his3-BstX1-R	5'-CTGCAGAACCAAGAGTTTGGCAGCCTT ATCGCTGTGCG-3'	*his3'$_{283}$*
S2	tRNA-his3-BstX1-F	5'-TTCTGCAGCCAAACTCTTGGAATCAAT ATAGGC-3'	
S3	sup3-R-BstX1	5'-CTGCAGAACCAAGAGTTTGGGAAAGG GTAAGGAGAGGACG-3'	***sup3e* (1) (Orientation 1)**
S4	sup3-F-BstX1	5'-TTCTGCAGCCAAACTCTTGGACGTTTCA TTGTCAAGTTCG-3'	
S5	2sup3-F-BstX1	5'-CTGCAGAACCAAGAGTTTGGACGTTTC ATTGTCAAGTTCG-3'	***sup3e* (2) (Orientation 2)**
S6	2sup3-R-BstX1	5'-TTCTGCAGCCAAACTCTTGGGAAAGGG TAAGGAGAGGACG-3'	

2.27 Two - dimensional gel DNA replication fork analysis

2.27.1 Cell preparation

S. pombe cells were grown in 400 ml YEL with adenine (200 mg / litre) to log phase, having a cell count of 1 x 10^7 cells /ml (or at $O.D_{600}$ 0.4). Then they were decanted into prechilled Winstons (250 ml volume flasks) and 'stopped' by adding sodium azide (NaN_3) to a final concentration of 0.1%. Ice flakes were added to fill flasks. The flasks were chilled by swirling on ice for about 1 minute then the cells were harvested at 3,000 g. for 10 minutes at 4°C. The supernatant was removed and the cell pellet was washed in ice cold water and pooled into a 50 ml Falcon tube and the tube was spun at 3,000 g for 10 minutes at 4°C (pellets were stored at -80°C, if it is not for immediate use).

2.27.2 Cell Lysis

Pellets were resuspended in 2 ml NIB Buffer [Nuclear isolation buffer; made of 50 mM MOPS (pH 7.2), 150 mM KAc, 2 mM $MgCl_2$] and transfered to cold 50 ml Falcon tubes, containing 2-3 ml of acid washed glass beads and vortexed for 15 cycles of 30 seconds and then put on ice for 30 seconds on ice. The broken cell suspension was removed from beads to cold Oakridge tubes and the beads washed x 3 with equal volumes of ice cold nuclear isolation buffer. The pooled suspension was centrifuged at 5,000 g for 30 minutes at 4°C. The supernatant was removed and the pellet resuspended in 4 ml TEN buffer (5 mM Tris, 50 mM

EDTA and 100 mM NaCl; pH8) containing sodium sarkosyl (1.5 %) and 20 mg/ml Proteinase K. Samples were then gently swirl mixed and incubated at 42°C for a minimum of 2 hours. Samples were then spun at 1,000 g for 5 minutes at 4°C and supernatant transferred to 15 ml Falcon tubes containing 4.2 g cesium chloride.

Samples were then loaded into Beckman Quick-Seal centrifuge tubes (part No 34212) together with 0.025% Hoescht Dye 33258 (5 µl of a 5 mg/ml stock solution in water) and centrifuged at 50,000 g (Beckmen L8-M ultracentrifuge) for 18 hours at 20°C. DNA was visualized on a long wave UV platform and the middle band collected via a syringe and the sample washed 5 times with an equal volume of 5:1 isopropanol: distilled water. This was mixed and the DNA spooled out after adding 2 volumes ice cold 70% ethanol (EtOH) and the precipitated DNA was washed in 3 ml 70% Ethanol (EtOH) and air dried and resuspended in 400 ul TE buffer (pH 8) at 4°C.

2.27.3 Restriction Enzyme Digestion of DNA

200 µl of the DNA was digested with appropriate enzymes and buffers (New England Biolabs) at 37°C overnight using 100,000 u enzyme. Digests were then phenol chloroform precipitated for minimum of 2 hours at -20°C, washed in 70% EtOH and then diluted in 1200 µl TE buffer (pH 8) and 300 µl 5 M NaCl. Digestion of *RTS1* element containing DNA was carried out using *Sac*1 enzyme with appropriate buffer, which produced a fragment size of 4552 bp (including the *RTS1* element insert). The *sup3-e* element containing DNA was digested using *Eco*R1 enzyme which had a fragment size of 4045 bp (including the *sup3-e* element).

2.27.4 BND cellulose purification

2 ml of prepared BND cellulose solution was added to a 3 ml syringe column (plugged with glass wool) and dripped to 0.5 ml off the column bottom. Then the column was washed with 2 ml x 1 M NET buffer. The DNA samples were then loaded and dripped through. Columns were then washed with 2 x 1 ml NET buffer (10 mM Tris, 1 mM EDTA and 1 M NaCl; pH 8). The DNA was then eluted with 2 x 750 µl 1.8% caffeine in 1 M NET (30°C), collected and spun at 10,000 r.p.m. for 10 minutes to remove residual BND cellulose. Samples were then transferred to fresh tubes containing 750 µl isopropanol precipitated at 4°C for 2 hours,

spun at 10,000 g for 60 minutes, washed in 70% EtOH and resuspended in about 15 µl TBE / loading dye buffer.

2.27.5 Electrophoresis

Samples were run in the first dimension in 0.5% Agarose / TBE for 16 hours, 20 V and in the second dimension in 1.2% agarose / TBE 0.5 µg/ml ethidium for 1.5 hours 200 V run in cold room and 0.5 µg/ ml Ethidium TBE buffer was used.

2.27.6 Southern Blot and the probe

Salt transfer protocol for Southern blot (GeneScreen NEN Life Science Products) (Refer 2.21) was applied for transfer onto GeneScreen membrane. DNA for the probe was generated by PCR of the *ade6* gene using, 1 µl wild type strain DNA, with 1 µl each of Primers;

ade6 A -5'-TGGTAGTACGCAGTTTAGACG-3' and

ade6 B -5'-GCAGCTTCAAGAGGGTTG-3', (using PCR program as in 2.32ii) and 25 ng of the purified product was labeled with P^{32} γ CTP using the Megaprime DNA Labelling System (Amersham Biosciences). The membrane was probed (Protocol by GeneScreen, NEN Life Science Products) and scanned on a phosphor imager.

2.27.7 Interpretation of 2D gel images

The results from the prepared 2D gel image are read. The interpretation of the image is as described in Figure 2.2.

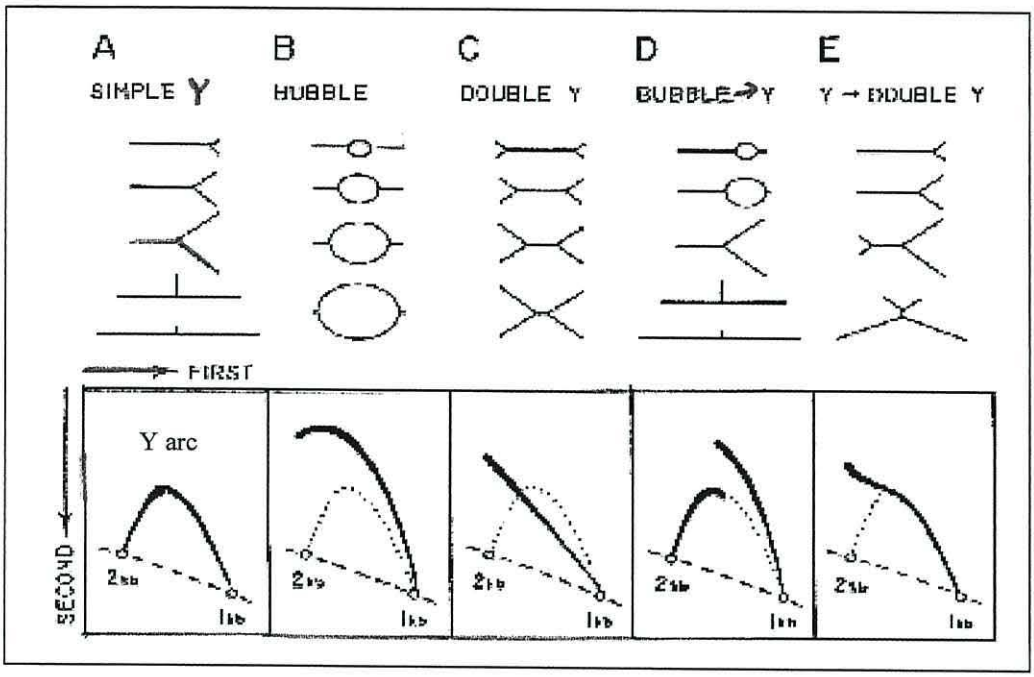


Figure 2. 2 Interpretation of two dimensional agarose gel electrophoresis (2D gel) analysis.

First dimension separation (arrow from left to right) of the replication intermediates is by size and second dimension separation (arrow from top to bottom on the left side of the image) by shape. The pre-digested DNA with restriction endonuleases cuts the replication bubble, producing replication intermediates (RIs) of various shapes. These appear as simple 'Y' (A), bubble (B), Double 'Y' (C), bubble or 'Y' (D) and 'Y' to double 'Y'.

A spot would be produced on the Y arc when a similar size replication intermediate accumulates at certain part of the replication fork; this indicates a replication fork is being blocked from progression.

2.28 Primers and sequencing:

All sequencing works were carried out, primers manufactured and supplied by MWG Biotech (Germany).

2.29 Media, chemicals and Reagents

Reagents for yeast and bacterial media and supplements were obtained from Difco (Becton Dickinson) and Sigma. All other common laboratory reagents were obtained from Sigma or BDH unless stated otherwise. Enzymes and buffers were from New England Biolabs (NEB), unless otherwise stated.

Agar was omitted for liquid media.

When minimal media like NB or EMM2 used, amino acid supplements were added to a final concentration of 200 mg/l.

1. YEA	**1 litre**
Yeast Extract	5 g
Glucose	30 g
Agar	14 g
2. YEA with Geneticin	**500 ml**
YEA	
Geneticin (50 mg/ml stock solution)	1 ml
500 mg geneticin was dissolved in 10 ml sterile distilled water for stock solution. The YEA+ Geneticin media was wrapped in aluminium foil and stored at 4°C.	
3. SPA	**500 ml**
Glucose	5 g
KH_2PO_4	0.5 g
Agar	15 g
*Vitamins (x1000)	0.5 ml
1 ml 1000 x Vitamins in every 500 ml *Added after autoclaving media	
4. EMM2	
Potassium Hydrogen Phalate	**1 litre**
Na_2HP0_4	3 g
NH_4Cl	2.2 g
Glucose	5 g
*Vitamins (x1000)	20 g
*Minerals (x 10,000)	1 ml
*Salts (x50)	0.1 ml
Agar	20 ml
	14 g
*To add after autoclaving media	
5. SALTS x 50	**500 ml**
$MgCl_2.6H_2O$	26.25 mg
$CaCl_2.2H_2O$	0.3675g
KCl	25 g
Na_2SO_4	1 g

6. VITAMINE X 1000	**500 ml**
Pantothenic acid	0.5 g
Nicotinic acid	5 g
Myo-inositol	5 g
Biotin	5 mg
7. MINERALS X 10,000	**500 ml**
Boric acid	2.5 g
$MnSO_4.7H_2O$	2 g
$FeCl_2.6H_2O$	1 g
KI	0.5 g
Molybdic acid	0.2 g
$CuSO_4$	0.2 g
Citric acid	5 g
*After autoclaving add a few drops of 1:1:2 chlorobenzene/ dichloroethane/ chlorobutane	
8. NITROGEN BASE AGAR (NBA)	**1 litre**
Nitrogen base	1.7 g
Glucose	10 g
$(NH4)_2\ SO_4$	5 g
Agar	24 g
9. 100 X DENHARDT'S SOLUTION	**50 ml**
Polyvinylpyrrolidone (MW40,000)	1 g
Bovine Serum Albumin	1 g
Ficoll 400	1 g
10. 20 X SSC	**1 LITRE**
3 M NaCl	175.35 g
0.3 M Sodium Citrate Dihydrate	88.23 g
11. 20 X SSPE	**1 LITRE**
3M NaCl	175.3 g
0.2 M NaH_2PO_4-H_20 27	6 g
0.02 M EDTA-Na (0.5 M stock solution)	40 ml
Distilled water	800 ml
Adjust pH to 7.4 with NaOH	
Add distilled water to make 1 litre	

Table 2.6 The *S. pombe* strains used in this project

Strain Number	Genotype	Source
BP90	*h⁻ ade6-M26 ura4-D18leu1-32*	McFarlane collection
BP743	*h⁻ rad 3-136*	McFarlane collection
BP1	*h⁻ wt strain* (Wild type)	McFarlane collection
BP8	*h⁺wt* strain (Wild type)	McFarlane collection
BP154	*h⁻ ade6 469*	McFarlane collection
BP1217	*h⁻ ade6-704 leu1-32 ura4-D18 rad9::ura4*	McFarlane collection
BP1219	*h⁻ ade6-{(GT)-1397}his3-D leu1-32 ura4D-18*	McFarlane collection
BP1220	*h⁻ade6-{(GT)-1397}his3-D leu1-32 ura4D-18 trax::kanMX6*	This study
BP1244	*h⁻ade6-{(GT)-1397} ura4D-18 leu1-32*	McFarlane collection
BP1089	*h⁻ade6-M26 ura4 -D18 leu1-32 translin::kanMX6*	McFarlane collection
BP1090	*h⁻ ade6-M26 ura4-D18 leu 1-32 trax::kanMX6*	This study
BP1200	*h⁻ ade6-M26 ura4-D18 leu 1-32* (pSRSB2)	This study
BP1201	*h⁻ ade6-M26 ura4-D18 leu 1-32* (pSRSB3)	This study
BP1204	*h⁻ ade6-M26 ura4-D18 leu 1-32* (pREP42X)	This study
BP1205	*h⁻ ade6-M26 ura4-D18 leu 1-32* (pREP4X)	This study
BP1134	*h⁺ ade6::his3'$_{785}$ his3-D1ura4-D18 lys1-37*	This study
BP1140	*h⁺ ade6::RTS1(1) his3-D1ura4-D18 lys1-37*	This study
BP1229	*h⁺ ade6::RTS1(2) his3-D1ura4-D18 lys1-37*	This study
BP1022	*h⁻ade6-52 ura4-D18 leu1-32*	McFarlane collection
BP1004	*ΔRHOR ade6-210 ura4-D18 swi1Δ::ura4*	McFarlane collection
BP1092	*h⁻ade6-704 sup3-5*	McFarlane collection
BP473	*h⁻ade6-M375 leu1-32 ura4D-18 zzz7-3::(ade6-469-ura4⁺)*	McFarlane collection
BP796	*h⁻ade6-M375 leu1-32 ura4-D18 zzz15-3::(ade6-469-ura4⁺)*	McFarlane collection

BP1135	*h⁺ade6:: his3'$_{283}$ his3-D1 ura4-D18 lys1-37*	This study
BP1177	*h⁺ade6 ::sup3-e (1) his3-D1 ura4-D18 lys1-37*	This study
BP1298	*h⁺ade6 ::sup3-e (2) his3-D1 ura4-D18 lys1-37*	This study
BP1303	*h⁺ade6 ::sup3-e (1) his3-D1 ura4-D18 lys1-37*(p*ade6-469*)	This study
BP1304	*h⁺ade6:: sup3-e (2) his3-D1 ura4-D18 lys1-37*(p*ade6-469)*	This study
BP1326	*h⁺ade6 :: his3'$_{283}$ his3-D1 ura4-D18 lys1-37*(p*ade6-469*)	This study
BP1327	*h⁺ade6:: his3'$_{283}$ his3-D1 ura4-D18 lys1-37 zzz7::ura4*	This study
BP1328	*h⁺ade6:: sup3e-1 his3-D1 ura4-D18 lys1-37 zzz7::ura4*	This study
BP1329	*h⁺ade6:: sup3e-2 his3-D1 ura4-D18 lys1-37 zzz7::ura4*	This study
BP1413	*h⁻ade6-M216 ura4-D18 leu1-32*	McFarlane collection
BP1427	*h⁻ade6::hi3'$_{283}$ his3-D1 ura4-D18 lys1-37 leu1-32 swi1::ura4*	This study
BP1428	*h⁻ade6::sup3e-1 his3-D1 ura4-D18 lys1-37 leu1-32 swi1::ura4*	This study
BP1429	*h⁻ ade6::sup3e-2 his3-D1 ura4-D18 lys1-37 leu1-32 swi1::ura4*	This study
BP1430	*h⁺ade6:: his3'$_{756}$ his3-D1 ura4-D18 lys1-37 zzz7::ura4*	This study
BP1431	*h⁺ade6:: RTS1(1)his3-D1 ura4-D18 lys1-37 zzz7::ura4*	This study
BP1432	*h⁺ade6:: RTS1(2) his3-D1 ura4-D18 lys1-37 zzz7::ura4*	This study
BP1433	*h⁺ade6:: his3'$_{756}$ his3-D1 ura4-D18 lys1-37 zzz15::ura4*	This study
BP1434	*h⁺ade6:: RTS1(1) his3-D1 ura4-D18 lys1-37 zzz15::ura4*	This study
BP1435	*h⁺ade6:: RTS1(2) his3-D1 ura4-D18 lys1-37 zzz15::ura4*	This study
BP1437	*h⁻ ade6 his3'$_{283}$ his3-D1 ura4-D18 lys1-37 leu1-32 swi1::ura4* (pDP13)	This study
BP1438	*h⁻ ade6 sup3-e (1) his3-D1 ura4-D18 lys1-37 leu1-32 swi1::ura4* (pDP13)	This study
BP1439	*h⁻ ade6 sup3-e (2) his3-D1 ura4-D18 lys1-37 leu1-32 swi1::ura4* (pDP13)	This study
BP1443	*h⁻ade6:: his3'$_{283}$ his3-D1 ura4-18 lys1-37 leu1-32*	This study
BP1444	*h⁻ade6:: sup3-e (1) his3-D ura4-18 lys1-37 leu1-32*	This study
BP1445	*h⁻ade6:: sup3-e (2) his3-D ura4-18 lys1-37 leu1-32*	This study
BP1521	*h⁻ade6:: his3'$_{283}$ his3-D ura4-18 lys1-37 leu1-32* (pSRS5)	This study
BP1522	*h⁻ade6:: sup3-e (1)his3-D ura4-18 lys1-37 leu1-32* (pSRS5)	This study
BP1523	*h-ade6:: sup3-e (2) his3-D ura4-18 lys1-37 leu1-32* (pSRS5)	This study
BP1524	*h⁻ade6:: his3'$_{283}$ his3-D ura4-18 lys1-37leu1-32 swi1::ura4* (pSRS5)	This study

BP1525	*h⁻ade6:: sup3-e (1) his3-D-ura4-18 lys1-37 leu1-32 swi1::ura4* (pSRS5)	This study
BP1526	*h⁻ade6:: sup3-e (2) his3-D-ura4-18 lys1-37 leu1-32 swi1::ura4* (pSRS5)	This study
BP1547	*h⁺rqh1Δ::kanMX6 ura4-D18 leu1-32*	McFarlane collection
BP1605	*h⁻ ade6:: his3$_{283}$ ura4-18 lys1-37 leu1-32 rqh1::kanMX6* (pSRS5)	This study
BP1606	*h⁻ ade6:: sup3-e (1) ura4-18 lys1-37 leu1-32 rqh1::kanMX6 (pSRS5)*	This study
BP1607	*h⁻ade6:: sup3-e (2) ura4-18 lys1-37 leu1-32 rqh1::kanMX6* (pSRS5)	This study
BP1621	*h⁻ade6:: his3'$_{283}$ his3-D1 ura4-D18 lys1-37 leu1-32 swi1::ura4*	This study
BP1622	*h⁻ade6::sup3-e (1) his3-D1 ura4-D18 lys1-37 leu1-32 swi1::ura4*	This study
BP1623	*h⁻ ade6::sup3-e (2) his3-D1 ura4-D18 lys1-37 leu1-32 swi1::ura4*	This study
BP1657	*h⁺ade6:: his3'$_{283}$ his3-D1 ura4-D18 lys1-37 zz15::ura4*	This study
BP1658	*h⁺ade6:: sup3-e (1) his3-D1 ura4-D18 lys1-37 zzz15::ura4*	This study
BP1659	*h⁺ade6:: sup3-e (2) his3-D1 ura4-D18 lys1-37 zzz15::ura4*	This study
BP1662	*h⁺ade6:: his3'$_{283}$ his3-D1 ura4-D18 lys1-37 leu1-32* (pSRS5)	This study
BP1663	*h⁺ade6:: RTS1(1) his3-D1 ura4-D18 lys1-37 leu1-32* (pSRS5)	This study
BP1664	*h⁺ade6:: RTS1(2) his3-D1 ura4-D18 lys1-37 leu1-32* (pSRS5)	This study
BP1696	*h⁻ade6:: his3'$_{756}$ his3-D1 ura4-D18 lys1-37 leu1-32 swi1:: ura4*	This study
BP1697	*h⁻ade6:: RTS1(1) his3-D1 ura4-D18 lys1-37 leu1-32 swi1::ura4*	This study
BP1698	*h⁻ade6:: RTS1 (2) his3-D1 ura4-D18 lys1-37 leu1-32 swi1::ura4*	This study
BP1695	*h⁺ade6:: sup3-e (2) his3-D1 ura4-D18 lys1-37 zzz7::ura4 swi1::ura4*	This study
BP1699	*h⁺ade6:: his3'$_{283}$ his3-D1 ura4-D18 lys1-37 zzz7::ura4 swi1::ura4*	This study
BP1775	*h⁺ade6:: sup3-e (1) his3-D1 ura4-D18 lys1-37 zzz7::ura4 swi1::ura4*	This study
BP1700	*h⁻ade6:: his3'$_{756}$ his3-D1 ura4-D18 lys1-37 leu1-32 swi1:: ura4* (pSRS5)	This study
BP1701	*h⁻ade6:: RTS1(1) his3-D1 ura4-D18 lys1-37leu1-32 swi1::ura4* (pSRS5)	This study
BP1702	*h⁻ade6:: RTS1(2) his3-D1 ura4-D18 lys1-37 leu1-32 swi1::ura4 (pSRS5)*	This study
BP1720	*h⁻ ade6:: his3'$_{756}$ his3-D1 ura4-18 lys1-37 leu1-32 rqh1::kanMX6* (pSRS5)	This study
BP1721	*h⁻ ade6:: RTS1(1) ura4-18 lys1-37 leu1-32 rqh1::kanMX6* (pSRS5)	This study
BP1722	*h⁻ade6:: RTS1(2) ura4-18 lys1-37 leu1-32 rqh1::kanMX6*	This study

	(pSRS5)	
BP1809	*h⁻ade6:: his3'$_{283}$ ura4-D18 lys1-37 leu1-32 swi1::ura4*	This study
BP1810	*h⁻ade6::sup3-e (1) his3-D1 ura4-D18 lys1-37 leu1-32 swi1::ura4*	This study
BP1811	*h⁻ ade6::sup3-e (2) his3-D1 ura4-D18 lys1-37 leu1-32 swi1::ura4*	This study
BP1812	*h⁻ade6:: his3'$_{283}$ his3-D1 ura4-D18 lys1-37 leu1-32 swi1::ura4* (pSRS5)	This study
BP1813	*h⁻ade6::sup3-e (1) his3-D1 ura4-D18 lys1-37 leu1-32 swi1::ura4* (pSRS5)	This study
BP1814	*h⁻ ade6::sup3-e (2) his3-D1 ura4-D18 lys1-37 leu1-32 swi1::ura4* (pSRS5)	This study

CHAPTER 3

GENERATION OF *traxΔ* STRAINS AND PRIMARY FUNCTIONAL ANALYSIS

3.1 Introduction

Translin is proposed to have functions in the generation of chromosomal translocations, mitotic cell division regulation and mRNA metabolism (Chennathukuzhi *et al.*, 2003a; Kanoe *et al.*, 1999). It has been shown that consensus DNA sequences at chromosomal break point junctions in lymphoid malignancies and sarcomas were specifically recognised by Translin, linking it to development of some cancers (Aoki *et al.*, 1995; Gajecka *et al.*, 2006b).

The Trax / Translin sequences are conserved from fission yeast to human, suggesting these molecules have an important conserved biological functions (Clauβen *et al.*, 2006; Laufman *et al.*, 2005; Jaendling *et al.*, 2008). Trax is a translin binding protein which shares conserved sequence similarities with Translin. Trax orthologs have been found in virtually all species that also have Translin (Clauβen *et al.*, 2006). However, no *S. cerevisiae* ortholog for either *trax* or *translin* genes have been found. The conservation of *trax* and *translin* genes in *S. pombe* made it an ideal yeast model for study of their function.

The idea that Translin and Trax may play a role in cell proliferation is supported by a variety of studies that investigated the effect of Translin or Trax depletion in different cell types. TB-RBP heterozygous primary mouse embryo fibroblasts (MEFs) have a reduced growth rate compared to MEFs derived from wild type littermates (Yang *et al.*, 2004a). The same authors also observed that Trax was post transcriptionally stabilised by Translin and both Trax and Translin were needed for normal cell proliferation. It was also demonstrated that reduction of Trax protein in HeLa cells slows growth rate and progression through G2 to M transition (Yang and Hecht, 2004b). Consistent with this observation, over-expression of Translin leads to acceleration of cell proliferation (Ishida *et al.*, 2002).

Despite the potential role in cell regulation and causation of cancer by Translin and Trax, very little is known about their biological function. After completion of the *S. pombe* genome sequence project, a pilot gene deletion project to assess the feasibility of a genome-wide deletion project and to estimate the percentage of essential genes was carried out by

Decottignies and colleagues (2003) and the *trax* gene was deleted. It was reported that *trax* was a non essential gene (Decottignies *et al.*, 2003). To date the function of the *trax* and *translin* genes remains unknown.

In order to study the functions of the *trax* gene in more detail, we first deleted the *trax* gene in *S. pombe*. In this chapter, the generation of *trax*Δ mutants which provided the basis for functional analysis of the *trax* gene is described. The effect of the *trax* deletion on cell proliferation and growth were studied. *trax* over-expression was also studied. Translin has been shown to post transcriptionally stabilize Trax in previous studies using MEFs. We also examined whether this function exists in lower eukaryotes.

3.2 Results

3.2.1 Amino acid sequence alignment of the human, mouse and the *S. pombe* Trax proteins.

The *trax* gene presents as a single copy in the human genome located at 1q42 (Devon *et al.*, 2000). The open reading frame of the *S. pombe trax* gene is 696 bp which codes for a 231amino acid predicted protein of 26.7 kD.

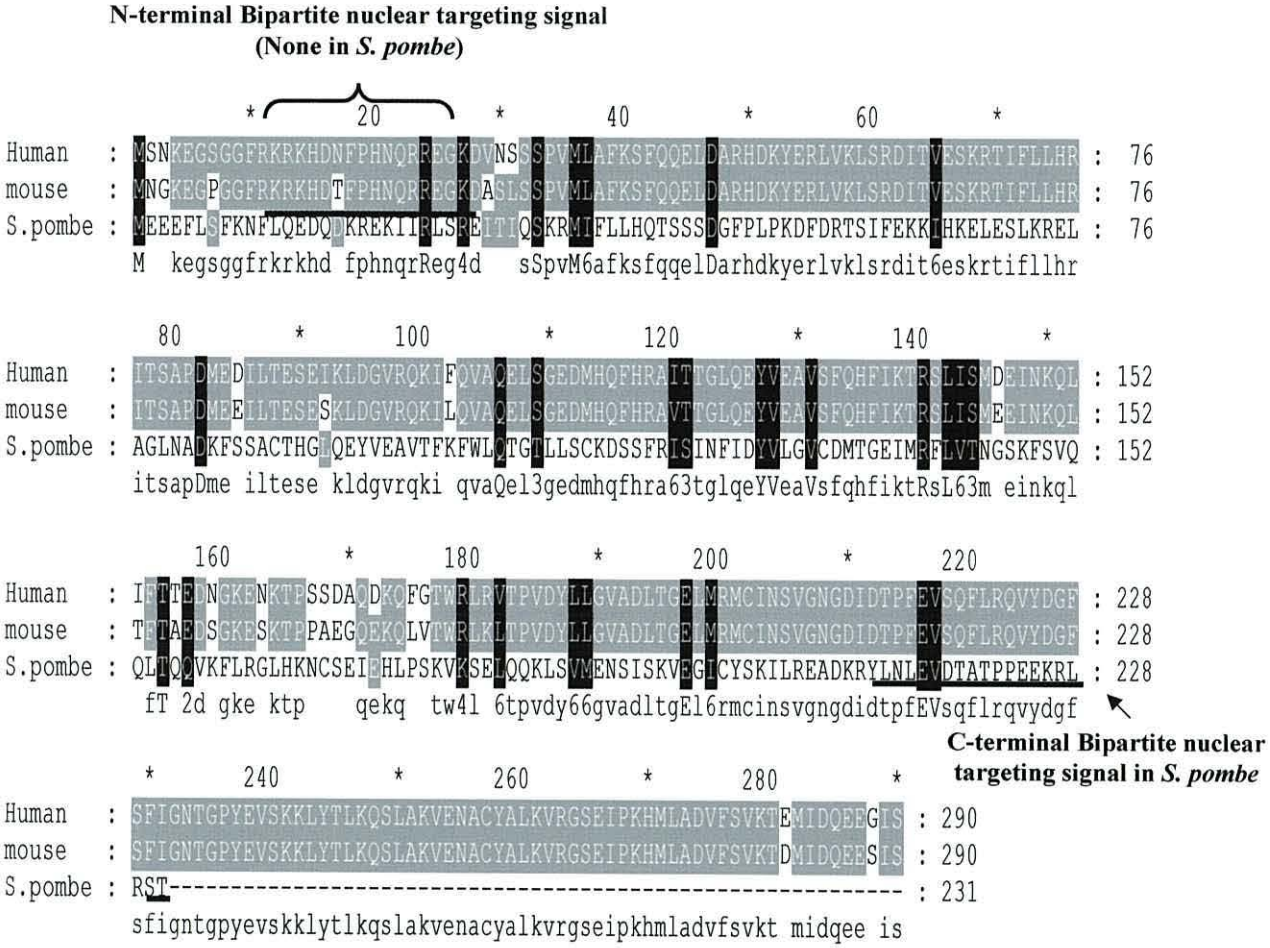


Figure 3.1 Amino acid sequence alignment of the human, mouse and the *S. pombe* Trax proteins

The program ClustalW was used to align the amino acid sequences of the human, mouse and *S. pombe* Trax proteins. The identical residues are highlighted in black. Similar residues are highlighted in grey. The bipartite nuclear targeting signal at N terminal missing in *S. pombe*. However, the C-terminus of the *S. pombe* Trax protein contains a putative bipartite nuclear targeting signal line (amino acids 210–229, KRYLNLEVDTATPPEEKRLR). Bipartite nuclear targeting signals are marked with black underline.

Alignment of amino acid sequences of human, mouse and *S. pombe* Trax proteins using ClustalW program (Figure 3.1), revealed that the sequences of 34 amino acids at the N-terminus of the mammalian protein, which include the bipartite nuclear targeting signal (amino acids 11–27, RKRKHDNFPHNQRREGK) (Aoki *et al.*, 1997b) is absent in the *S. pombe* Trax protein. However, the C-terminus of the *S. pombe* Trax protein does contain a putative bipartite nuclear targeting signal (amino acids 210–229, KRYLNLEVDTATPPEEKRLR).

3.2.2 Deletion of the *S. pombe trax* gene

To delete the *trax* gene we used the PCR based method of Bähler and co workers (1998) (See 2.1, Materials and Methods). In brief, the *kanMX6,* (Kanamycin resistance) cassette was amplified using primers with extensive homology to regions flanking the *trax* open reading frame (ORF) (Figure 3.2).

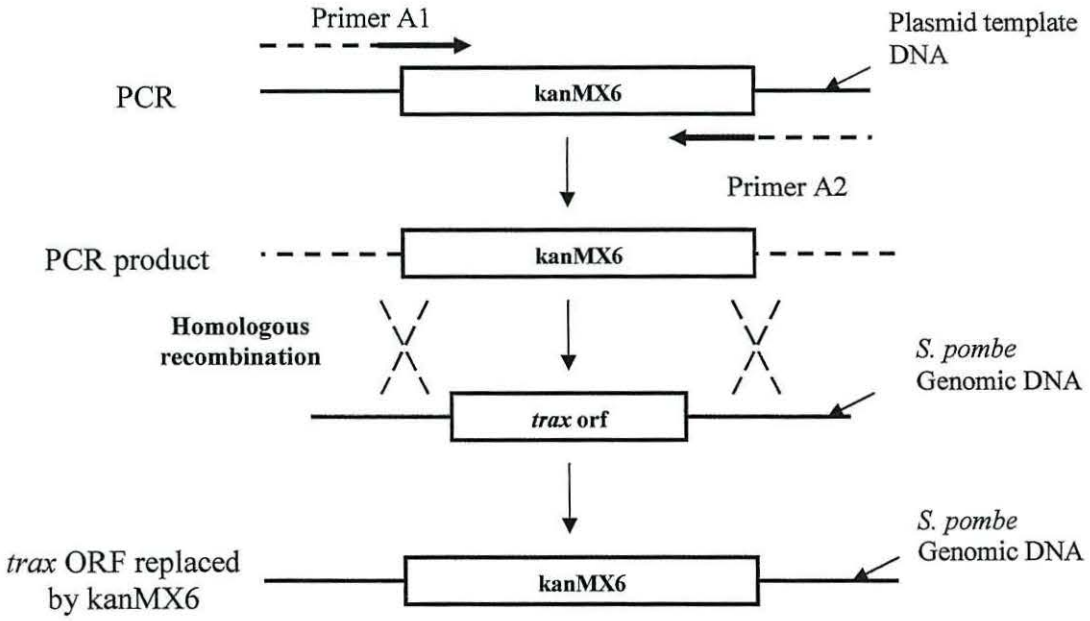


Figure 3.2 Schematic for *trax* gene deletion

kanMX6 cassette was obtained by PCR using primers with extensive homology to regions flanking the *trax* open reading frame (marked with broken lines). The purified PCR product was transformed into *S. pombe.* The *trax* ORF was substituted by *kanMX6* cassette, by homologous recombination.

The candidate *traxΔ* isolates were initially screened by PCR checking of *kanMX6* using specially designed primers (see Table 2.1 Materials and, Methods for sequence). Two candidates were selected for detail characterization. They were designated strain number 2 and 8. The DNA used was extracted from the respective *S. pombe* strains (See 2.5 Methods and Material). The TRAX Check forward primer (P1) was designed to prime from 98 bp upstream of the start codon of the *trax* gene and the TRAX Check reverse primer (P2) was designed to prime 166 bp down stream of the stop codon. The internal primers, TRAX intF (P3) were designed to prime 49 bp down stream of the start codon within the *trax* gene; whereas the TRAX intR primer (P4) was designed to prime 20 bp up stream of the stop codon within the *trax* gene. The TRAX midF primer (P5) was designed to 136 bps down stream of the start codon within the *trax* gene open reading frame (Figure 3.3).

Primers P1 and P2 are expected to produce a PCR product of approximately ~1.8 kb in *traxΔ* strains, compared to 964 bp in the $trax^{+}$ strain. Further to this, the strains were tested by nested PCR using various primers (refer to Figure 3.3 A and B for approximate primer positions). Primers were designed from within the *trax* gene and nested PCR performed to show that the *trax* gene has been completely deleted.

In the *trax+* control strain (BP90, which was originally used as the parental strain for the *trax* deletion) the primers P1+P4, P2+P3, P2+P5 and P3+P4 produced PCR product of approximately 800 bp, 800 bp, 700 bp and 600 bp respectively, which were the predicted values for *trax+* (Figure 3.3 A, Figure 3.4 A). There was no band produced when the same set of primers were tested with the *traxΔ* candidate strains 2 and 8 (Figure 3.3 B).

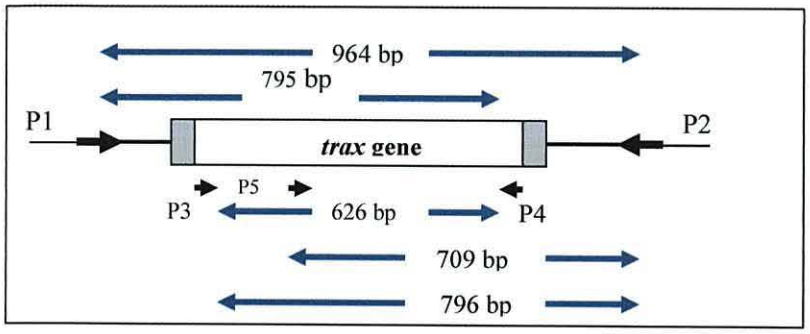
The PCR was carried out with a different set of primers to confirm presence of the *kanMX6* cassette in place of the *trax* gene. Primers P1+P2, P2+P7 and P2+P8 produced a product of approximately ~1.8 kbp, ~1.2 kb and ~ 600 bp respectively in the *traxΔ* candidate strains but not in the parental *trax+* strain (BP90) suggesting that the candidate *traxΔ* strains have *kanMX6* in place of the *trax* open reading frame in their genome (refer to Figure 3.3 B). I concluded that candidates (2) and (8) were likely to be legitimate *traxΔ* strains.

3.2.3 Southern blot of *traxΔ* strains

The genomic DNA of the *traxΔ* candidate strains, 2 and 8, as analysed by Southern blot to confirm that the *trax* gene has been replaced by a single copy of the *kanMX6* (Refer 2.13 Methods and Material). Restriction enzymes were selected using the Jelly Fish program to determine cut sites on the *kanMX6* cassette. *Pst*1 and *Spe*1 enzymes were used to digest the genomic DNA. The blotted digests were probed with a 1051 bp sized *kanMX6* probe (Refer to 2.13, Methods and Material for details).

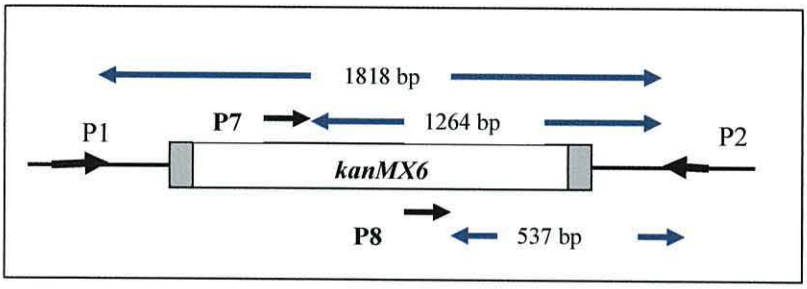
The restriction endonuclease sites are as shown in Figure 3.5 A. The *Pst*1 has one site within the *kanMX6* insert, as well as in genomic DNA proximal to the *kanMX6* insertion site. *Spe*1, on the other hand has one site distal to the *kanMX6* stop codon as well as proximal to the start codon of the *kanMX6* insert. The predicted *Pst*1 and *Spe*1 digested *kanMX6* is shown in Figure 3.5 A. The genomic digest with *Pst*1 and *Spe*1 produced *kanMX6* containing two partially digested fragments, size of ~2.4 kb and ~1.7 and a fully digested fragment size of ~1.3 kb (Figure 3.5 B). These were close to the expected fragments sizes of 2.370, 1.715, and 1.270 Kb in size (Figure 3.5 A). For reasons which remain unclear, the DNA preparations made faild to digest fully using these restriction enzymes. Given that western blot and PCR analysis indicated the protein was not present and the gene fully deleted this was not taken further.

A



Predicted Size (bp)	Approximate Observed Size (bp)
P1+P2= 964	1000
P1+P4= 795	800
P3+P4= 626	600
P5+P2= 709	700
P3+P2= 796	800

B



P1+P2= 1818	1800
P7+P2= 1264	1200
P8+P2= 537	600

Homologous region

Figure 3.3: Predicted and observed PCR product size obtained from PCR analysis of wild type and *traxΔ* candidates

DNA from the two candidate *traxΔ* strains (2 and 8) subjected to PCR using various primer combinations (Refer to Table 2.1 for primer sequence).
A. Approximate position of primers (P) on the *trax* gene used for PCR check of the *trax*$^{+}$ strain. The predicted size of the product is as illustrated at the right side of the figure.

B. The primer position on suspected *traxΔ* strains and expected PCR product size are shown (blue arrows). The predicted size of the product is as listed on the right hand side of the figure.

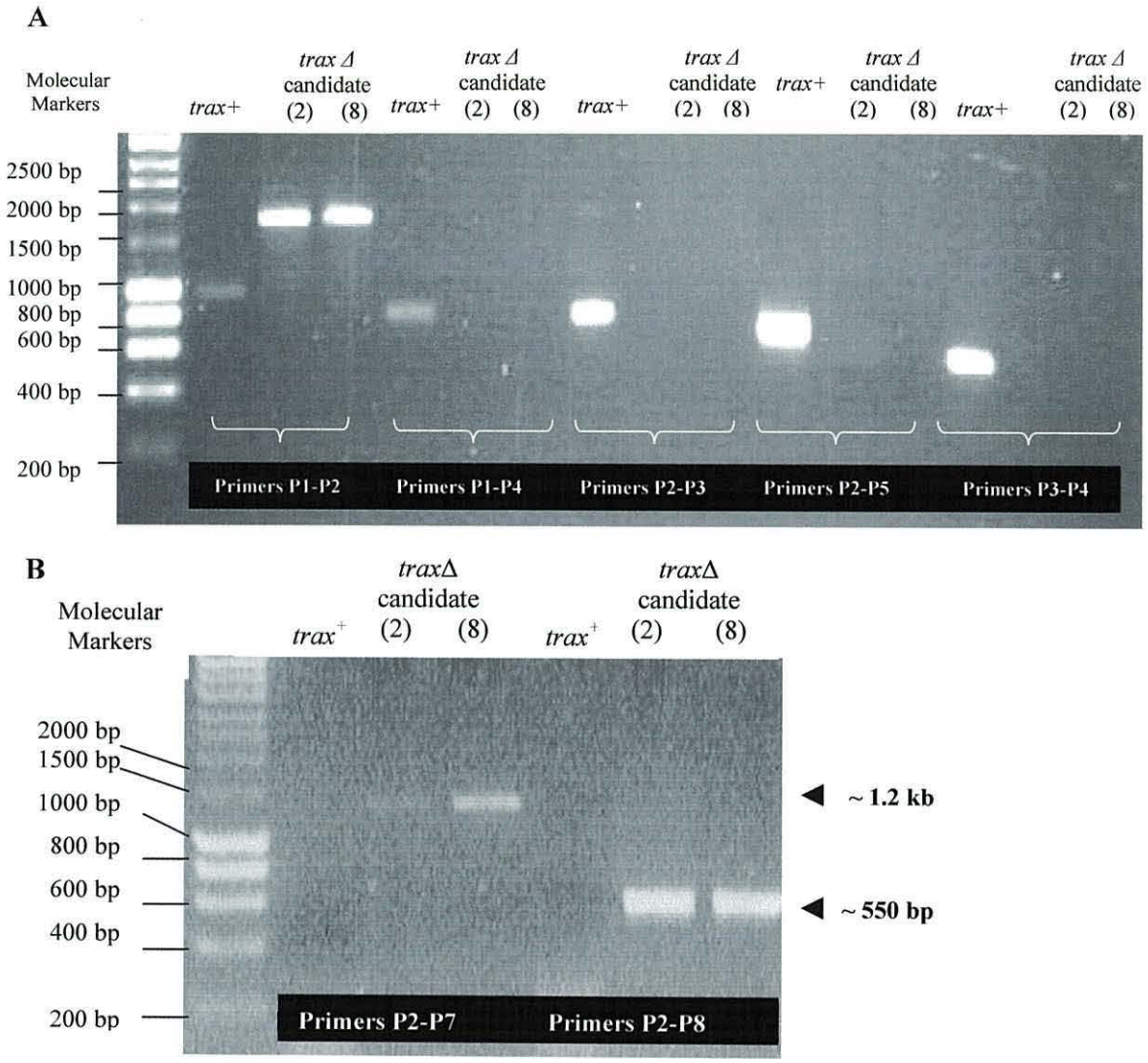


Figure 3.4 PCR results shown in agarose gel analysis

A. An agarose gel analysis of the PCR product of DNA from *trax*$^+$ and candidate *traxΔ* strains 2 and 8. PCR using primers P1 and P2 produced a product size of ~1 kb in the wild type strain and ~1.8 kb in candidate *traxΔ* strains as predicted. Primers P1-P4, P2 –P3, P2- P5 and P3-P4 produced ~800 bp, ~800 bp, ~700 bp, and ~600 bp products respectively in the *trax*$^+$ strain. No product was generated in the suspected *traxΔ* strains tested with the same set of primers.

B PCR showing that candidate t*raxΔ* strains 2 and 8 have the *kanMX6* cassette in place of the *trax* open reading frame (ORF). The PCR with primers P2 and P7 and primers P2 and P8 (Refer to Figure 3.3 B) produced a PCR product of ~1.2 kb and ~600 bp respectively in candidate *traxΔ* strains as predicted. No product was generated in the *trax*$^+$ strain. This further confirms that *kanMX6* has replaced the *trax* in the *traxΔ* strains 2 and 8.

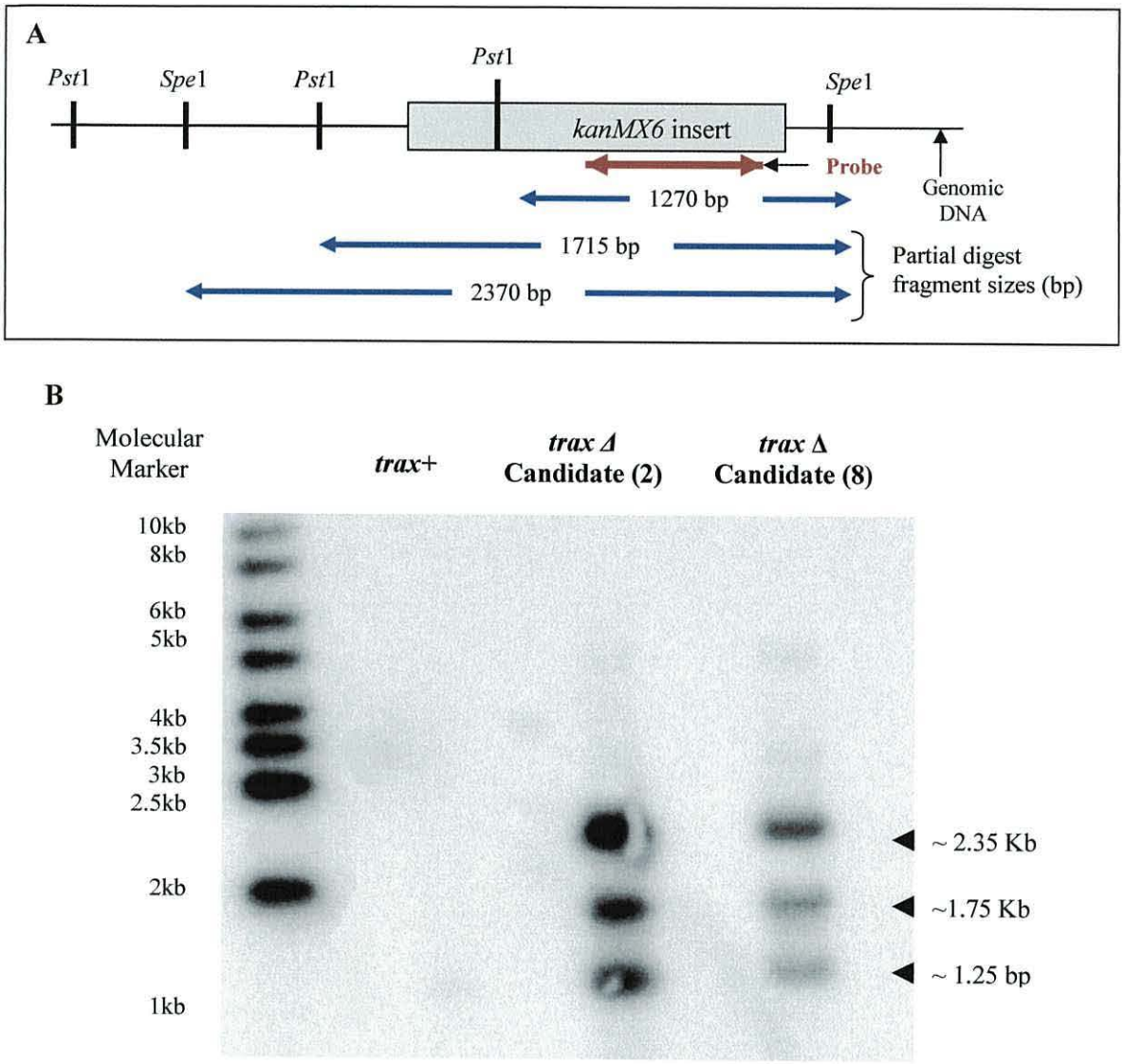


Figure 3.5 Southern blot of candidate *traxΔ* strains 2 and 8 showing presence of a single copy of *kanMX6* is inserted in the genome of each candidate.

A. This diagram represents digest sites of enzymes *Pst*1 and *Spe*1 and the expected band sizes from the Southern blot. The brown line indicates the approximate position of the probe used.

B. Enzyme *Pst*1 and *Spe*1 digested fragments of *traxΔ* candidate 2 and 8 genomic DNA shows the expected band sizes of ~2.35 kb (partial digest) ~1.7kb (partial digest) and ~1.25 kb (full digest) detected by Southern blot.

3.2.4 Western blot of *traxΔ* strains

PCR analysis and Southern blotting indicate strains 2 and 8 have the *trax* gene deleted. To further verify that there is no Trax protein being expressed by these strains, we carried out Western blot analysis (see 2.14; Materials and Methods) using polyclonal antibodies we raised against Trax peptides. Two different sets of polyclonal antibodies were raised against different Trax peptides (see 2.14; Materials and Methods for peptide sequence used). Both gave identical results in the wild type and did not produce any bands in the *traxΔ* strains (Results for antibody raised against the C terminal shown in Figure 3.6).

A specific band with mobility of approximately 27 kD was apparent in the *trax*+ whole cell extracts (WCEs) (Figure 3.6). This molecular weight is a close match to the predicted weight for Trax of 26.7 kD. No Trax specific bands were detectable in the *traxΔ* candidate 2 and 8. The membrane was also probed with anti-tubulin antibodies, to ensure total loaded protein was almost equal in each well (Figure 3.6).

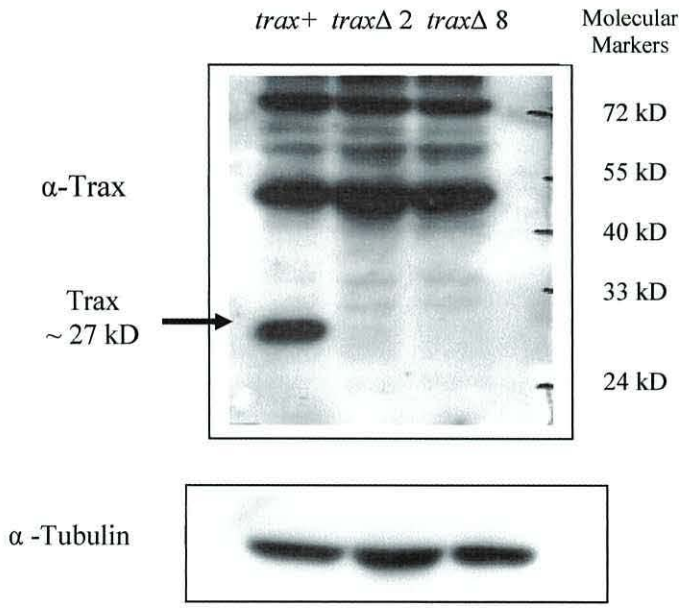


Figure 3.6: Western blots of candidate *traxΔ* strains 2 & 8 showing that they do not produce Trax protein.

The protein extract from wild type and candidate *traxΔ* cultures were probed with polyclonal anti-Trax antbodies. A band migrating with a molecular weight of approximately 27 kD is observed in the wild type but not in the two *traxΔ* strains. This molecular weight matches the predicted weight for Trax protein (26.7 kD). Polyclonal antibody used for this membrane was raised against C terminal Trax peptides.

The membrane was stripped and re-probed with anti tubulin antibodies to verify if similar amount of protein was loaded on each lane (lower image).

3.2.5 Trax does not play a primary role in regulating cell viability, proliferation or promote changes in the cell morphology in fission yeast.

3.2.5.a. Growth Rate

I have demonstrated that haploid *traxΔ* mutants are viable. This is consistent with previous studies (Decottignies *et al.*, 2003; Laufman *et al.*, 2005). Translin and Trax deficient MEFs have been shown to have a slow growth phenotype, suggesting that Translin and Trax function in controlling mitotic cell proliferation in metazoans (Yang *et al.*, 2004a). To determine whether loss of *trax* has any adverse effect on cell proliferation and cell morphology, we analysed growth rate and morphology in exponentially growing mutant and wild type cells. The *traxΔ* strains were streaked and incubated at various temperatures; 25°C, 30°C and 33°C, to look for any possible temperature sensitive growth phenotype. No difference was observed (data not shown).

To determine whether the deletion of the *trax* gene resulted in any growth defect, wild type and the *traxΔ* strains were grown exponentially in EMM2 liquid media with appropriate supplements at 30°C. Cultures were sampled at intervals of 1.5 hours. The samples collected were measured in a spectrophotometer (OD_{600}). Total cell counts were determined by counts using a haemocytometer. Viable cell counts were determined by plating serial dilutions of cultures onto YEA media. The cells were also stained with Calcofluor and DAPI stain and cell morphology and septation characterization was observed.

Growth rate, as measured by OD_{600}, did not show any significant difference between wild type and the *traxΔ* mutant (Figure 3.7 A). This was consistent with microscopic and viable cell counts obtained from this experiment in which no difference was observed between wild type and *traxΔ* strains (Figure 3.7 B and C). Proportion of cells with a septum (septation index) in all three samples was between 10-15% of a total of 500 cells examined at each interval (data not shown). This demonstrates that the cells were all growing exponentially during this experiment.

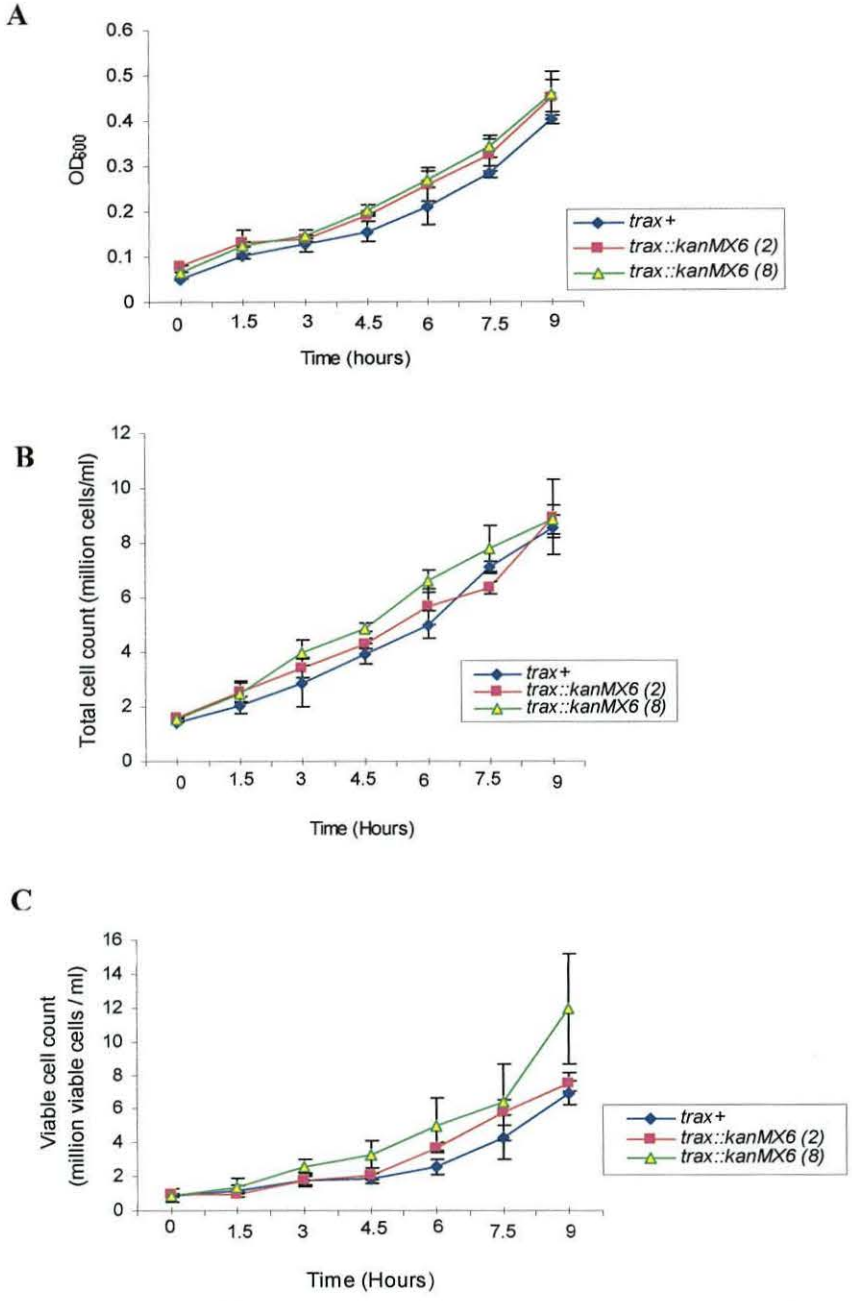


Figure 3.7 Growth analyses of wild type (*trax+*) and *traxΔ* strains grown at 30°C.

Growth curve was plotted by growing *trax+* and *traxΔ* cells in EMM2 media with appropriate supplements over nine hours. Samples were colleted at 1.5 hours intervals and measured by OD_{600}, microscopic cell count and viable cell counts. A minimum of three independent cultures were used for each strain. Error bars shown are standard deviations (SD).

A. Growth curve by O.D. measurements ($O.D_{600}$) of *trax+* and *traxΔ* strains 2 and 8.
B. Growth curve by total cell counts of cells grown in EMM2 of the same cultures.
C. Growth curve by viable cell counts of cells grown on solid media of the same cultures.

b. Loss of Trax does not affect cell morphology

I wanted to examine if the loss of *trax* leads to any abnormality in appearance of *S. pombe* cells. The wild type cells were compared with the *traxΔ* cells by microscopic examination. I did not find any difference in the morphology of *traxΔ* strains in comparison to the wild type. This is consistent with previous studies in *S. pombe* (Laufman *et al.*, 2005).

I concluded that *traxΔ* strain does not have any defect in growth rate or cell morphology of *S. pombe* cells at the temperatures tested (25°C, 30°C and 33°C).

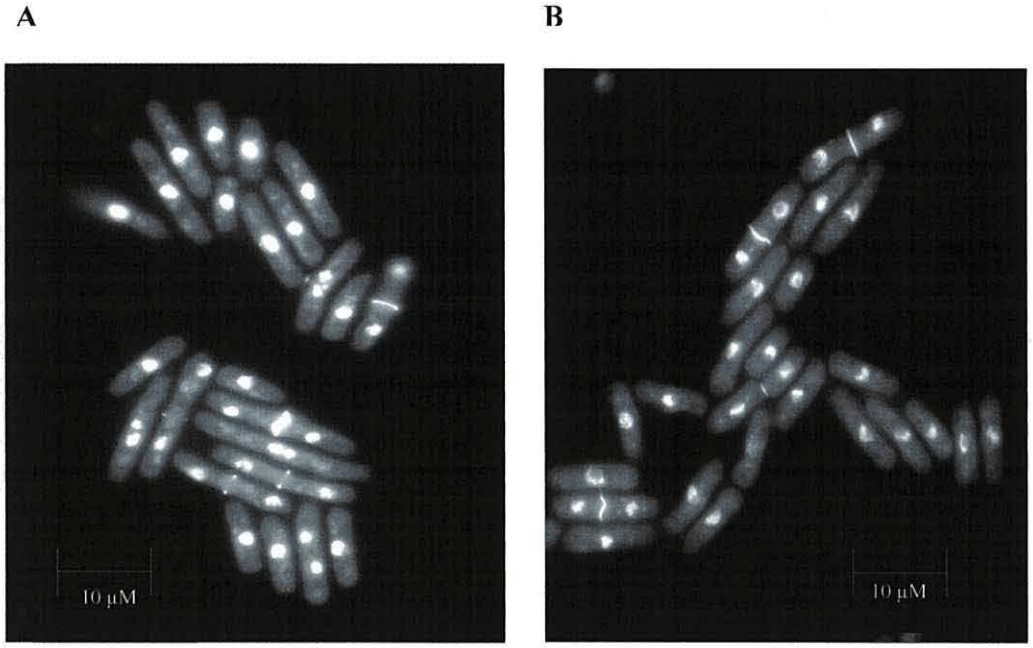


Figure 3.8 Examination of cell morphology and nuclear structure by Calcuflour and DAPI stain.

The *S. pombe* cells grown in the EMM2 media were fixed in 70% ethanol stained with Calcofluor (septa) and DAPI (nuclei) and examined under the fluorescent microscope. No measurable difference in size or morphology is observed between *trax*Δ and *trax*+ strains.

A. *trax*$^{+}$ strain (BP90) seen under the Fluorescent microscope
B. *trax*Δ (strain 8).

3.2.6 Over expression of *trax* does not result in changes in cell proliferation or morphology.

a. Cloning of the *trax* ORF into the pREP vector for *trax* over expression.

Previous reports have found that over-expression of translin accelerates cell proliferation in higher eukaryotes and that depletion of Translin and Trax reduces cell proliferation (Yang *et al.*, 2004a; Yang and Hecht, 2004b). I wanted to examine if over expression of *trax* has any effect on *S. pombe* cell proliferation and morphology. The *trax* open reading frame (ORF) was cloned into an *S. pombe* plasmid vector, pREP series placing the *trax* gene under the control of the inducible *nmt* (no message thiamine) promoter (Maundrell, 1993). Expression levels are said to be medium (pREP42X) and high (pREP4X).

*Bam*HI enzyme was used to cut the cloning site adjacent to the *nmt* promoter on the respective plasmids. The *trax* ORF was obtained by PCR with *Bam*HI restriction sites on both forward and reverse primers (Refer to 2.16 Methods and Material). The PCR fragment was digested with *Bam*HI enzymes and cloned into the plasmids with high (pREP4X) and medium (pREP42X) expression *nmt* inducible promoters (Refer to Figure 3.9). The ORF containing plasmids were transformed into electro competent *E. coli* cells and the required plasmids were isolated and sequenced. The plasmids with correct inserts of $trax^{+}$ ORF (the sequence given below) were transformed by electroporation method into *S. pombe* wild type cells (BP90). The medium and high expression *nmt* inducible plasmids containing *trax* ORF were called pSRSB2 and pSRSB3 respectively. Similarly, empty plasmids were also transformed into the parental cells to be used as control strains.

ATGGAAGAGGAATTCCTCTCATTTAAAAATTTTTTACAGG AGGATCAAGATAAGAGGGAGAAAATTATTCGTCTTTCTCGT
GAAATCACAATTCAAAGTAAACGAATGATCTTTCTTTTACATCAAACATCCTCTTCTGATGGATTTCCTCTACCCAAGGATT
TCGACAGAACATCCATTTTTGAAAAAAAAATTCACAAAGAACTAGAAAGTCTGAAGAGAGAGTTGGCTGGATTAAATGCAG
ACAAGTTCTCTTCTGCTTGTACACAC GGCCTTCAAGAGTATGTTGAAGCTGTCACTTTCAAATTCTGGTTACAAACAGGTA
CGTTACTTTCTTGTAAAGATAGTAGCTTTAGAATTTCTATTAATTTCATCGACTATGTACTCGGTGTTTGTGACATGACTGG
TGAAATAATGAGATTCCTTGTAACCAACGGCTCTAAGTTCTCTGTCCAACAACTCACTCAGCAAGTTAAATTTCTTCGTGGA
CTACACAAAAACTGCTCAGAAATTGAACACTACCATCTAAGGTCAAGTCAGAATTACAGCAGAAATTATCAGTAATGGAGAA
CAGCATTTCAAAAGTTGAAGGAATTTGCTATAGTAAAATTTTAAGAGAAGCAGATAAA AGATATTTAAATTTGGAAGTGGA
TACAGCAACTCCTCCCGAGGAGAAACGATTACGGTCCACATAA

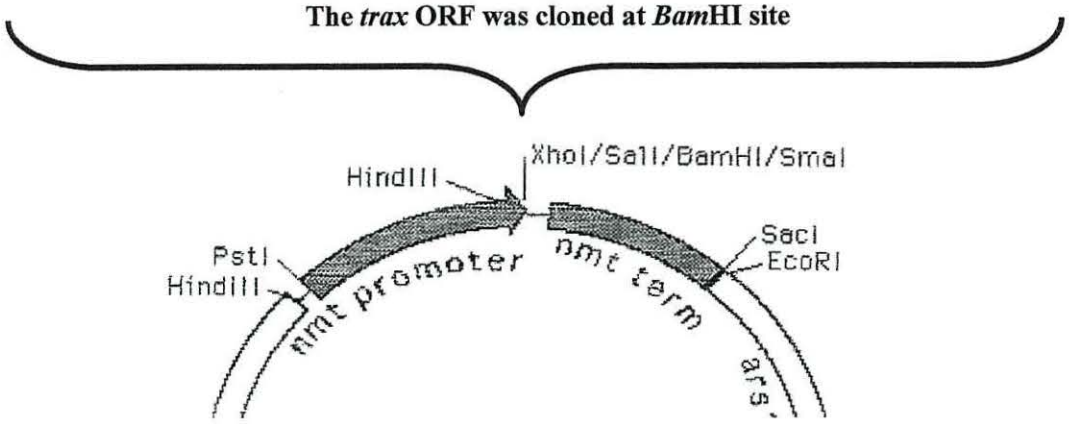


Figure 3.9 The *trax* ORF was cloned in the plasmid at the multi cloning site

Partial diagram of the pREP back bone is shown above. *Bam*HI was used to cut the site adjacent to the *nmt* promoter. The *trax* ORF was cloned at this cloning site. The *trax* ORF (sequence is shown above) was cloned in two plasmids, pREP4X and pREP42X having high and medium strength repressible promoters respectively. At the concentration of 15 mM thiamine in EMM2 media, the promoter is suppressed and when the thiamine is removed the promoter is activated.

Source: Forsburg Lab pombe pages: Index of fission yeast vectors

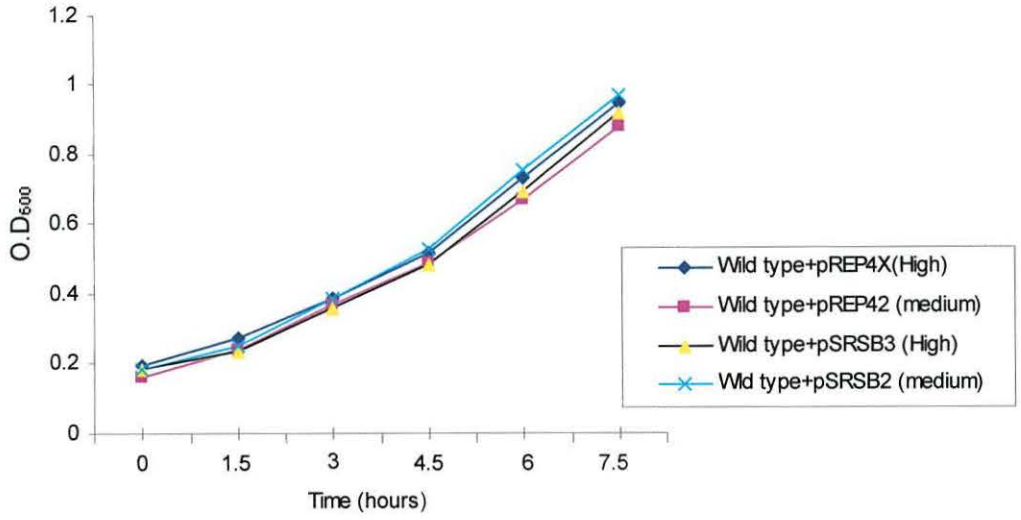
b. Over expression of *trax* does not affect the proliferation and morphology of the cells.

The expression from *nmt* promoters was first suppressed by growing cells in EMM2 supplemented with thiamine at a concentration of 15 mM. It has been reported that removal of thiamine from the medium produces the first detectable message after 10-16 hours (Maundrell, 1990). Proliferation of cells was examined by OD as well as microscopic examination after 16 hours post thiamine removal at intervals of 1.5 hours.

Deletion of *trax* did not seem to stimulate any growth in my previous study (as shown in Figure 3.7 A, B and C). Preliminary results carried out with cells having empty plasmids (pREP4X, pREP42) and plasmids over expression of *trax* (pSRSB3, pSRSB2) showed that

trax over-expression did not affect the cell proliferation (as in Figure 3.10A) or morphology. The same tests were repeated using triplicate samples for viable cell counts using the *trax+* strain having empty high expression plasmid (pREP4X) and a *trax*$^{+}$ strain with the high expression plasmid containing *trax* ORF (pSRSB3). All the cells with empty plasmid and cloned *trax* (*trax* over expressed) showed very similar growth kinetics, suggesting over-expression of the *trax* gene does not influence cell proliferation in *S. pombe* (Refer to Figure 3.10). This finding is contrary to the earlier studies showing that Trax depletion affects growth of HeLa cells (Yang and Hecht, 2004b; Yang *et al.*, 2004a). The same authors also demonstrated that TB-RBP–deficient MEFs exhibited reduced growth rate in comparison with the wild type littermates.

A



B

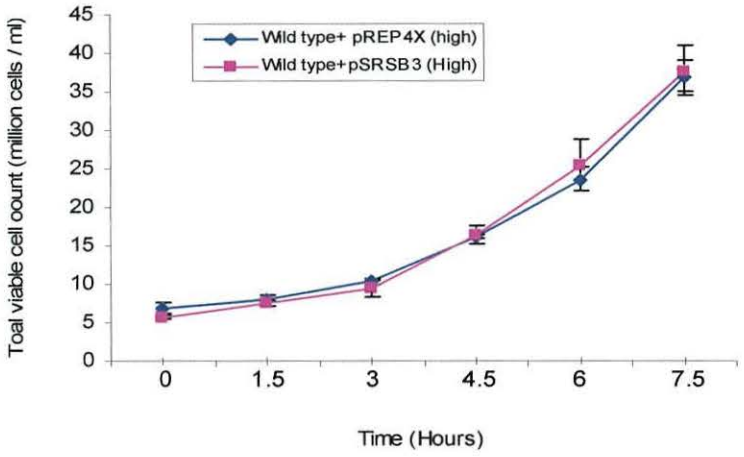


Figure 3.10 Over expression of *trax* gene does not alter growth rate.

The wild type cells (BP90) with empty pREP4X / pREP42 plasmids (high expression *nmt* plasmid and medium expression *nmt*) were cultured in EMM2 with appropriate supplements along with BP90 strain having high and medium *nmt* expression which contain *trax* ORF producing the effect of Trax protein over-expression. Samples of cultures were collected at intervals of 1.5 hours

A. Preliminary results showing the growth curves of the *S. pombe* cells measured by $O.D_{600}$. No difference in proliferation rate seen between the *trax* over-expressed and control cells
B. Graph showing the growth curves by total viable cell culture in YEA. No significant difference seen in viable cell count between *trax* over-expressed and control cells. Error bars shown represent the standard deviation.

Examination of cells under the microscope having *trax* over-expression or without *trax* over-expression did not reveal any differences in cell size or cell morphology. However, a small proportion of cells having *trax* over expressed appeared to have a double septum (approximately 5 in 500 cells examined). This phenomenon was also observed in cells with empty plasmids at similar levels. However, cells with double septa were not observed in *traxΔ* cells without any plasmid.

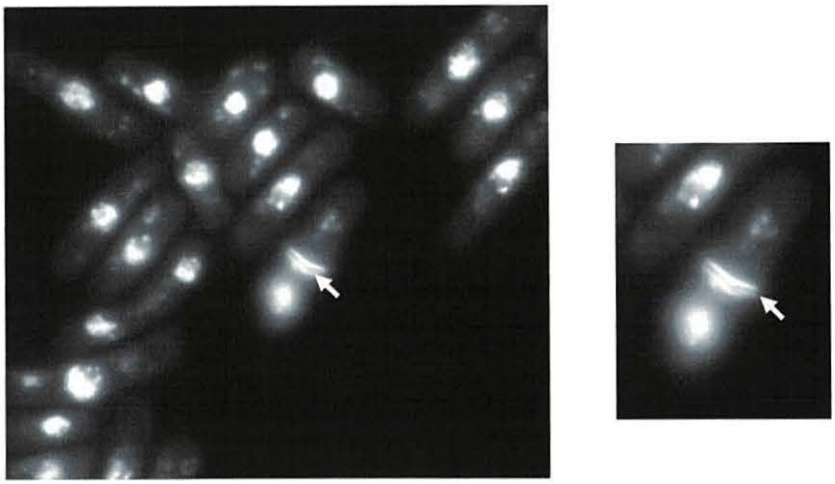


Figure 3.11 *S. pombe* cells with plasmids have a double septum.

A small number of *S. pombe* cells with plasmid having *nmt* repressible promoters show presence of double septum (shown in white arrow). These appeared in both cells empty plasmids (pREP4X and pREP42X) and plasmids with *trax* ORF (pSRSB3 and pSRSB2). However, this was not observed in *traxΔ* strains.

Whist I saw an increase in *trax* mRNA in strains carrying pSRSB2/3, I did not observe concomitant elevation of protein (see below). Therefore the effect of over expression of *trax* may not be providing insight into function. This phenomenon was also observed by other authors working with mice (Chennathukuzhi *et al.*, 2003a) in MEFs (Yang *et al.*, 2004a) and in *Drosophila* (Clauβen *et al.*, 2006). The lack of elevated Trax protein, when the *trax* mRNA is greatly elevated indicates that there is a post transcriptional regulatory pathway controlling the levels Trax expression in *S. pombe*. Given that Translin is required for regulation of Trax levels, I explored the possibility that elevated Translin was required for over expression of Trax protein. To achieve this, I used co-transformed wild-type cells with

plasmids over-expressing *trax*+ (pSRSB3) and *translin*+ (pAJ1). pREP4X and pREP3X empty vectors were used as negative controls respectively. I found that the over-expression of *trax* and *translin* simultaneously also did not have any effect in cell proliferation or morphology (Figure 3.12).

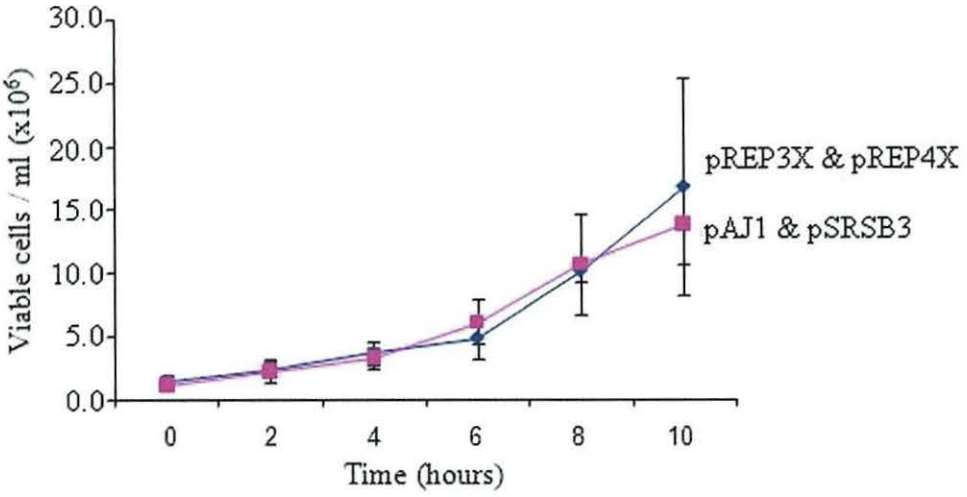


Figure 3. 12 Effect of co-over expression of *translin* and *trax* on cell proliferation.

translin and *trax* were over expressed using the pAJ1 and pSRSB3 plasmids respectively. pREP3X and pREP4X empty vector were used as a negative controls. Exponential growth at 33°C was followed over 10 hours, and the OD_{600} and viable cell count measured for four cultures of each strain.

The standard deviations between the three measurements are shown as error bars. No difference in growth rates or cell morphology was seen. (The strains were supplied by A. Jaendling)

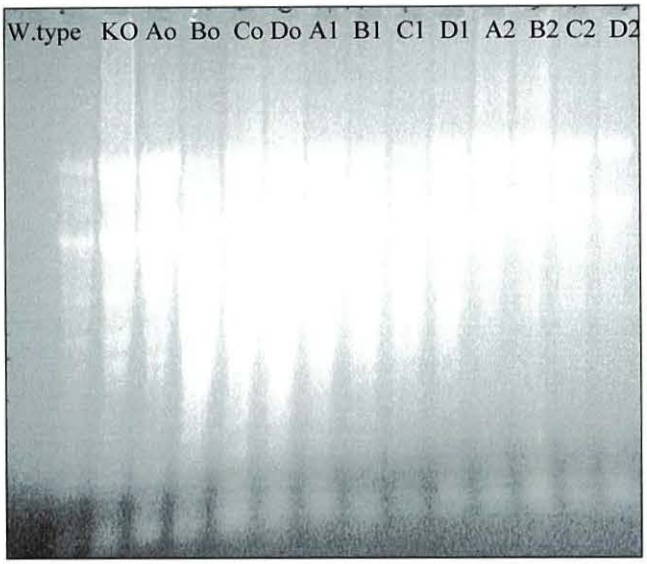
3.2.7 Demonstration of *nmt* regulated *trax* over-expression

To test whether the *trax* gene was being over expressed Northern and Western blot analysis was carried out in cells containing *nmt-trax* plasmids. A total volume of 400 ml cultures of each strain was grown and samples were collected at intervals of 16 hours (Time 1), 20 hours (Time 2) and 24 hours (Time 3) post thiamine removal for analysis of RNA and whole cell protein extraction. A sample culture was also taken earlier at the point of removal of the cultures from thiamine representing a sample with the *nmt* promoter totally suppressed (Time 0). In Figure 3.13 the column headings A and B refer to the cells with empty plasmids (pREP4X-high expression and pREP42X-medium expression) C refers to the cells with high *nmt* expression plasmid (pSRSB3) and D to the cells with a medium expression plasmid (pSRSB2). Samples of cultures collected in presence of thiamine were called '0'. 1 refers to 16 hours after growth without thiamine, and 2 refer to 20 hours. The *trax*+ strain (BP90) and *traxΔ* (strain 8) were used as positive and negative controls respectively.

First, I wanted to demonstrate that the *trax* gene was being over expressed by the *nmt* plasmid. In order to do this, we isolated total RNA from the strains and analysed them by Northern blot (Refer to 2.19 Methods and Material). A picture of the agarose gel (Ethidium bromide stained) was taken prior to the RNA blotting. This gave an image representing the quantity of RNA material loaded in the gel (Figure 3.13 A). The RNA was probed with P^{32} radioisotope labelled *trax* DNA (see 2.19 Materials and Methods). The image developed after exposure, using a Phosphor imager (BIO-RAD Molecular Images FX) (see Figure 3.13 B).

The *trax* RNA levels were raised in cells having the plasmid cloned with *trax* ORF as compared to cells having empty plasmids. There is a clear difference in the band intensity observed between the medium and high expression level *nmt*, further confirming that the promoter was functioning well. The wild type levels of *trax* RNA were not detectable relative to the over-expressing cells (Figure 3.13).

A



B

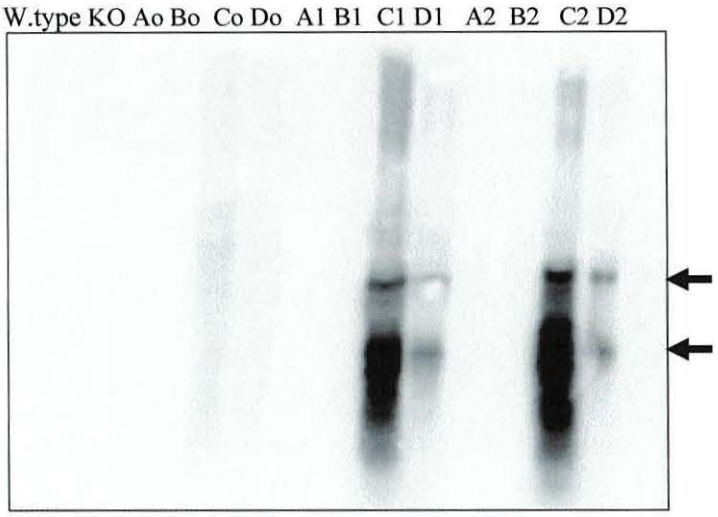


Figure 3.13 Northern blots of RNA from strains with the *trax* gene over expressed over a period of time.

A. Agarose gel image showing the RNA preparation prior to transfer onto membrane. This shows the levels of total loaded RNA (stained with ethidium bromide).

B. Northern blot performed using *trax* probes showing two prominent bands (black arrows) of approximately 1.0 and 1.5 kb. The bands are darker with high expression *nmt* in comparison with the medium *trax* RNA in *trax*+ (BP90).

W.type = *trax*+
K.O = *traxΔ* strain 8
A, B = *trax*+ with high and medium expression empty plasmids respectively; (pREP4X and pREP42X).

C, D = *trax*+ with high and medium expression plasmid having *trax* ORF
0, 1, 2 = Time cells sampled after removal from Thiamine; 0= immediately after; 1= 16 hours later; 2= 20 hours later

3.2.8 Western blot examination shows limited / no increase in Trax protein levels in cells over-expressing *trax*.

We demonstrated that the *trax* over expression was occurring (section 3.2.7). Next, we wanted to examine if the increase in the *trax* mRNA levels (as in 3.2.7) corresponds with an increase in Trax protein. We preformed a Western blot experiment using WCEs from the same cultures used for Northern analysis. (Refer to 2.14 Materials and Methods). Trax protein levels do not increase proportionate to the large increase in the mRNA levels (Figure 3.13 and 3.14). Probing with anti tubulin antibodies shows that total loaded protein is not the factor causing this. Figure 3.14 shows two sets of results. Set I does not show results of D2 (*trax*+ having plasmid pSRSB2 which is a medium expression plasmid) as the gel was damaged. Set II has complete results although the total loaded protein was less in amount. However, both the sets show similar results. Higher levels of Trax protein is seen in cultures grown after removal of thiamine, compared to the cultures grown in presence of thiamine. However, these increased levels are noted in both *S. pombe* strains with empty plasmid as well as plasmid with *nmt* repressible *trax*. Therefore, it could be a factor caused by change of medium.

These analyses indicate that the results obtained from experiments with *trax* over-expression must be cautiously interpreted as they do not represent significant over-expression of Trax protein despite the mRNA being elevated.

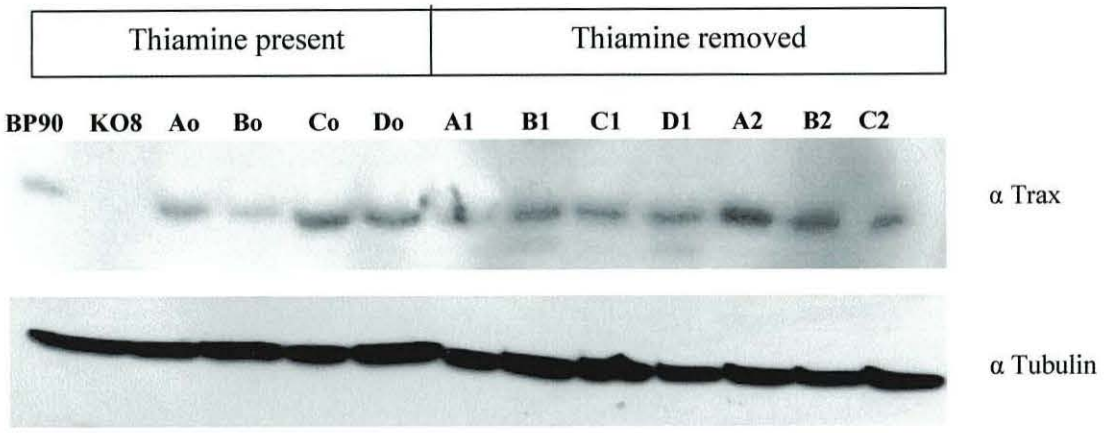
3.2.9 Regulation of Trax levels by Translin is functionally conserved in *S. pombe.*

Previously, it was reported that levels of Trax in MEFs and *Drosophila* cells were dependent upon Translin (Yang *et al.*, 2004a; Chennathukuzhi *et al.*, 2003a; Claußen *et al.*, 2006). The data we have obtained here are in agreement with these findings. We examined *trax* over expressed samples for mRNA levels using Northern blots which show that the *trax* mRNA levels are elevated to high levels (Figure 3.13 B), meanwhile the Trax protein levels are not proportionately elevated and remain almost constant (Figure 3.14). This could be due to excessive quantities of *trax* mRNA present in the over expressed strains relative to the quantities of Translin available for stabilization of Trax protein. This also implies that the Trax is post transcriptionally regulated by Translin.

To explore the relationship between Trax and Translin further, I analyzed Trax levels in the *translinΔ* mutant (*translinΔ* mutant provided by Alessa Jaendling). In short exposures, no Trax was detectable in the Translin-deficient strain (Figure 3.15A). However, on prolonged exposure, a Trax-specific band was observed in the *translinΔ* mutant indicating that low levels of Trax are present in the Translin-deficient cells (Figure 3.15B). This species is not detectable in a prolonged exposure of a Western blot of WCE from the *traxΔ* mutant. Prolonged exposure to the WCEs of a $trax^{+}$ strain revealed at least three Trax specific bands which migrate with higher molecular weights (black arrows in Figure 3.15B) indicating low levels of modified forms of Trax exist in mitotically proliferating wild type cells.

To confirm that the influence of Translin on Trax expression was post transcriptional in nature I examined *trax* mRNA levels in the *translinΔ* strain. I found that *trax* mRNA levels to be indistinguishable between *translinΔ* and wild type cells indicating that the reduced Trax protein in *translinΔ* strains is due to loss of post transcriptional regulation (Figure 3.15C).

SET I



SET II

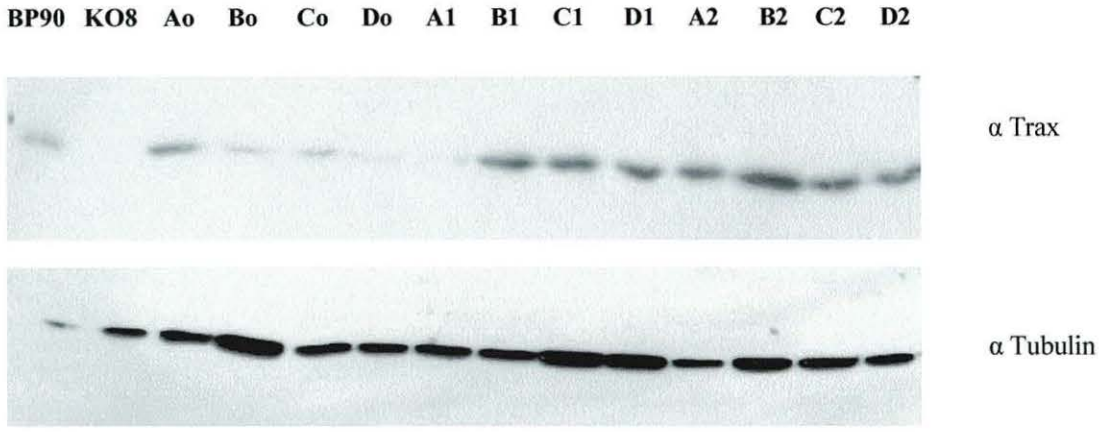


Figure 3.14 The quantity of Trax protein detected appeared constant in all strains, despite having *nmt1* promoter induced over expression of Trax.

The expressions of protein by cells with and without the induction of expression promoter are almost at constant levels. BP90 is the *trax*+ parental strain into which the plasmids were cloned. KO8 refers to the *traxΔ* strain 8, showing no Trax protein being detected. (Band D2 gel in set I was damaged - no resulted noted). Results of two repeats are shown.

A: *trax*+ with empty plasmid pREP4X.
B: *trax*+ with empty plasmid pREP42X.
C: *trax*+ (BP90) with (plasmid pSRSB3) high expression plasmid;
D: *trax*+ (plasmid pSRSB2 medium expression plasmid.

0; Refers to the time thiamine was removed (no induction of *nmt* 1 promoter)
1; Refers to the time interval of 16 hours after removal of thiamine.
2; Refers to the time interval of 20 hours after removal of thiamine.

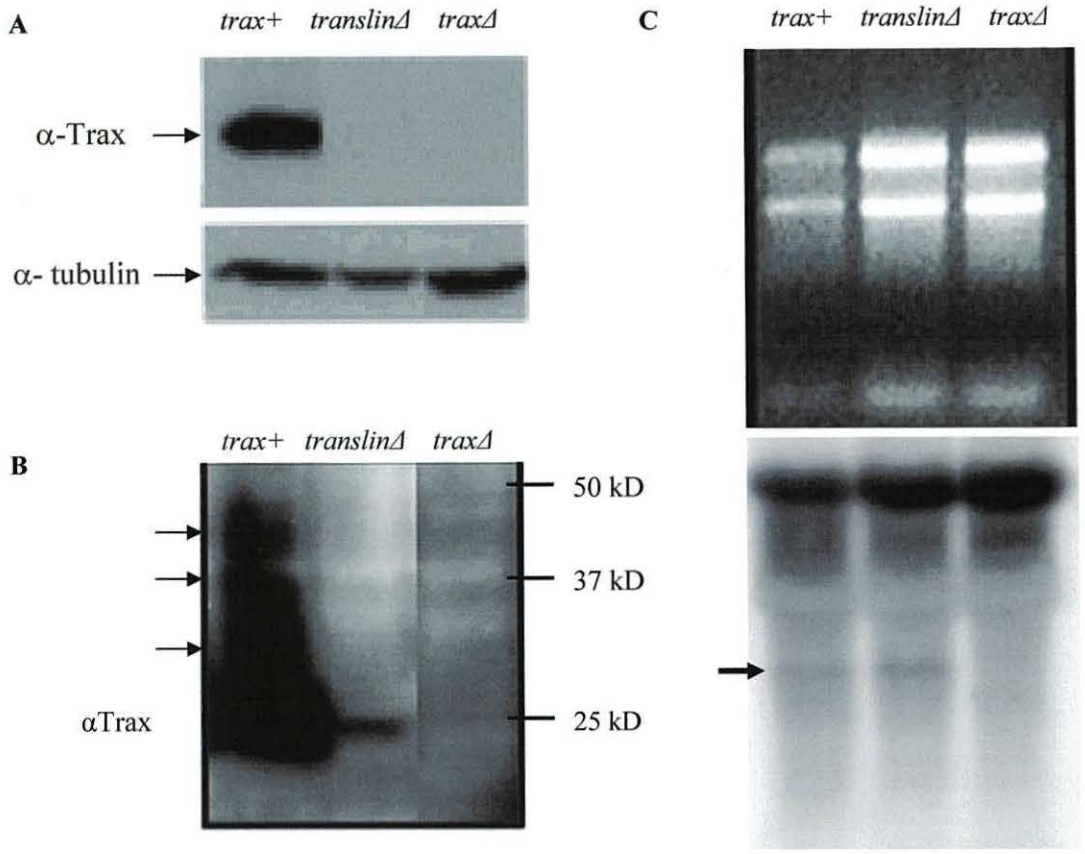


Figure 3.15 In *translinΔ S. pombe* the Trax protein is not expressed although the *trax* mRNA levels are not altered.

A, Trax protein is not detected in *translinΔ* or *traxΔ* mutants (on low exposure).

B. Greatly prolonged exposure results in detection of a Trax-specif band in the *translinΔ* mutant, which is not seen in the *traxΔ* mutant. At least three Trax-specific bands with different molecular weights are observed in the wild-type (black arrows); indicating modified forms of Trax exist in mitotically proliferating wild type cells.

C Northern blot of *trax*$^{+}$, *translinΔ* and *traxΔ* strains showing the *trax* mRNA levels are not altered in *translinΔ* strain and not present in *traxΔ* strain (Black arrow). Upper panel shows total RNA stained with ethidium bromide indicating uniform loading of total RNA.

3.3 Discussion

3.3.1 Deletion of the *trax* gene

I have successfully isolated two *traxΔ* strains using a PCR-based method (Bähler *et al.*, 1998). This was confirmed by PCR, Southern and Western blotting methods, which demonstrate that the *trax* gene was deleted completely in both strains.

The construction of these strains is centrally important to this study as it permits further analysis of the phenotype caused by the loss of a functional *trax* gene (see Chapters 3 and 4) . During the development of the *traxΔ* strains and testing of the anti-Trax antibodies, we noted that there was some cross reactivity for the detection of higher molecular weight proteins with both antibody sets. This might suggest that these antibodies might not be good candidates for use in immunofluorescence experiments, although they provide an excellent tool for Western blot analysis.

3.3.2 Trax does not influence morphology and cell proliferation.

The property of a protein influencing the cell to change in morphology or replication characteristics could point to an important functional characteristics relating to the causation of cancer. A gene, when deleted, causing increase rate of cell proliferation could point to a tumour suppressor gene. Meanwhile, if the gene causes increased cell proliferation when over-expressed, it could mean it is an oncogene.

Translin and Trax have been implicated in the regulation of proliferation of cells. Yang and co workers (2004) demonstrated that in MEFs both Trax and TB-RBP are needed for normal cell proliferation. They showed that Translin-deficient homologous had a slow growth phenotype in comparison with MEFs from their wild type and heterozygous litter mates. The cell proliferation defect was reversed with introduction of TB-RBP, suggesting that Translin (TB-RBP) functions in controlling mitotic cell proliferation in metazoans. However, studies have shown that the Trax protein was absent in the TB-RBP deficient MEFs despite normal Trax mRNA levels (Yang *et al.*, 2004a; Chennathukuzhi *et al.*, 2003a). Therefore, the effect could be contributed by lack of functional Trax instead of / or as well as due to loss of Translin. Using RNA interference, Yang and co workers (2004a) proved that Trax was also

needed for normal cell proliferation in MEFs. Studies in HeLa cells also reported that reduction of Trax causes reduced cell proliferation (Yang and Hecht, 2004b).

However, it is worth noting that the reduction in cell proliferation rate for TB-RBP deficient MEFs are rather small (1- to 2-fold increase) and could very well fall within the experimental error ranges. This is particularly true given that the method used was based on cell count numbers, where the margin of error could be considerable. Yang and Hecht also demonstrated that HeLa cells with reduced Trax levels had slower growth and the proportion of cells in the G2/M phase was increased. The results would be more convincing if the experiments were carried out in normal human cell lines with reproducible results. Mammalian cells have more complex cell and metabolic cycles and thus it is more difficult to draw definitive conclusion from experiments giving results with a rather small variation compared to the Trax-proficient cells. Moreover, the *trax* gene was not completely removed but the levels were reduced in Trax-proficient cells in the experimental system.

Contrary to these findings, our experiments in *S. pombe* did not find any significant difference in proliferation between the *traxΔ* strains relative to the wild type. I found that the *traxΔ* mutants were viable, appear normal in size and morphology and have cell proliferation rates similar to that of *trax*$^{+}$ strains (Figure 3. 10). We also found that the *trax-translin* double mutants too, did not have any effect on cell viability, morphology, size and proliferation rate (Figure 3. 12). The results I obtained show that the Trax and Translin do not have any primary effect on *S. pombe* growth control. I found similar results with both the *trax* null mutants and the *trax* over expressed cells, in relation to the morphology, viability and proliferation of the cells. Thus, I conclude that the absence of Trax does not have any effect in growth regulation in *S. pombe* in optimal laboratory conditions. However, the absence of increased levels of Trax protein corresponding to over expression of *trax*, makes the finding inconclusive in strains having over expressed *trax*.

It is possible that the Trax and Translin in mammalian cells could have more complex and distinct function (s) than that of a unicellular eukaryote like *S. pomb*.

3.3.3 Regulation of Trax levels by Translin is functionally conserved in *S. pombe*

Previously it was reported that the level of Trax in higher eukaryotes was dependent upon Translin (Clauβen *et al.*, 2006; Chennathukuzhi *et al.* 2003a; Yang *et al.*, 2004a). Consistent with these findings, I found that this biochemical function is conserved even in unicellular lower eukaryote. High levels of Trax protein could not be detected in *translinΔ* cells. However, on prolonged chemiluminescent exposure of the Western blot probed with anti-Trax antibodies, a Trax-specific band was observed in the *translinΔ* mutant, indicating low levels of Trax are present. This band was not seen in *traxΔ* mutants (Figure 3.15A; B) even on prolonged exposure. It was also observed that prolonged chemiluminescent exposure of the Western blot probed with anti-Trax antibodies revealed three Trax-specific species which migrate with molecular weights in the approximate range of 30-48 kD, suggesting that some cellular Trax exists in modified forms. Whilst a reduction of Trax in Translin-deficient cells has been reported in MEFs and *Drosophila* cells, the presence of residual Trax has not been widely observed (Chennathukuzhi *et al.*, 2003a; Yang *et al.*, 2004a; Clauβen *et al.*, 2006).

The regulation of Trax protein levels has been shown to be post transcriptional in nature in mammals (Yang *et al.*, 2004a; Chennathukuzhi *et al.*, 2003a); consistent with this I find no change in *trax+* mRNA levels in the *translinΔ S. pombe* strain, indicating the post transcriptional regulation is also apparent in a unicellular lower eukaryotes. This was demonstrated clearly as I could not detect increase levels of Trax in *trax* over-expressed cells in contrast to dramatic increase in the levels of *trax* mRNA (Figure 3.13 and 3.14). It was previously reported that Trax protein levels in TB-RBP (mouse Translin) deficient MEFs were restored when they were treated with the mammalian protease inhibitor (MG-132) (Yang *et al.*, 2004a). This indicates that Trax is regulated by ubiquitin mediated proteolysis and in support of this ubiquitinated Trax has been identified in MEFs (Yang *et al.*, 2004a).

To test if a similar process is involved in *S. pombe*, Jaendling *et al.* (2008) used a *translinΔ* mutant which carried a temperature sensitive allele of the gene coding for Mts3 protein, which is an essential component of *S. pombe* proteosome (Gordon *et al.*, 1996). They demonstrated that, whilst no Trax protein was detectable in the *traxΔ* strain at the *mts-3-1* non-permissive temperature (37°C), Trax was detectable in the *translinΔ* strain at the *mts3-1*

double mutant at this temperature. Consistent with this there was a slight elevation in Trax levels in the *mts3-1* single mutant at the restrictive temperature. However, the levels of Trax protein in a *translin*Δ background are not restored to *translin*+ levels by the inhibition of proteosome activity, indicating that another Translin-dependent, proteosome-independent pathway controls levels of Trax.

Over-exposure of WCE Western blots from wild-type *S. pombe* cells probed with anti-Trax antibodies in this study, revealed a ladder of higher molecular weight Trax-specific species (Figure 3.15 B); whilst the molecular weight shift between these species was less than that of expected for ubiquitin monomers, 8.4 kDa. It is possible that one of these species is a ubiquitinated form of Trax and that inhibition of proteasome in cells with no Translin (*translin*Δ) might restore levels of Trax in the same fashion for MG-32 treated MEFs.

So I conclude that the regulation of Trax protein levels by Translin has been highly conserved, this implies this biochemical function could have a critical role in the cells, which remain unidentified. Yang and co-workers (2004a) speculate that Trax alone without Translin may be toxic to MEF cells. Therefore, increased levels of Trax may be deleterious to normal cell survival, thus mutations that stabilize Trax protein without TB-RBP interaction may lead to genetic disorders. However, they did not provide any evidence in support of this argument.

Many of the studies carried out on Trax and Translin have associated these proteins with brain/neuron and testis function(s) (Taira *et al.*, 1998; Chennathukuzhi *et al.* 2003a). Some authors have associated the role of these proteins with nervous system in mice (Devon *et al.*, 2000; Li *et al.*, 2008), suggesting a tissue specific function. The fact that a unicellular organism like *S. pombe* does not have these functions suggests that these proteins do not function soley in a tissue specific fashion, or have evolved some tissue specific activity in more complex eukaryotes. It is also possible that the role played by Trax and Translin is more fundamental and not likely to be tissue-specific as they are present in all tissues and organs.

The existence of the regulatory function between Translin and Trax which seems to be present in both lower eukaryotes as well as mammals clearly implies that it is an important conserved function, thus more research needs to be done to explore these.

3.4 Summary of Main Findings

1. Two *trax* deleted *S. pombe* strains were successfully generated using the PCR based gene deletion technique (Bähler *et al.*, 1998).
2. *trax* deleted strains were viable and were able to grow and appeared visibly normal under all conditions tested.
3. Deletion of the *trax* gene did not affect the morphology or the proliferation of cells.
4. Trax is post transcritionally stabilised by Translin in *S. pombe*; a function of Translin which is conserved in eukaryotes including mammals.
5. Some Trax proteins exist in modified form in mitotically proliferating cells.

CHAPTER 4

EXAMINATION OF A POTENTIAL ROLE OF TRAX IN DNA DAMAGE RECOVERY AND GT REPEAT STABILITY

4.1 Introduction

Translin and Trax have been proposed to be involved in the DNA damage response (Kasai *et al.,* 1997), regulation of cell proliferation (Ishida *et al.*, 2002; Yang *et al.*, 2004a; Yang and Hecht, 2004b), chromosomal translocation formation (Chalk *et al.*, 1997; Kasai *et al.*, 1994) and mRNA transport / translation (Wu and Hecht, 2000; Castro *et al.*; 2000; Morales *et al.*, 2002; Finkenstadt *et al.*, 2002). Translin has also been shown to specifically bind to the single-stranded microsatellite repeats $d(GT)_n$ and the corresponding transcripts $(GU)_n$ (Aharoni *et al.*, 1993; Jacob *et al.*, 2004; Laufman *et al.*, 2005), thus it has been proposed that translin might play an important role in microsatellites stabilization.

Both human and mouse Trax can form heterodiamers with Translin / TB-RBP (Aoki *et al.*, 1997a; Taira *et al.*, 1998). The Trax / Translin heterodimer is capable of binding to both single-stranded DNA and RNA, raising the possibility that the heterodimer is multifunctional. Translin forms an octomeric toroidal structure (Kasai *et al.*, 1997; VanLoock *et al.*, 2001) which has similarities to the structures of other protein complexes associated with DNA replication, repair and recombination (Hingorani and O'Donnell, 2000). Other than the association with breakpoint sequences, several lines of evidence also implicate Translin and Trax functions in maintaining genome stability, particularly in response to DNA damage; for example the murine translin interacts with GADD34, a protein implicated in the DNA damage response (Hasegawa and Isobe, 1999).

Treatment of HeLa cells with the chemotherapeutic DNA damaging agents Mitomycin C and Cisplatin was shown to result in an elevation in the levels of nuclear translin (Kasai *et al.*, 1997). What function Translin may have in the nucleus is as yet unclear. However, in contrast MEFs derived from Translin-deficient mouse embryos exhibited no measurable difference in cell survival in response to DNA damaging agents relative to Translin proficient MEFs (Yang and Hecht, 2004b). Further evidence for a role in the DNA damage response comes from the finding that Trax has been identified as a DNA damage-dependent

interacting partner of the C1D protein (Erdemir *et al.*, 2002b) which regulates homologous recombination (HR) and non-homologous DNA end joining (NHEJ) (Erdemir *et al.*, 2002a). In response to DNA damage caused by γ-irradiation, CID is induced and targets DNA-dependent protein kinase (DNA-PK) to specific nuclear regions.

Whilst Translin and Trax have been implicated in DNA damage recovery, recent work by Clauβen and co-workers (2006) found that Translin and Trax defective *Drosophila* embryos had no increased sensitivity to DNA damaging agents and that meiotic cross over recombination was normal. These findings, and the observation that Translin-deficient MEFs show no sensitivity to DNA damaging agents (Yang *et al.*, 2004a), argued against a primary role for Translin and Trax in DNA damage recovery.

Previous work in higher eukaryotes has to some degree, implicated both Trax and Translin in the response to DNA damage (see Chapter 1). However, no direct evidence exists to indicate that either protein is required for cell recovery from DNA damage, although previous studies have explored only limited DNA damage response pathways.

I took advantage of the fact that subtle sensitivities to DNA damaging agents can be readily tested using *S. pombe*. I tested the sensitivity of both the *traxΔ* mutants generated, (2 and 8) to a range of different DNA damaging agents, since it is unknown if Trax/ Translin function in a specific repair pathway. Each DNA damaging agent is known to cause different types of lesions which are targeted by different DNA repair pathways. The summary of the lesions caused by different DNA damaging agents that I tested and the DNA repair pathway that target them are as shown below;

DNA damaging agent	Effect	Main repair pathway	References
UV radiation	Causes Pyrimidine dimerisation	Photolyses UVER or NER	Bowman *et al.*,1994 de Laat *et al.*, 1999
Camptothecin	Topo I inhibitor	NER or HR	Wan *et al.*, 1999
MMS	Alkylating agent		Fung *et al.*, 2002 Morishita *et al.*, 2002
Hydroxyurea	Causes NTP depletion	NER or HR	Enoch *et al.*, 1992
Mitomycin C	DNA crosslinker	NER or HR	Jachymczyk *et al.*, 1981 McHugh *et al.*, 2001
Phleomycin	Causes DSBs	HR or NHEJ	Kostrub *et al.*, 1997

I assayed the sensitivity of the *traxΔ* mutant to a range of DNA damaging agents [UV light, methyl methane sulfonate (MMS), mitomycin C (MMC), phleomycin, campthothecin] and the DNA replication inhibitor hydroxyurea (HU); which enabled me to explore a range of DNA damage recovery pathways.

Further to this, I also employed the *S. pombe* model system to determine if the loss of *trax* has any effect in microsatellite stability and the DNA damage response.

4.2 RESULTS

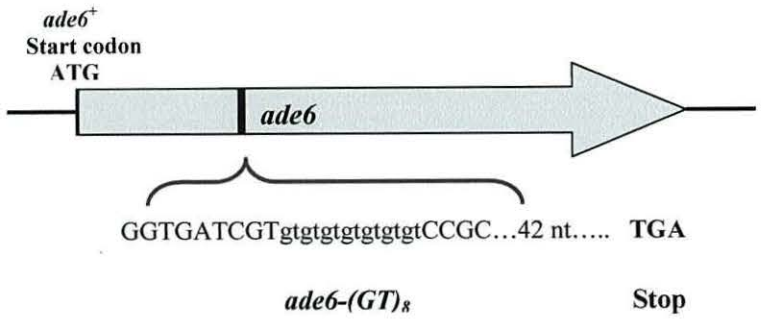
4.2.1 *traxΔ* cells do not exhibit any GT microsatellite repeat instability

S. pombe Translin was shown to bind selectively to single-stranded $d(GT)_n$ and $d(GTT)_n$ DNA repeats *in vitro* (Laufman *et al.*, 2005). It has a high affinity for single-stranded microsatellite GT repeats, $d(GT)_n$, and G-strand telomeric repeats, $d(TTAGGG)_n$, but not to corresponding double-stranded DNAs (Aharoni *et al.*, 1993; Jacob *et al.*, 2004).

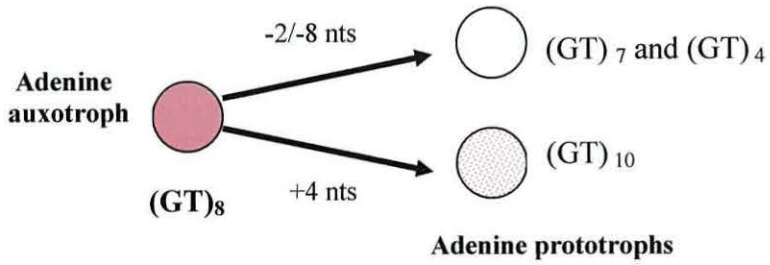
However, unlike the human protein, it has higher affinities for the homologous RNA sequences $(GU)_n$ and $(GUU)_n$ (Laufman *et al.*, 2005). Affinity of proteins like Translin to microsatellite could have the effect of stabilizing the microsatellite repeats, thus play a role in certain disease processes. Alterations to the stability of microsatellite repeat sequences can result in human genetic disease associated with neurological disorders (Pearson *et al.*, 2005) and cancers (Woener *et al.*, 2006). Microsatellite repeat instability has been linked to impaired mismatch repair (MMR) systems in *S. pombe* (Mansour *et al.*, 2001).

Given that the Trax is a Translin binding partner, I employed the system of Mansour and co-workers (2001) to study $(GT)_8$ repeat stability in *traxΔ* cells compared to wild type cells, to determine whether the selective binding of Translin to $d(GT)_n$ repeats (Laufman *et al.*, 2005), reflected a function in regulation of microsatellite repeat stability and whether this is regulated by Trax. In brief, the assay system consists of eight GT repeats (GT_8) inserted within the *ade6* open reading frame resulting in inactivation of the *ade6* gene (Figure 4.1 A); changes in the number of GT repeats can result in an in frame open reading frame for *ade6* which restores adenine prototrophy; this occurs with loss of one [$(GT)_7$; 2 nucleotides] or four [$(GT)_4$; 8 nucleotides] repeats or gain of two repeats [$(GT)_{10}$; 4 nucleotides] (Figure 4.1 B).

A



B



C

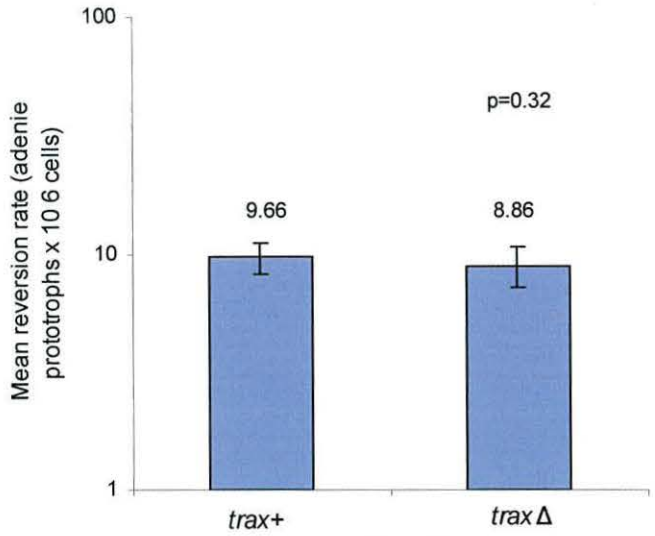


Figure 4.1: *traxΔ* cells do not exhibit any change in GT microsatellite repeat instability.

A. $[GT]_8$ repeat was created by integration of 7[GT] units (letters in lower case) at an existing GT site at nucleotide 522 within the *ade6* open reading frame (ORF).
B. Frame shifts, due to loss or gain of GT repeats, can result in expression of a functional *ade6* protein capable of conferring adenine prototrophy; loss of one [GT] or $4[GT]_4$ repeats or gain of two $[GT]_2$ repeats results in adenine prototrophy.

Adapted from (Mansour *et al.*, 2001, Jaendling *et al.*, 2008)

C. No significant increase in the rate of GT microsatellite instability is observed in *traxΔ* cells ($P = 0.32$; Student's *t*-test; strain 8) relative to *trax*$^+$. Error bars are standard deviation. The value shown above the bar chart is the mean reversion rate.

Fluctuation analysis (Refer to 2.23 Materials and Methods) was carried out for the *trax*Δ and *trax*$^+$ strain 8 with GT_8 repeats inserts in the *ade6* gene. The rate of adenine prototrophy formation for both the *trax*Δ (BP1220) and *trax*$^+$ strain (BP1244) carrying $(GT)_8$ repeats were calculated as described by Lea and Coulson (Lea and Coulson, 1949). There was no significant difference in frequency of the GT_8 instability observed between mutant and wild-type strains. The wild type had a mutation rate of 9.66 compared to 8.86 in the *trax*Δ strain, ($P = 0.32$ Students *t*-test) (Figure 4.1 C). The *translin*Δ strain was reported to exhibit a small increase in GT microsatellite repeat instability (Jaendling *et al.*, 2008) but it was not statistically significant, although a broader range of values was observed for the mutants, possibly indicating a subtle underlying effect, which was not apparent in the *trax*Δ strains.

4.2.2 *trax* deleted mutants exhibit no overt defect in response to a range of DNA damaging agents.

In view of Trax having been implicated in DNA repair pathways, I wanted to check if *trax*Δ strains show sensitivity to DNA damaging agents. Both strains 2 and 8 were tested. We used standard established procedures for this. Strains to be tested were grown overnight in YEL to approximately 1 x 10^7 (log phase). Serial dilutions up to 10^{-4} dilution of the cultures were used. 10 µl of the serial dilutions from 10^{-1} to 10^{-4} of the *S. pombe* cultures were dropped in descending order onto Petri dishes containing YEA with 200 mg/litre adenine and different DNA damaging agents (see 2.20 Methods and Material). Control plates containing no damaging agent were prepared and the cultures were dropped as in the DNA damaging agent containing plates. Plates were incubated at 25°C, 30°C and 37°C for 3-4 days and levels of growth recorded. A *rad3-136* mutant was used as a positive control strain, as this strain exhibits sensitivity to a broad range of DNA damaging agents.

All plates containing genotoxic chemicals were prepared at various concentrations and used on the day of preparation. For example, Methyl Methane Sulphonate (MMS) an alkylating agent was tested at concentrations, of 0.0075%, 0.005% and 0.0025 %; the hydroxy urea (HU) sensitivity was tested at concentrations of 10 mM and 5 mM; camptothecine was tested at a concentration of 0.5 µg/ml. The strains were also exposed to UV light.

Both *trax*Δ mutants (2 and 8) show no sensitivity to MMS at concentration of 0.0025%, 0.005% and 0.0075% at the different temperatures studied. Results are shown in Figure 4.2. In plates with 0.005% MMS, incubated at 37°C, growth is inhibited in all strains (see Figure 4.2). A similar finding was also noted for in plates containing Hydroxyurea (see Figure 4.3) and Camptothecin (Figure 4.4). Exposure of the same strains to ultraviolet irradiation did not result in any measurable sensitivity (Figure 4.5)

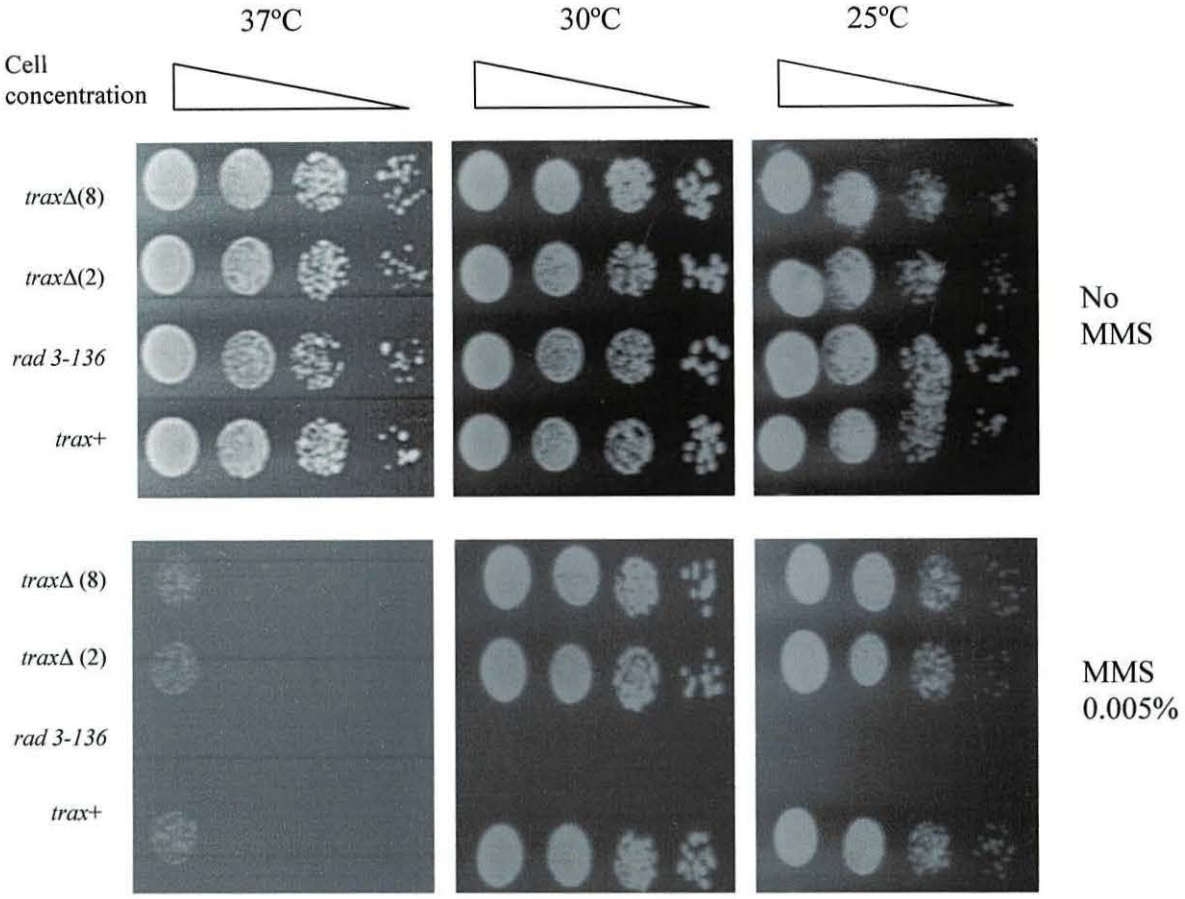


Figure 4.2: Effect of Methyl Methane Sulphonate (MMS 0.005%) on *traxΔ* strains

traxΔ mutants exhibit no sensitivity to 0.005% MMS at all temperatures tested. MMS sensitivity was also tested at concentrations of 0.075% and 0.025%. However, all doses produced similar results; except that no cells grew in plates with MMS 0.075%, concentration, incubated at 37°C. *rad3-136* is a positive control strain that shows sensitivity to MMS.

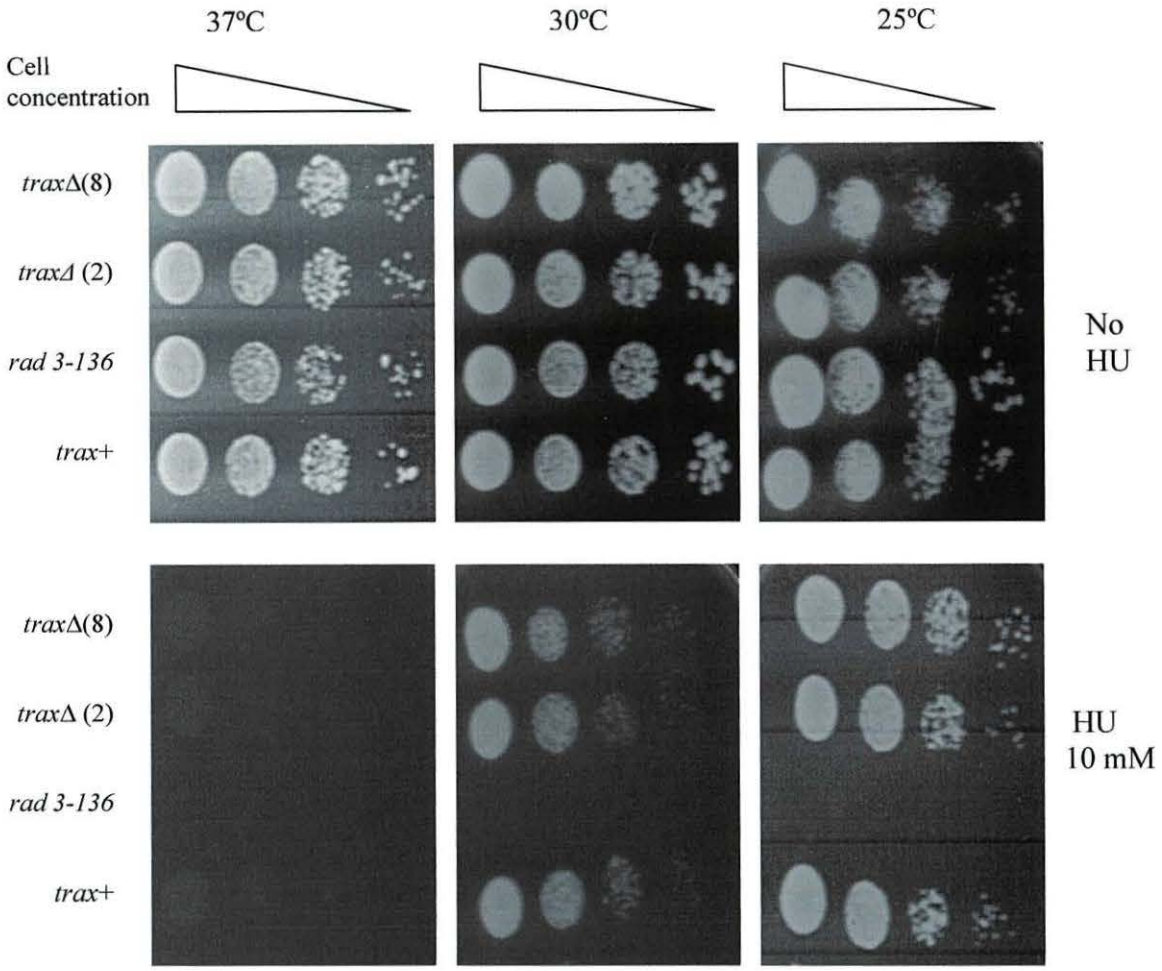


Figure 4.3 *traxΔ* strains exhibit no sensitivity to 10 mM Hydroxy urea (HU)

Dropped cultures of *traxΔ* strains did not show any sensitivity as compared to the control strain following exposure to of 10 mM Hydroxyurea. Concentration of 10 mM and 5 mM yielded similar results.
(Data for 5 mM not shown)

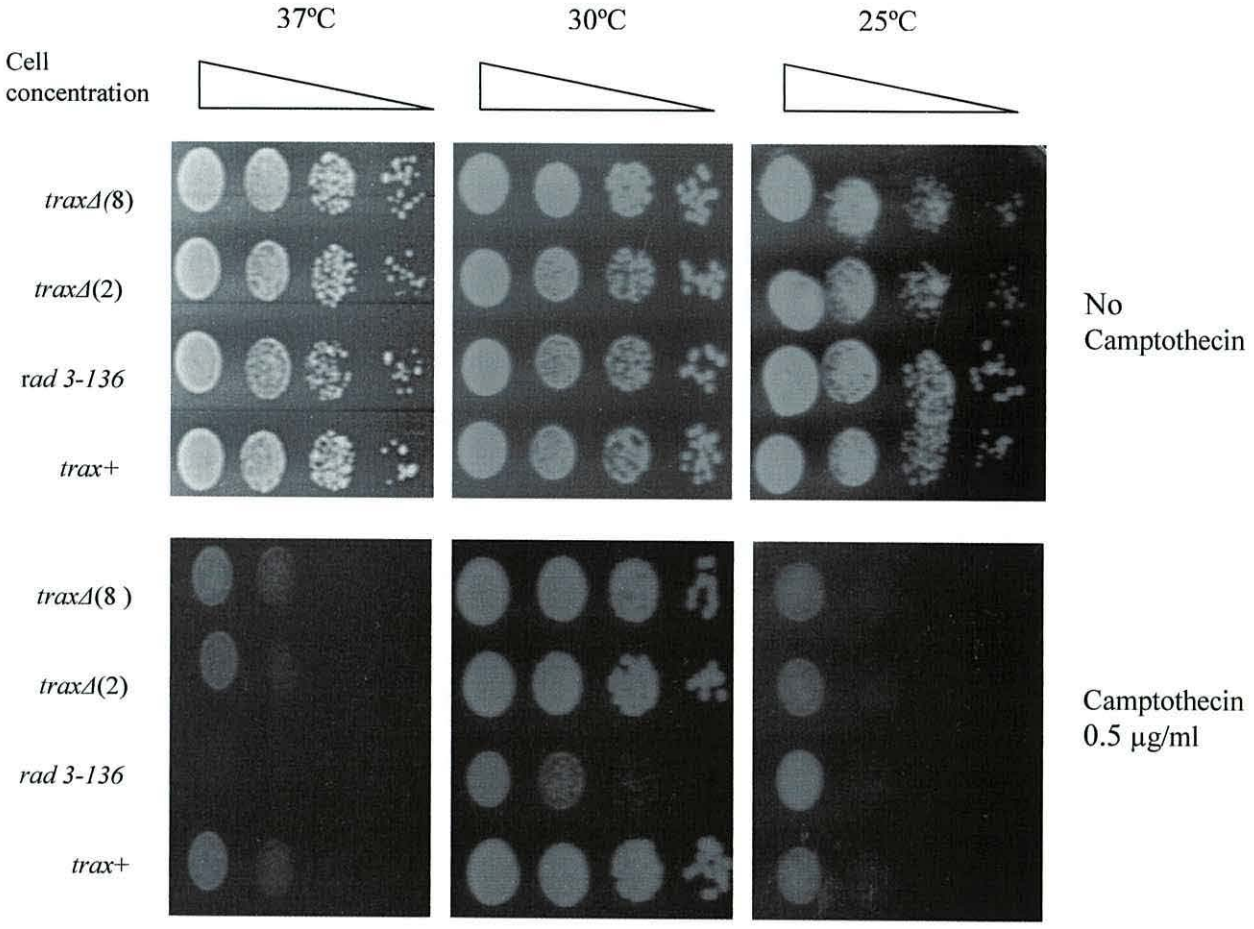


Figure 4.4 *traxΔ* mutants exhibit no sensitivity to 0.5 µg/ml camptothecine.

traxΔ strains have a similar sensitivity to 0.5 µg/ml camptothecine as *trax*$^{+}$ strains. Cultures show non specific sensitivity to the drug at low temperature of 25°C and high temperature of 37°C; most likely due to the stress of non ideal temperature provided for growth.

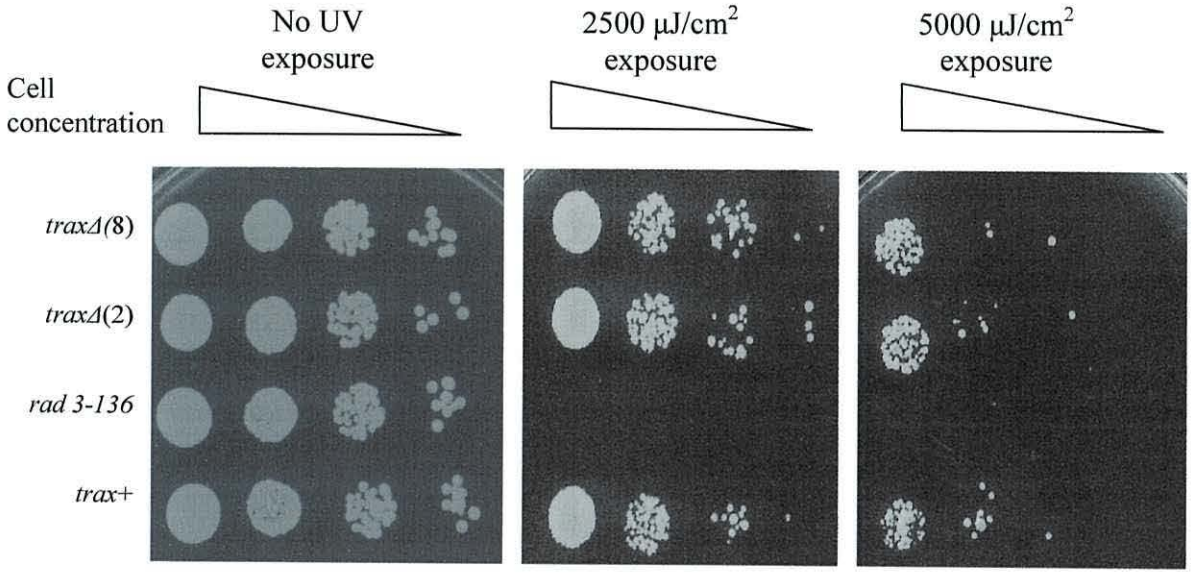


Figure 4.5 *traxΔ* strains show no sensitivity to ultra violet exposure.

Drops of serially diluted cultures plated on YEA with growth supplements were dried at 30°C in an incubator for 30 minutes. The plates were then exposed to different doses of UV light wave length; 395 nm. The results show that the wild type and the *traxΔ* strains were affected in similar manner. At 5000 μJ/cm^2 and 2500 μJ/cm^2. The UV-sensitive *rad3-136* mutant strain was used as positive control.

4.2.3 DNA damaging agents show no effect on G1 arrested *S. pombe* cells

Due to the possible role of Translin in generating chromosomal translocations, it has been suggested that Translin might function in non-homologous DNA end joining (NHEJ) and / or homologous recombination (HR) repair pathways. The nuclear matrix protein C1D is an activator of the DNA-dependent protein kinase (DNA-PK) which is essential for the repair of DNA DSBs and V(D)J recombination. C1D interacts specifically with Trax in mammalian cells (Erdemir *et al.*, 2002b). I wanted to examine if Trax could have a role in NHEJ, which is a double-strand break repair pathway used when sister chromosomes are not available.

In the previous tests with DNA damaging agents, a synchronous culture were grown in YEL medium, in which cells that are in G1 phase would constitute approximately 10-15% of the total. To ensure that there was no G1-specific repair requirement we used nitrogen starved cells to obtain G1 phase arrested cultures and repeated the tests using DNA damaging agents. The same set of *trax+* and *traxΔ* strains were grown in 5 ml EMM2 media, without nitrogen, with appropriate supplements and were drop tested as in 4.4.

We subjected G1 arrested cells to sensitivity tests against MMS; 0.0075%, 0.005% and 0.0025%, Phleomycin; 10 μg/ml, Mitomycin C at 0.15 mM concentration and UV light). No sensitivity was observed for MMS (Figure 4.6), Phleomycin (Figure 4.7), Mitomycin C (Figure 4.8) or to UV irradiation (Figure 4.9). These results are similar to sensitivity tests carried out on cultures grown in media permitting asynchronous growth.

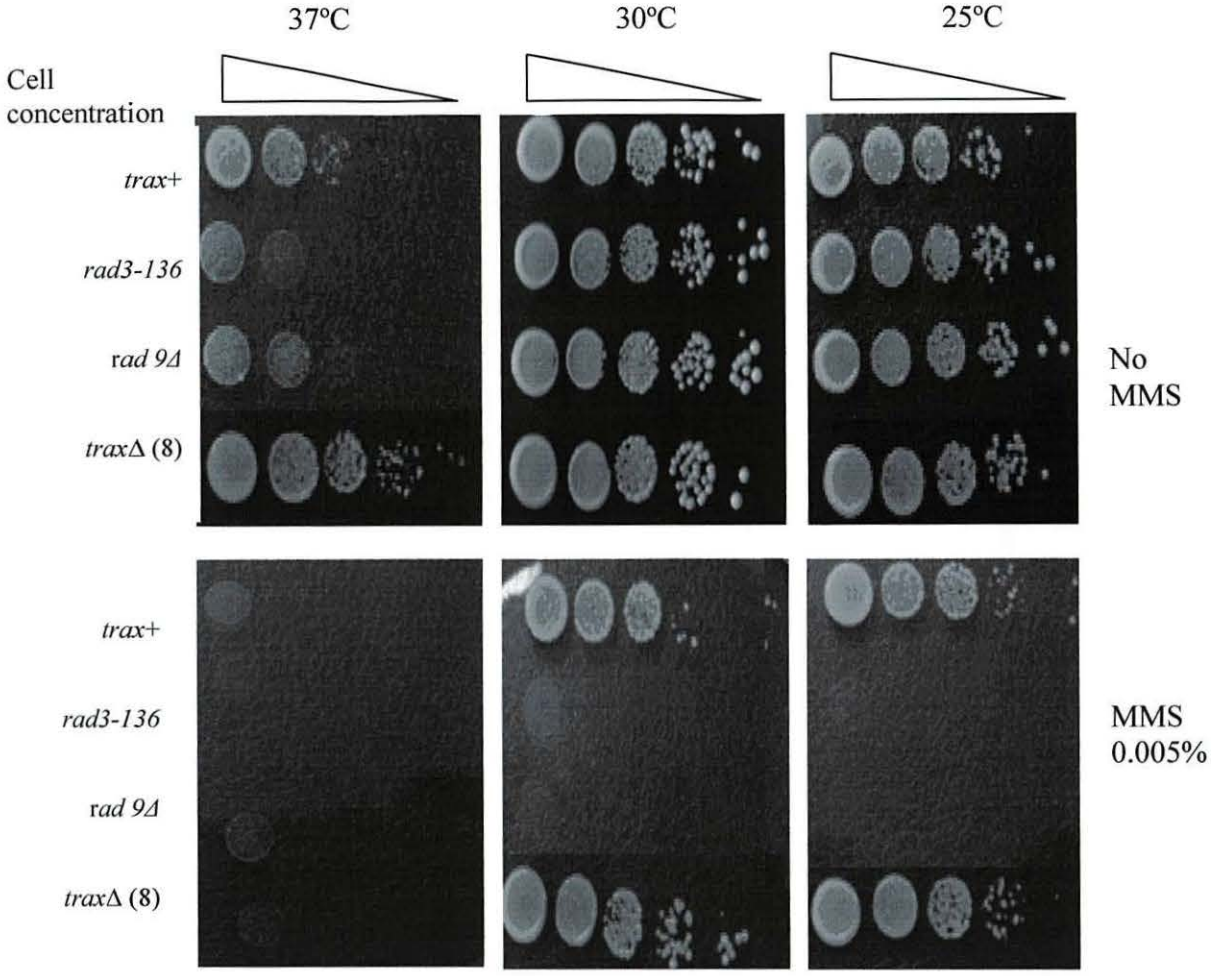


Figure 4.6 *traxΔ* cells arrested in G1 show no sensitivity to MMS 0.005%.

traxΔ mutants show no sensitivity to MMS when grown in nitrogen free media, which arrests cells in G1 phase. These cells have no sister chromatid to repair the damage by homologous recombination, thus NHEJ would be a choice repair mechanism for DSBs. MMS at concentration of 0.007%, 0.005% and 0.0025% were tested. No sensitivity to MMS was seen at all temperatures compared to the wild type.

All strain tested show sensitivity at 37°C, probably due to temperature stress. Only strain 8 was tested. All strains did not grow in MMS concentration of 0.0075% incubated at 37°C. *rad3-136Δ* and *rad9Δ* mutants were used as positive controls.

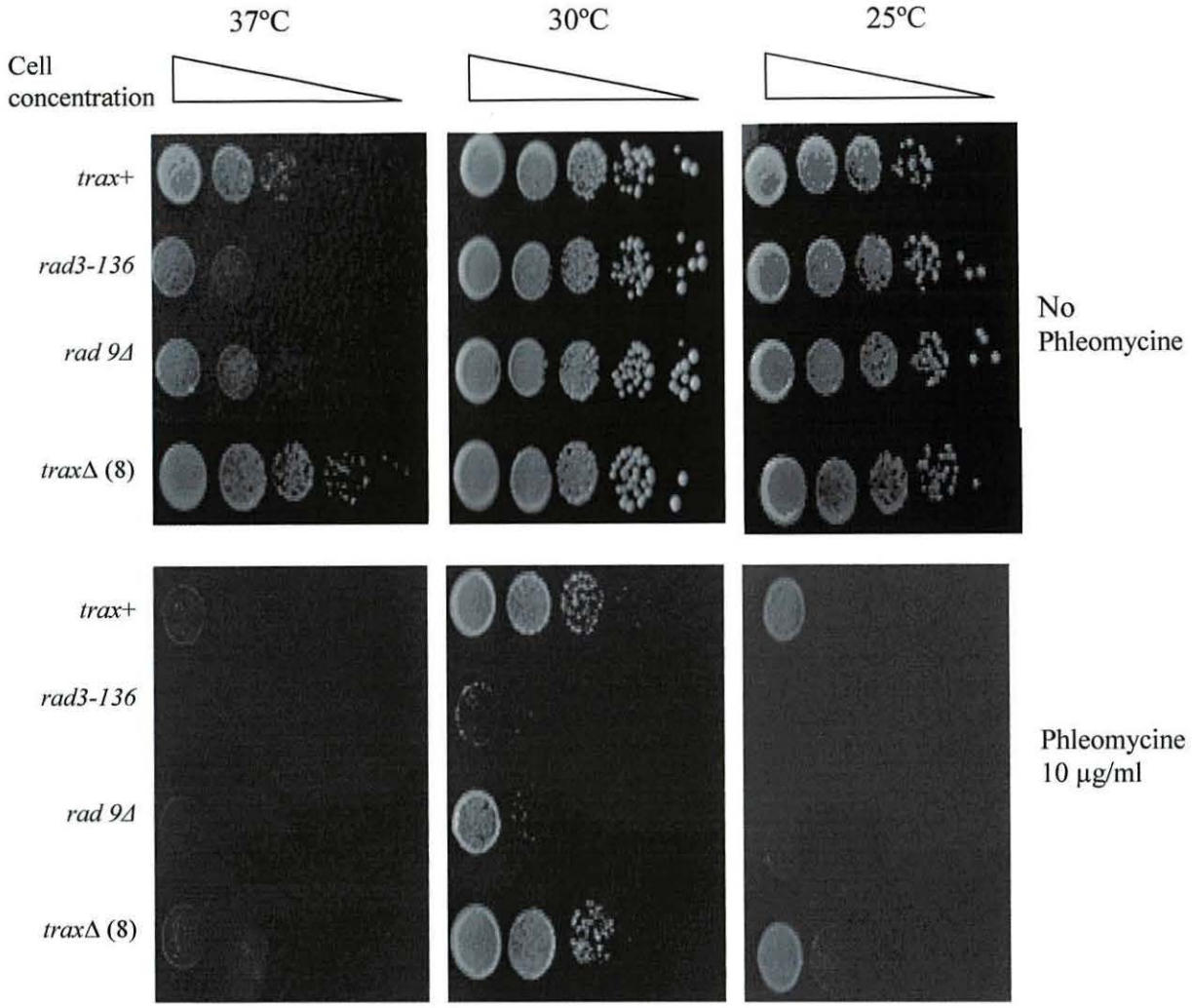


Figure 4.7 *traxΔ* cells arrested in G1 exhibit no sensitivity to Phleomycine (10 µg/ml).

Both *trax* $^{+}$ and *traxΔ* mutants show no sensitivity to phleomycin (10 µg/ml). General sensitivity is shown in all strains incubated at 37°C in plates containing 10 µg/ml. Sensitivity tests were also carried out for phleomycin at 1, 2.5, and 5 µg/ml concentrations. However, at concentrations below 5 µg/ml, *rad3-136Δ* and *rad9Δ* control strains did not exhibit any inhibition.

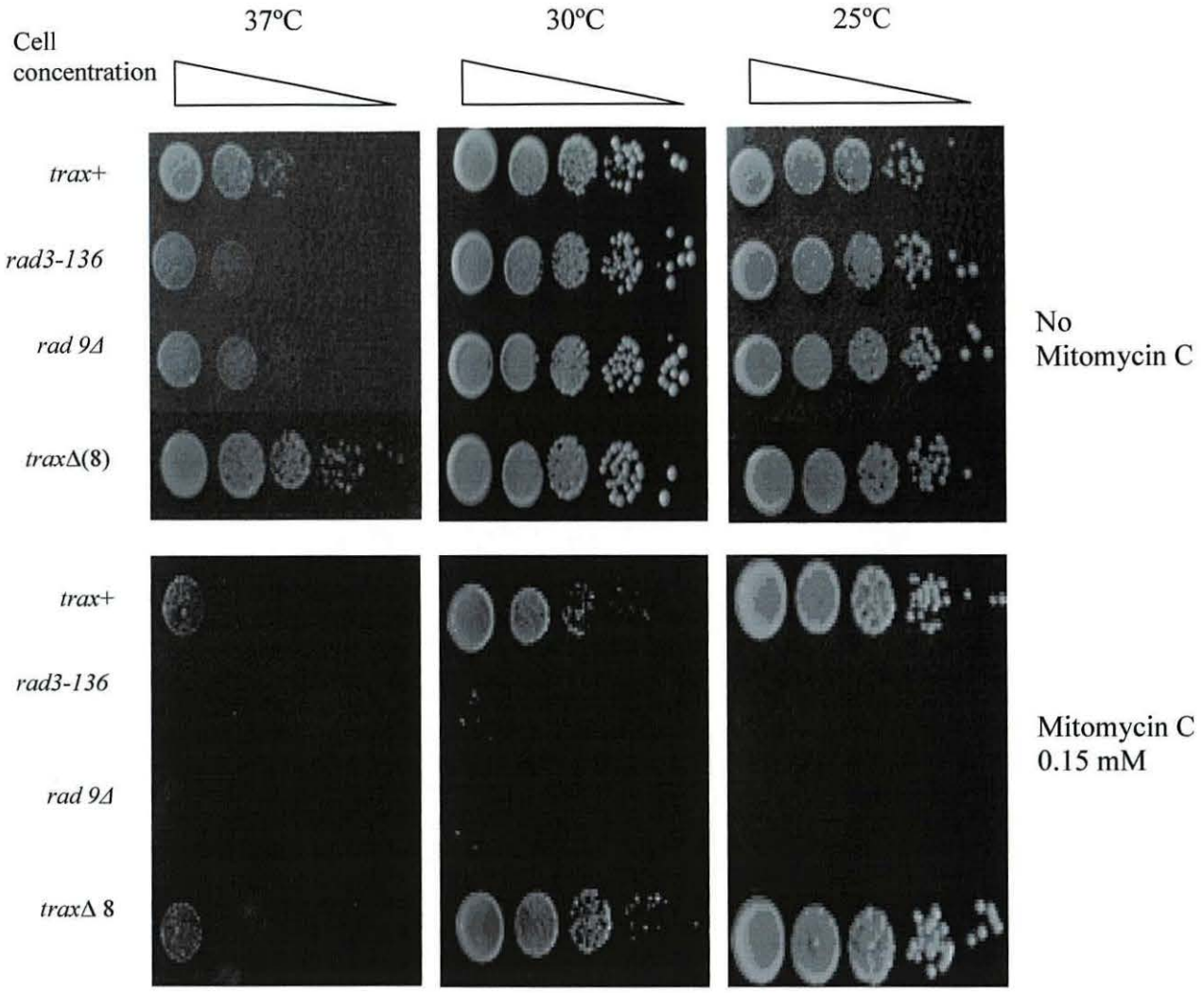


Figure 4.8 *traxΔ* strains arrested in G1 show no effect when treated with Mitomycin C (0.15 mM)

There is no difference in sensitivity to mitomycin C (0.15 mM) shown by the wild type compared to the *traxΔ* strains at 30°C and 25°C.

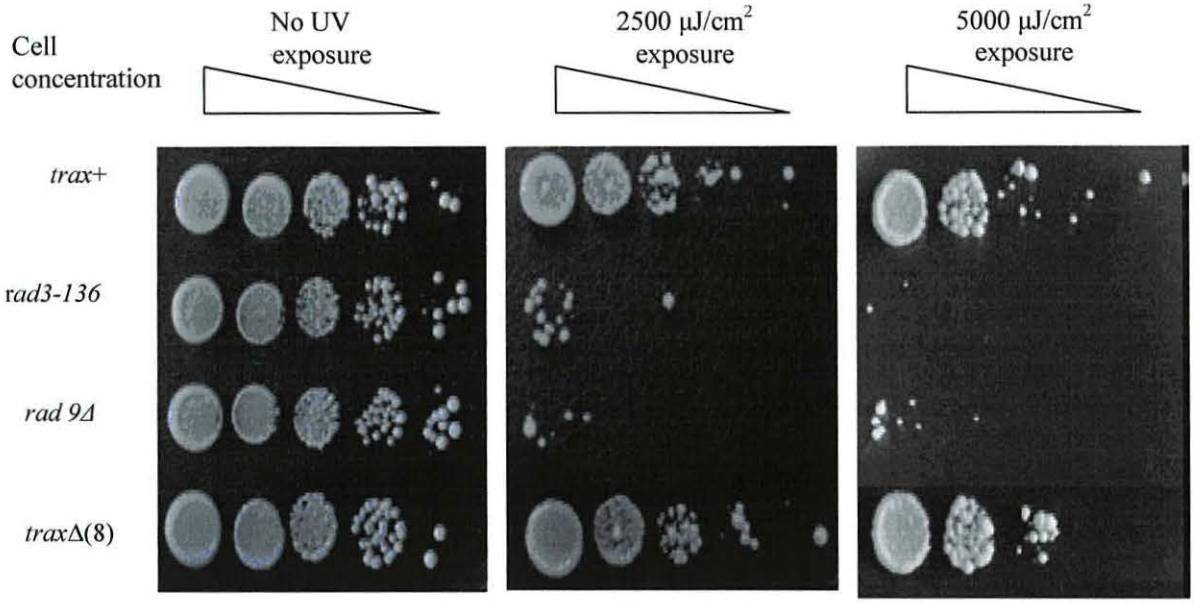


Figure 4.9 Exposure to UV (395 nm) light does not confer sensitivity to G1 arrested *traxΔ* strain. Cultures were incubated at 30°C.

The *traxΔ* strain is not sensitive to 2500 μJ/cm^2 and 5000 μJ/cm^2 of UV light.

4.3 Discussion

4.3.1 Loss of Trax does not cause increase in GT microsatellite repeat instability

Proteins can bind to single-stranded DNA (ssDNA) with no specific preferences to certain sequences; for example the ssDNA binding protein (SSB) in *E. coli* (Lohman and Ferrari, 1994) and the eukaryotic replication protein A (RPA) (Wold, 1997) or bind with high specificity or strong preferences for particular sequences of ssDNA. The *Oxytricha nova* telomere end-binding protein (TEBP) and the *S. cerevisiea* Cdc13 are known to associate with single-stranded telomeric overhangs in these organisms (Fang *et al.*, 1993, Nugent *et al.*, 1996).

S. pombe Translin binds selectively to single-stranded $d(GT)_n$ and $d(GTT)_n$ DNA repeats *in vitro* (Laufman *et al.*, 2005). In this study, GT_8 microsatellite repeats were equally stable in wild type and the *trax*Δ mutant. This means Trax has no effect on the stability of the microsatellite repeats tested. Previous studies on GT repeat stability in *S. pombe* showed that loss of Swi4 and Exo1 functions gave relatively mild increases in GT repeat stability; in both cases the effect was between 3- and 4-fold (Mansour *et al.*, 2001).

Slightly increased level of GT_8 microstellite instability (but not significant) was observed in *translinΔ* mutants (Jaendling *et al.*, 2008). From my research I conclude that Trax and Translin do not seem to have any effect on maintaining stability of the GT microsatellites.

4.3.2 A role for Trax in DNA damage recovery

I have found no evidence to show that Trax have a primary role in DNA damage recovery, in *S. pombe*. The findings that Translin binds to breakpoints of lymphoma- and leukaemia-associated translocation junctions (Aoki *et al.*, 1995; Kanoe *et al.*, 1999; Gajecka *et al.*, 2006b) and single-stranded DNA ends (Kasai *et al.*, 1997) implied this protein may have a role in the maintenance of genome stability. Other than the association with breakpoint sequences, several lines of evidence also implicate Translin and Trax functions in maintaining genome stability, particularly in response to DNA damage. TB-RBP interacts with GADD34, a protein that is implicated in the DNA damage response (Hasegawa and Isobe, 1999).

I used UV irradiation which is capable of generating covalent bonding within DNA such as pyrimidine diamers. Photolyses can split the covalent bonds of pyrimidine dimmers by utilizing light energy (350-450 nm). In *S. pombe*, UV dimers are targeted by the nucleotide exision repair (NER) pathway and UVER (Bowman *et al.,* 1994; de Laat *et al.,* 1999). None of the *trax*Δ mutants I tested showed sensitivity to UV radiation.

Camptothecin (CPT), which is a topoisomerase I inhibitor, leads to SSBs by trapping Top1-DNA intermediates and inhibiting the enzymes religation activity. None of the *trax*Δ mutants tested showed any sensitivity to camptothecin. Phleomycin is structurally related to bleomycin. Bleomycin is known to cause AP sites, SSBs and DSBs in DNA (Kostrub *et al.,* 1997). Phleomycin is known to cause DNA lesion but the exact nature of these lesions is unclear. The *traxΔ* mutants also failed to show any sensitivity to phleomycin. Mitomycin C (MMC) which causes cross linking of DNA also failed to induce any sensitivity in *traxΔ* mutants. Mitomycin C induced intrstrand cross-links are repaired by either NER or HR repair (Jachymczyk *et al.,* 1981, McHugh *et al.*, 2000).

MMS is an alkylating agent that modifies both guanine (to 7-methyguanine), and adenine (to 3-methyladenine) to cause base mispairing and replication blocks respectively. Damage caused by alkylating agents is predominantly repaired by BER and DNA alkyltranferase. It is thought that the spontaneous hydrolysis of alkylated bases to AP sites during BER could lead to DSBs formation (Fung *et al.*, 2002; Morishita *et al.,* 2002) HU which causes a range of lesions mostly well known to cause depletion of the supply of nucleotides due to inhibition of ribonuclotide reductase, leading to an S-phase arrest (Enoch *et al.*, 1992). This results in replication fork stalling and DSBs formation, which are targeted by HR or NHEJ for repair. *traxΔ* mutant cells failed to exhibit any sensitivity to this agent .

Whilst Translin and Trax have been implicated in DNA damage recovery, recent work by Clauβen *et al.,* 2006, with Translin and Trax defective *Drosophila* embryo did not show any increase in sensitivity when exposed to ionizing radiation and produced normal meiotic cross over levels. These findings and observations are in agreement with the fact that Translin-deficient MEFs showing no sensitivity to DNA damaging agents (Yang *et*

al., 2004a) arguing against a primary role for Translin and Trax in DNA damage recovery. These findings suggest that Trax may not have any primary role in DNA damage recovery in the pathways mentioned above.

Others within the group have generated double mutants of *traxΔ* and *translinΔ* with *rhp14* (XPA), *rad32* (MRE11) and other genes known to be required for DNA damage recovery. *rhp14Δ traxΔ, rhp14Δ translinΔ, rad32Δ traxΔ* and *rad32Δ translinΔ* double mutants do exhibit additive sensitivity to a range of agents (not shown). These new findings implicate Trax and Translin in a redundant DNA repair pathway possibly in early damage recognition.

The presence of Translin in irradiated hematopoietic cells has been reported to contribute to regeneration by acting as sensor protein for radiation induced damage (Fukuda *et al.*, 2008). Fukuda and co-workers (2008) demonstrated that Translin deficiency in mice delayed hematopoietic regeneration after exposure to a sub lethal dose of ionizing radiation. This suggests that Translin might have other pathways or methods of protecting cells from damage or help in DNA damage recovery that need to be explored.

The high level of conservation of the Trax and Translin proteins suggest that they play an important biological function which provides a selective advantage. However, my preliminary functional characterization did not reveal any clear occurrence of a fundamental function. Loss of Trax did not change the cell proliferation, or cause any morphological change to the cells. The *traxΔ* mutants behaved similar to the original parental strain when tested for GT microsatellite stability as well as to various genotoxic agents; more over the mutant generated normal asci after meiosis (data not shown).

Previous studies by Laufman *et al.* (2005) reported that the *trax* and *translin* genes appear to be non essential for *S. pombe* cell growth and division. Studies in *Drosophila* by Clauβen and co workers (2007) also demonstrated that the *trax* and *translin* single and double mutants were viable, fertile and phenotypically normal. However, *Drosophila translin* mutants were reported to exhibit an impaired motor response which is sex specific and suggest a possible role in neuronal development and behaviour

(Suseendranathan *et al.*, 2007). The *trax* and *translin* could be also involved in pathways other than DNA damage recovery.

4.4 Summary of main findings

1. GT microsatellite stability is not affected by absence of Trax protein in *S. pombe*
2. No sensitivity to DNA damaging agents was observed in *traxΔ* mutant strains tested against MMS, Hydroxyurea, Mitomycin C, Phleomycin as well as ultraviolet light. The tests were repeated using the same set of strains in G1 phase arrested cells (grown in nitrogen depleted media) and produced similar results.

CHAPTER 5

THE RECOMBINATION POTENTIAL OF DNA REPLICATION FORK BARRIERS IS NOT UNIVERSAL.

5.1 Introduction

Eukaryotic cells regulate the progression of DNA replication forks in a highly complex manner to preserve genome stability (Aguilera and Gomez-Gonzales, 2008; Hodgson *et al.*, 2007). Replication fork stalling can have detrimental effect on organisms, as stalled replication forks can lead to elevated intra and ectopic recombination promoting site-specific gross chromosomal rearrangements (Lambert *et al.*, 2005b; Ahn *et al.*, 2005). In the eukaryotic genome, replication fork barriers (RFBs) and / or replication fork pauses (RFP) can be caused by DNA-protein complexes, secondary DNA structures, DNA damaging agents and replication inhibitors (Rothstein *et al.*, 2000).

The processing of stalled/paused/collapsed DNA replication forks can result in increased recombination, chromosomal instability and rearrangements including translocations and hence they are potentially oncogenic (Labib and Hodgson, 2007; Admire *et al.*, 2006; Lambert *et al.*, 2005b; Rothstein *et al.*, 2000). Homologous recombination is believed to play important role in processing stalled/blocked replication forks in eukaryotes (Michel, 2000; Ahn *et al.*, 2005). In total, such potentially harmful rearrangements can be mediated by homologous recombination, non-homologous DNA end joining or DNA replication slipage, creating new chromosomal structures, including translocations, inversions, duplication and/or deletions.

Current theories suggest, genomic replication pausing/termination takes place "randomly" in regions between active origins when forks converge or they can also occur at certain specialised loci; e.g. rDNA barriers in *S. cerevisiae* and *S. pombe* (TTF1, Reb1, Fob1) (Krings and Bastia, 2004; Brewer *et al.*, 1992), replication slow zones (Cha and Kleckner, 2002), tRNA genes (Deshpande and Newlon, 1996; Admire *et al.*, 2006) and *RTS1* (replication termination sequence) in *S. pombe* (Dalgaard and Klar, 2001).

The *S. pombe RTS1* element is a well characterized site-specific replication terminator. In wild-type *S. pombe,* the *RTS1* element is located proximal to the mating type locus, *mat1*

and acts to block DNA replication fork progression in an orientation-dependent manner, allowing recombination to be initiated during the mating type switching process (Codlin and Dalgaard, 2003) (refer to Chapter 1.9). Many features of the *RTS1* element are similar to the mammalian rDNA barriers located in the RNA polymerase 1 transcription termination region (Cardin *et al.*, 2001). Previous studies have shown that *RTS1* can function as a RFB when placed in the *ade6* gene and this correlates with a recombination stimulation (Ahn *et al.*, 2005; Lambert *et al.*, 2005b), providing evidence that RFBs in mitotically dividing cells can drive genetic change.

Some biological processes in eukaryotes, such as the conjoining of homologous chromosomes during meiosis I, V(D)J recombination in mammals and mating type switching in yeast, require the initiation of recombination in a highly programmed manner, where the sites of recombination and the partner choice are governed to give specific functional outcomes. However, sites which mediated harmful gross chromosomal rearrangements are not programmed to initiate recombination. They have some inherent instability or become unstable due to exogenous factors, such as DNA damage, generating an unscheduled recombinogenic potential (Aguilera and Gomez-Gonzales, 2008).

Studies in *S. cerevisiae* aimed at identifying naturally occurring fragile chromosomal regions in mitotically proliferating cells have found that such sites can be complex in nature. They can consist of aggregates of distinct genetic elements, including transposons, long terminal repeats (LTRs) of transposons and tRNA genes (Admire *et al.*, 2006). Interestingly, these sites exhibit higher levels of instability when DNA replication is compromise, suggesting that the inherent instability of these sites are related to DNA replication. This is consistent with the proposal that perturbations in DNA replication may serve as one of the primary oncogenic stresses in tumour formation (Labib and Hodgson, 2007; Admire *et al.*, 2006; Lambert *et al.*, 2005b; Rothstein *et al.*, 2000). It is proposed that these regions provide particularly poor substrates for the replication machinery, making them more susceptible to DNA replication fork progression failures, which ultimately could increase the frequency of the formation of recombination initiating lesions. Consistent with this model, tRNA genes, which are located in some

DNA replication-related fragile sites, have been shown to have potent RFB activity, which is thought to arise due to the replication machinery, the repliosome, colliding head-to-head with RNA polymerase III, which mediates tRNA gene transcription (Deshpande and Newlon, 1996).

I wanted to examine the possible role played by the genetic elements *RTS1* and a tRNA gene cassette, which consist of tandemly arranged $tRNA^{SER}$- $tRNA^{MET}$ genes as a RFB or a hotspot for mitotic recombination in *S. pombe*. Three of four tRNA dimers present in the *S. pombe* genome belong to this species (Hottinger-Werlen *et al.*, 1985). The $tRNA^{SER}$ co-transcribes $tRNA^{MET}$ sequence. In *S. pombe,* mutated tRNA "*sup*" (suppressor) strains are available. The suppressor tRNA gene *sup3-e* is a mutated version of the tandem serine-methionine coding tRNA gene ($tRNA^{SER}$- $tRNA^{MET}$), located on the right arm of chromosome I. The *sup3-e* mutant tRNA recognises the UGA (opal) stop codon and inserts a serine amino acid, which can lead to suppression of auxotrophic phenotypes of the *ade6-704, ade1-40, lys1-37, glu1-57* or *leu3-155* mutants (Willis *et al.*, 1986).

tRNA genes are widely distributed in genome, and pol III is the enzyme responsible for the transcription of all known eukaryotic nuclear tRNA genes. Other RNAs transcribed by pol III include the non-protein coding (nc)RNA genes, small RNAs, microRNAs, short interspersed nuclear element-encoded or tRNA-derived RNAs and novel classes of ncRNAS (Dieci *et al.*, 2007). The tRNA genes could have enormous potential for genome regulation as collision between replication and transcription leads to genomic rearrangements (Aguilera, 2002).

The aim of this chapter is to investigate the effect on recombination when DNA replication encounters a RFB within a "general" genomic locus. Thereby, testing the hypothesis which proposes that RFBs stimulate recombination universally. To achieve this aim I created strains which contain either the *RTS1* or tRNA genes (*sup3-e*) integrated into the *ade6* gene located on Chromosome III and then assayed recombination using inter molecular recombination assays.

5.2 Results

5.2.1 Construction of *S. pombe* strains with *RTS1* or tRNA (*sup3-e*) and spacer (*his3'$_{283}$/his3'$_{756}$*) inserts in the *ade6* recombination reporter gene.

The fission yeast *RTS1* element is a polar RFB. I integrated separate linear DNA fragments of the *ade6* gene containing *RTS1* in either orientation or a spacer control (*his3'$_{756}$*) and transformed them into *ade6*$^{-}$ *S. pombe* cells to integrate the elements into the genomic *ade6* locus at *Bst*XI site (See 2.25 Methods and Material for details). Two *ade6::RTS1* strains were isolated, each with *RTS1* in a distinct orientation; *ade6:: RTS1(1)* (orientation 1) and *ade6::RTS1(2)* (orientation 2). A third strain was also isolated containing the spacer DNA (756 bp) inserted into the same position in *ade6* (*ade6::his3'$_{756}$*).

Simlarly, two distinct strains with *sup3-e* inserted in two orientations [(*ade6::sup3-e(1)* (orientation 1) and *ade6::sup3-e(2)* (orientation 2)] were also obtained along with a spacer control (*ade6::his3'$_{283}$*). All integrated cassettes were checked by PCR and DNA sequence analysis. The integration of *RTS1* or *sup3-e* element inactivates the *ade6* gene rendering the strains auxotrophic to adenine. A schematic representation of the *ade6* ORF integrated with the genetic elements *RTS1* and *sup3-e* are as in Figure 5.1.

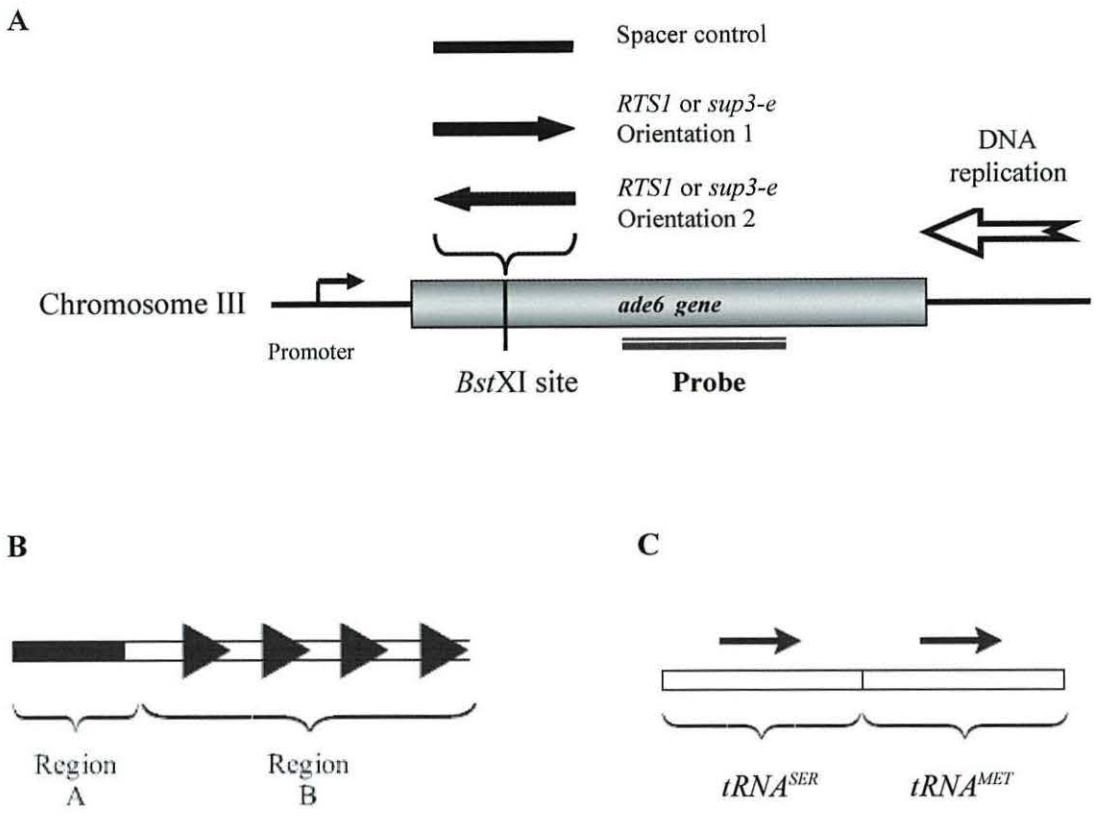


Figure 5.1 Position of the genetic elements and spacer controls integrated within the *ade6* gene of *S. pombe.*

A. The genetic elements (either *RTS1* or *sup3-e*) were inserted into *ade6* ORF at the *Bst*XI site in two orientations (indicated by the black arrows above the *Bst*XI site). The predominant direction of DNA replication from right to left is indicated by the large open arrow. Two distinct space controls, consisting of an origin-free stretches of the *his3* open reading frame, were also inserted at this site. The spacer controls were similar to the size of the genetic elements integrated. The *ade6* open reading frame is expressed from left to right and the promoter is indicated by the angular arrow.

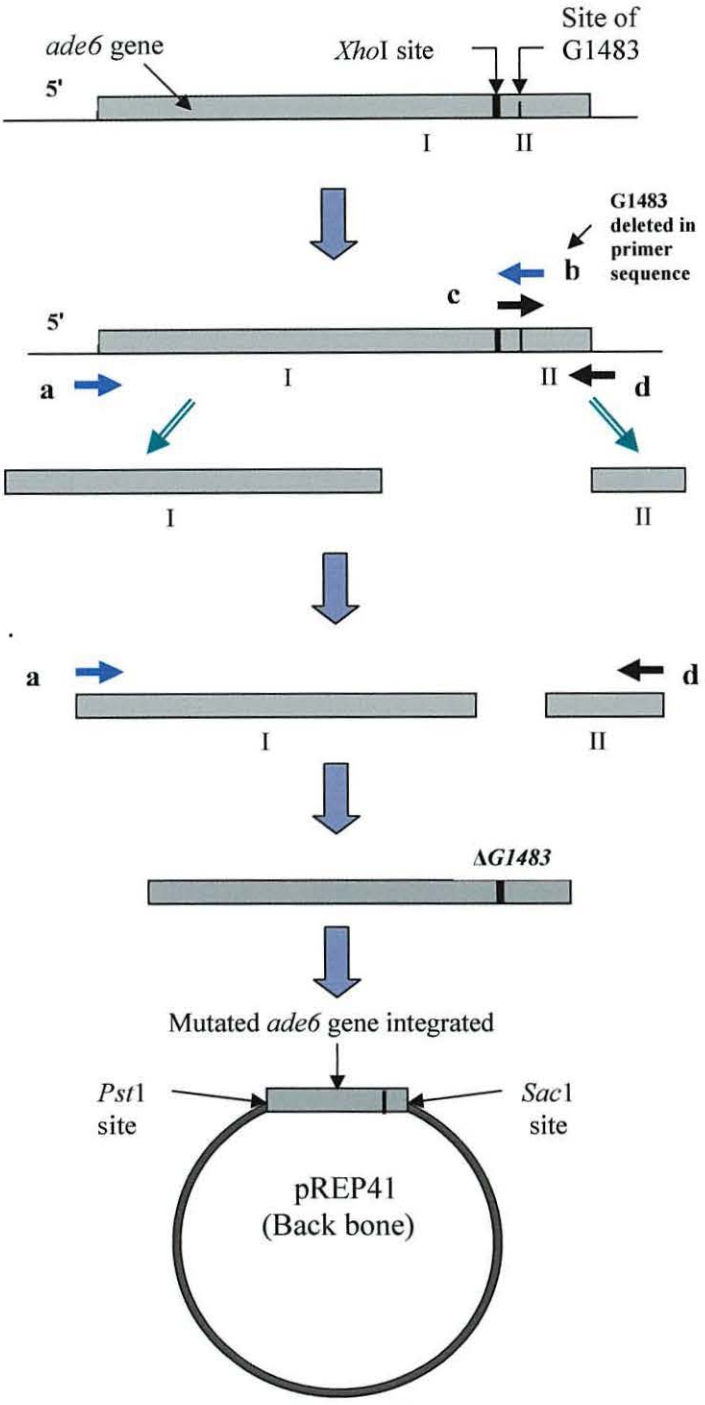
B. A schematic representation of the *RTS1* element (from Codlin & Dalgaard, 2003). The element consists of two regions. The polarity of the *RTS1* barrier is indicated by the direction of the black arrow heads and a RFB is generated when the replication fork approaches Region A first (left to right in the diagram). The element is shown in orientation 1 (which will not generate a RFB as replication is from right to left in this case).

C. A schematic representation of the *sup3-e* element. This is made up of two tandemly arranged tRNA genes, $tRNA^{SER}$-$tRNA^{MET}$. The direction of transcription is indicated by the black arrows and the element shown is in orientation 1, so this orientation would be expected to generate a head-to-head collision between the replisome and RNA polymerase III as replication is predominantly from right to left in the case.

5.2.2 Construction of plasmid pSRS5 for plasmid-by-chromosome recombination assay.

To test whether a single *RTS1* RFB can drive inter molecular homologous recombination, I established a plasmid-by-chromosome recombination reported system we have previously employed (Jaendling *et al.*, 2008). I required a plasmid carrying an *ade6* marker mutation which was not suppressed by *sup3-e*. The construction of the plasmid pSRS5 is diagrammatically presented in Figure 5.2. Briefly, one *ade6-Δ1483* allele was created in which guanine at nucleotide position 1483 (taking the A of the ATG start codon as position 1) within the *ade6* open reading frame (ORF) was deleted by PCR method (see 2.24 Materials and Methods for details). The *ade6* allele has the Guanine 1483 (G1483) mutation engineered at the 3′ end of the gene, distal to the site into which the *RTS1* or *sup3-e* element has been inserted (Figure 5.2). This was cloned into the pREP41 backbone to create pSRS5. pREP41 is an expression vector and a *Pst*1/*Sac*1 digest was used to remove the inducible *nmt* promoter, the polyinker site and the transcription termination site.

In the plasmid-by-chromosome assay, plasmid pSRS5 was transformed into the *S. pombe* strains which carry altered genomic *ade6* alleles (prepared in 5.2.1). These strains are adenine auxotrophs. A set of three strains generated with *RTS1* [*ade6::RTS1(1)*, *ade6::RTS1(2)* and *ade6::his3'*$_{756}$] and *sup3-e* [*ade6::sup3-e(1), ade6::sup3-e(2)* and *ade6::his3'*$_{283}$] were then simultaneously subjected to fluctuation analysis. Homologous recombination-mediated gene conversion (Figure 5.3) (with or without crossing over) of either *ade6* allele generates adenine prototrophs and the frequency of their production is a measure of recombination frequency (adenine prototrophs per million viable cells).



Primers were constructed having the *ade6* gene mutated by deletion of nucleotide G at the 1483 nt. PCR of mutated *ade6* gene with primers **a**, **b** and primers **c** and **d**, were carried out in separate reactions.

Fragments I and II were mixed together and used as templates for PCR with primers **a** and **d**

ade6 mutated ORF PCR product was synthesized

The mutated *ade6* ORF fragment was cloned into plasmid vector, pREP41 back bone.

Figure 5.2 Construction of plasmid vector with mutated *ade6* ORF (pSRS5).

One 'G' nt deleted *ade6* ORF was synthesized using the PCR method and integrated into the plasmid pREP41 back bone between the *Pst*1 and *Sac*1 site. The multicloning site and the *nmt* promoter region from the plasmid were removed. This plasmid with mutated *ade6* ORF is called pSRS5 (see 2.24 Materials and Methods for details and the primer sequences). The mutated *ade6* insert was sequenced to verify the presence of the mutation.

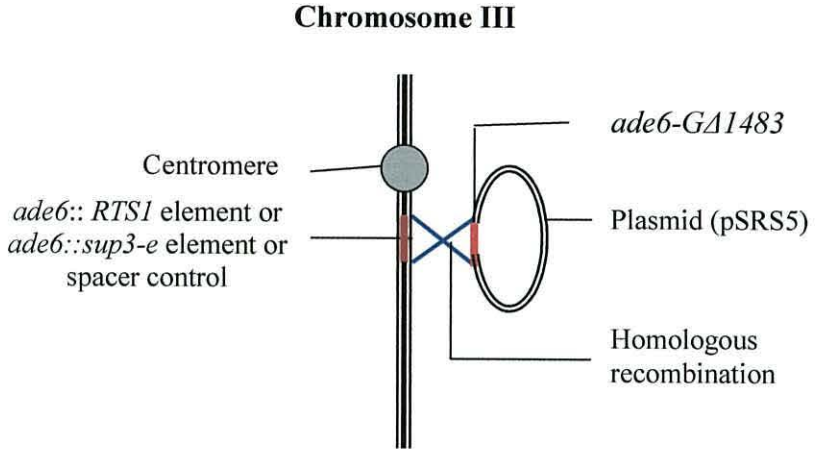


Figure 5.3 Schematic diagram of plasmid-by-chromosome assay.

Homologous recombination between the plasmid bearing *ade6* allele (*ade6-GΔ1483*) and the chromosomal allele (*ade6::*insert) can result in prototrophs generation by gene conversion. The frequency of the prototroph production is a measure of recombination frequency.

5.2.3 *RTS1* RFB activity is associated with increased inter molecular mitotic recombination.

I used strains that had the *RTS1* (orientation 1 and 2) and *his3'*$_{756}$ spacer (control) integrated in the *ade6* gene to verify if the *RTS1,* which functions as a RFB in an orientation-specific fashion, drives recombination. Plasmid pSRS5 was transformed into these cells and they were subjected to fluctuation analysis to measure plasmid-by-chromosome recombination frequency. The recombination frequency for *ade6::RTS1* orientation 2 [*(ade6::RTS1(2)*] was significantly higher by approximately 20-fold compared to the spacer control (Refer to Figure 5.4), confirming that an orientation-dependent recombination hotspot was created by *RTS1*.

To determine whether the expected RFB was present at *RTS1(2)*, I employed two dimension gel electrophoresis (2D gel) using total cellular DNA digested with restriction enzyme. Two dimensional agarose gel electrophoresis is used to analyze DNA replication and recombination intermediates (RIs). Briefly, digested genomic DNA is run on an electrophoresis gel in two dimensions. The first dimension gel is intentionally run at low voltage in low percentage agarose (0.4%) to separate DNA molecules in proportion to their mass. The second dimension is run at high voltage in a gel of higher agarose concentration (1.2%) in the presence of ethidium bromide so that the mobility of a non-linear molecule is drastically influenced by its shape. The gel is blotted onto a membrane and probed with P^{32} labeled probe. RFP sites, which cause the accumulation of RIs of a particular size, were detected by 2D gel electrophoresis as intense regions of hybridization along an arc of Y-shaped RIs. See Materials and Methods 27.4.6 (page 67) for interpretations of 2D gel patterns obtained for different replication intermediates.

The results confirm the presence of fork stalling (a dark spot on arc of 2D gel) for the *RTS1(2)* insert and not in *RTS1(1)* or the spacer control (Figure 5.5). Both spacer control and *RTS1* gave similar levels of inter molecular recombination (Figure 5.4 B). This further confirms that the *RTS1* functions as replication fork barrier that is an orientation dependent recombination hotspot when located within the *ade6* gene.

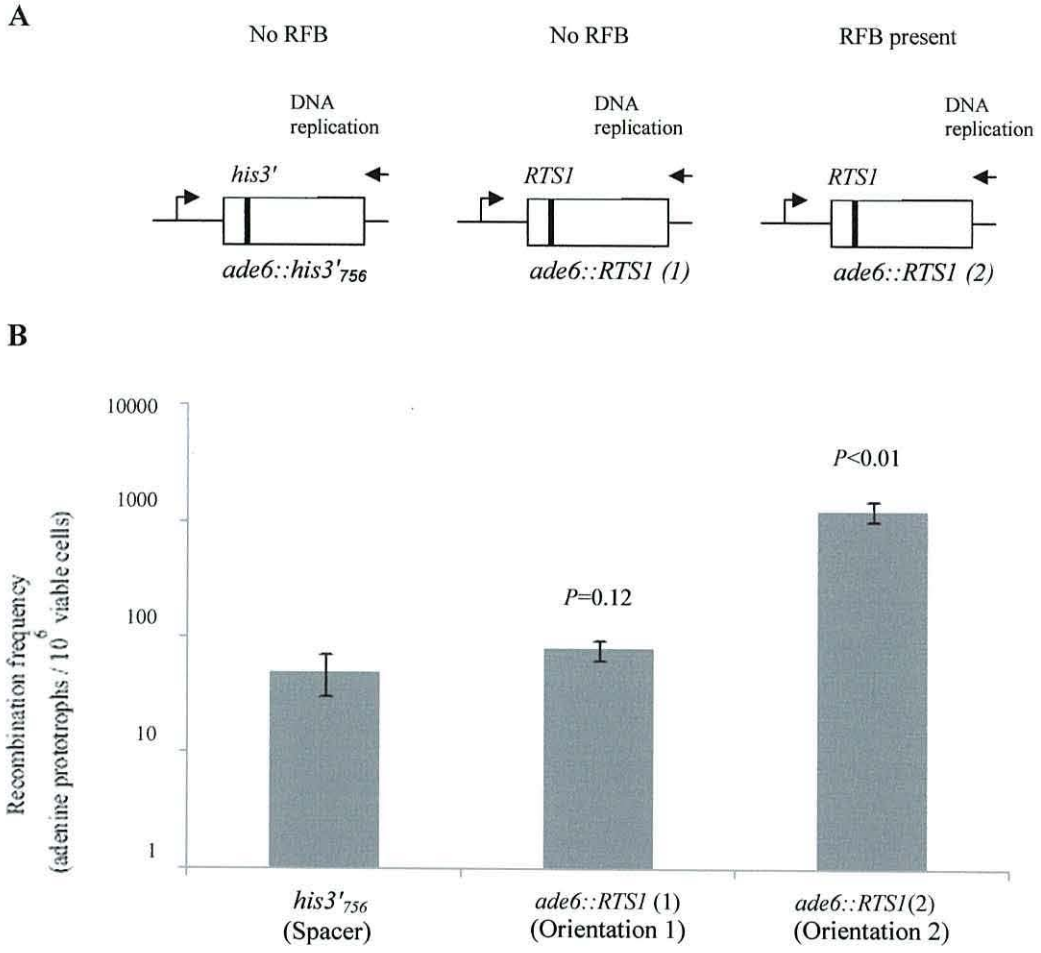


Figure 5.4 Plasmid (pSRS5) –by-chromosome recombination assay for *RTS1* within *ade6* shows higher recombination in *RTS1* orientation 2.

A. Top panel shows integration of spacer control (*his3'*$_{756}$) and *RTS1(1)* into *ade6* produce no RFB effect, where as the *RTS1(2)* produces RFB effect by opposing the predominant direction of the DNA replication.

B. *RTS1(2),* generates a significantly increased the recombination frequency almost two orders of magnitude higher than either orientation 1 or space control ($P<0.01$; Student *t*-test). This indicates that the *RTS1(2)* generates a mitotic recombination hotspot. Error bars are standard deviations. *P* values are generated by Student's *t*-test of pairwise comparison of spacer control with the *RTS1* allele.

(The data for plasmid (pSRS5)-by-chromosome fluctuation analyses for *RTS1* and recombination frequency calculation is in supplementary material 1)

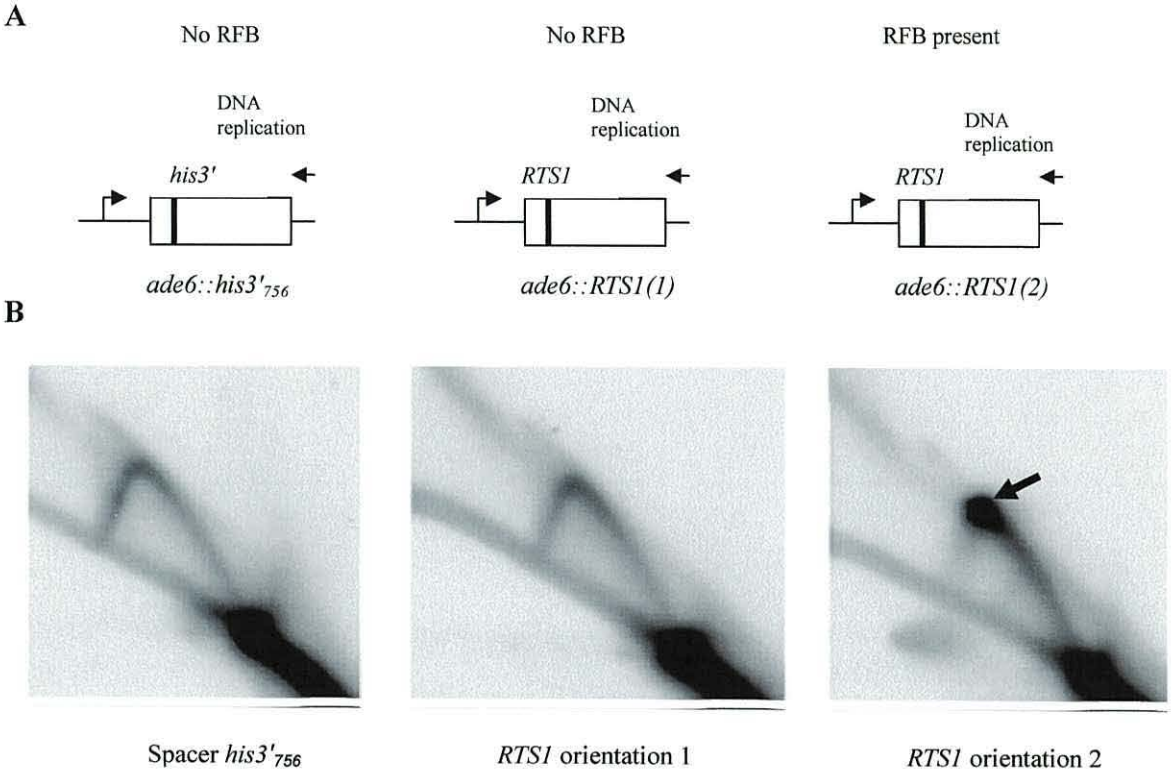


Figure 5.5 2D gel examination of DNA from strains having spacer control, *RTS1* (1) and *RTS1*(2) integrated into *ade6* .

A. Top panel diagrammatically shows the integration of spacer control and *RTS1* in the *ade6* gene. *RTS1*(2) causes RFB. This is confirmed by the 2D gel analysis shown below.

B. Panel shows the 2D gel results carried out for the strains with control spacer *his3'*$_{756}$ (left), *RTS1(1)*(middle) and *RTS1(2)*(right). Strains having the *RTS1(2)* clearly shows a prominent spot (marked with arrow) at the arc indicating occurrence of a 'replication fork barrier'. The strains used for this analysis did not carry the plasmid pSRS5. The DNA was digested with *Sac*I restriction enzyme.

5.2.4 *sup3-e* does not generate a mitotic recombination hotspot at *ade6*

Integration of *sup3-e* in orientation 1 is predicted to cause a block to the replication fork progression, due to a head on collision with RNA polymerase III, thus I predicted it would have to have increased recombination at *sup3-e* in orientation 1 [*sup3-e(1)*]. However, *sup3-e(1)* only produced a slight increase in recombination of 3-fold, which was just significant; (P=0.03; Student's t-test) in recombinants compared to the spacer control (Figure 5.7). Both spacer control and *sup3-e(2)* produced similar recombination frequencies. I find that *sup3-e* only generated a mild orientation-dependent inter molecular recombination hotspot. The mild hot spot activity shown in Figure 5.7 is not consistently reproducible, leading us to conclude that any activity, if present, is limited.

Next I carried out 2D gel electrophoresis analysis on strains containing *ade6::sup3-e* to examine if the low levels of recombination frequency are associated with a RFB. I find that *sup3-e* in both orientation 1 and 2 produced a RFB (Figure 5.6) although the intensity is less strong than that observed for the polar RFB generated by *RTS1* (Figure 5.5). The RFB does not correlate with a high recombination frequency and no increase in recombination at *sup3-e(2)* was observed compared to the spacer control. This suggests the *sup3-e* does not act as an effective recombination hot spot although it generates a non-polar RFB. The lack of polarity observed differs to the polarity observed for other tRNA genes (Deshpande and Newlon, 1996).

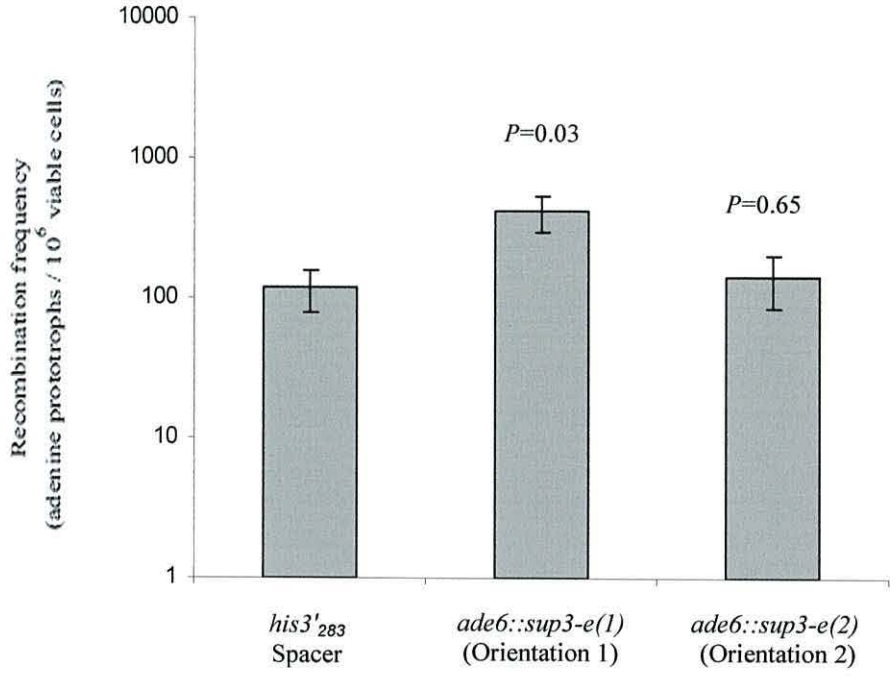


Figure 5.6 Plasmid (pSRS5) -by-chromosome recombination assay for *sup3-e* and spacer control within *ade6.*

The histograms show that, there is a significant, increase in recombination frequency in *sup3-e(1)* (P=0.03 Student's t-test) compared to the *sup3-e(2)*. These suggest the *sup3-e* does not produce a very high recombination frequency when integrated within *ade6*. *sup3-e(2)* did not produce significantly raised recombination frequency compared to the spacer insert, although RFB activity is seen. Error bars shown are standard deviations.
(The data for plasmid (pSRS5)-by-chromosome fluctuation analyses for *sup3-e* and recombination frequency calculation is in supplementary material 2).

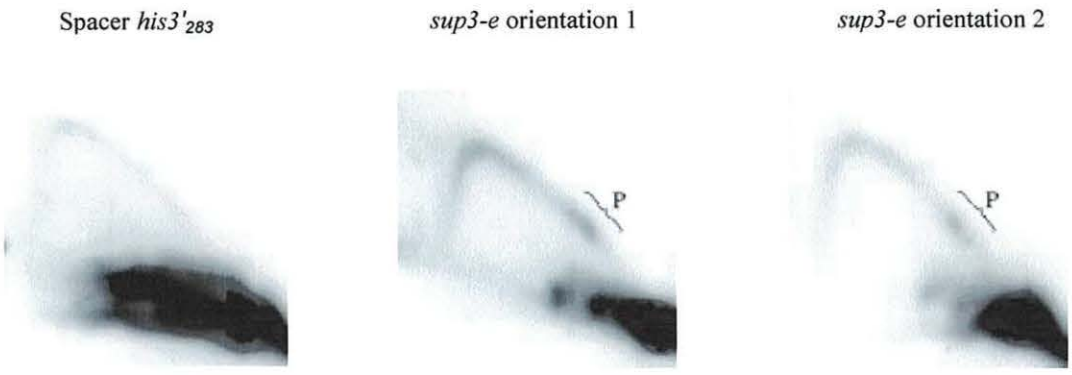


Figure 5.7 Two dimensional (2D) agarose gel electrophoresis used to analyze DNA replication of strains integrated with *sup3-e* and spacer control.

2D gel images of DNA from *sup3-e* and spacer control show that there are spots on the replication arc indicating a pause site (P) was created by the *sup3-e* in orientation 1 and orientation 2. No pause site was seen in spacer control. DNA for *sup3-e* was digested with *Eco*RI.

The pause is located at a different position on the arc relative to the pause generated by *RTS1* (Figure 5.5). This is because the DNA was digested with different restriction enzymes for each analysis.

5.2.5 Ectopic recombination initiated by a RFB is region specific

To further these analyses and determine whether a single *RTS1* or *sup3-e*-mediated RFB could stimulate recombination between non-homologous chromosomes at short homologous sequences, I employed a system based on that of Virgin and co-workers (Virgin *et al.*, 2001). They inserted mutant *ade6-469* alleles at ectopic sites either on chromosome I (*zzz15*) or chromosome II (*zzz7*). Ectopic recombination frequency was measured for strains constructed with *RTS1* or *sup3-e* having an additional *ade6-469* in the genome. The strains which have additional *ade6-469* in chromosomes I are called *zzz15* and those with an *ade6-469* allele in chromosome II are called, *zzz7* (Figure 5.8 A and B).

S. pombe has three chromosomes and the *ade6* locus is normally located on chromosome III (Figure 5.8). Recombination between *ade6* alleles in strains carrying a mutant *ade6* allele at the normal locus (on chromosome III) and one of the two *zzz ade6* alleles will result in adenine prototrophs (Figure 5.8 C) and the frequency of prototroph production (recombination) was measured by fluctuation analysis.

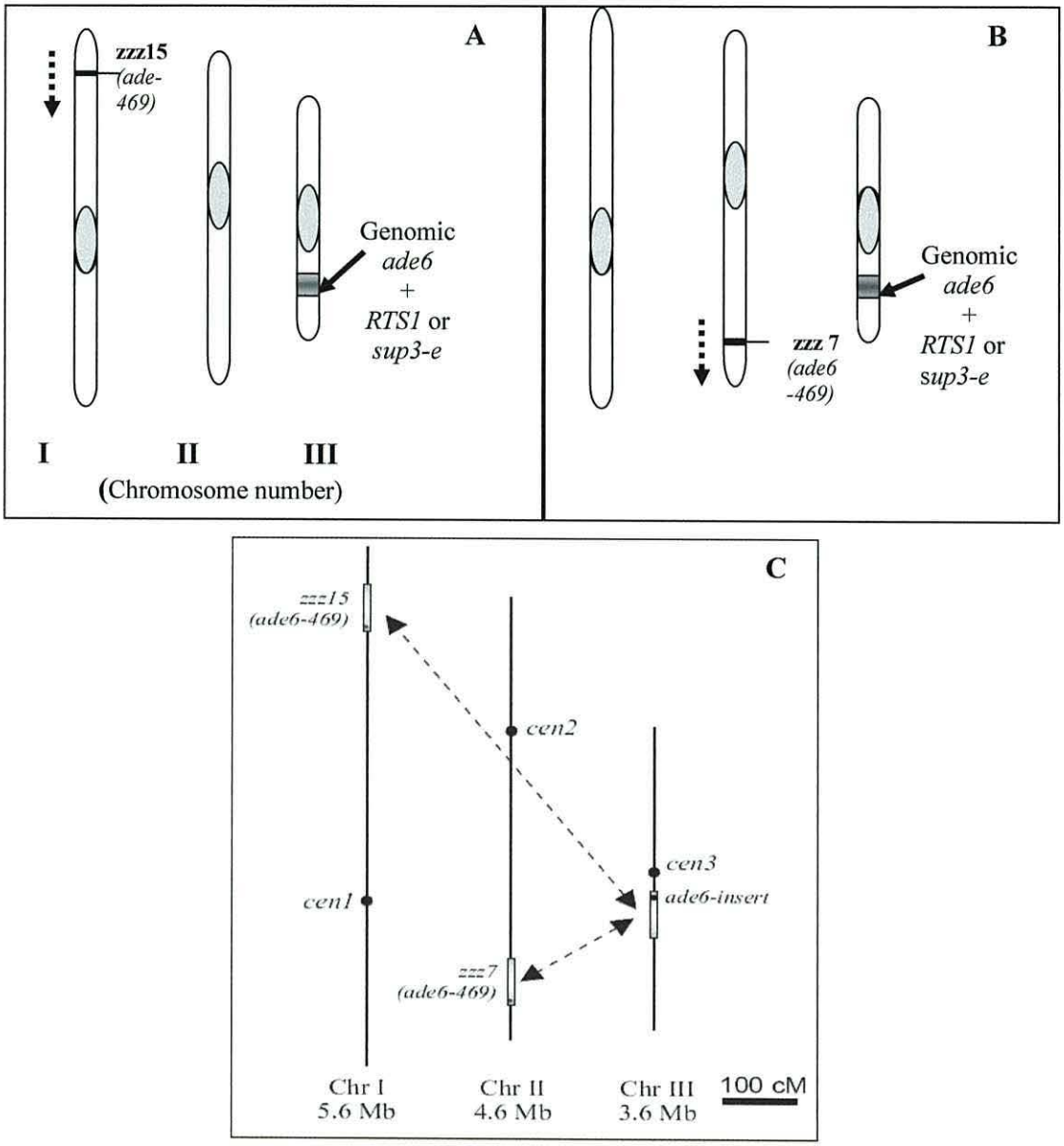


Figure 5.8 Assay system used to monitor replication fork barrier-associated mitotic recombination in the fission yeast model system.

A. *S. pombe* integrated with additional *ade6-469* in Chromosome I is the *zzz15* allele.

B. *S. pombe* integrated with additional *ade6-469* in Chromosome II is the *zzz7* allele.
The dashed arrows indicate the inferred orientations of the *ade6* genes at the different integration sites (Virgin and Bailey, 1998).

C. Schematic representation of chromosome-by-chromosome recombination assay was used to measure ectopic inter molecular recombination. Diagram showing the three *S. pombe* chromosomes with the approximate positions of the additional *ade6-469 (zzz7 and zzz15)* relative to the genomic *ade6* in chromosome III. Dashed arrows indicate ectopic recombination. The genomic *ade6* is integrated with *RTS1* or *sup3-e* making the strain auxotrophic to adenine.

5.2.6 *RTS1* stimulates orientation-dependent, ectopic recombination in a region specific fashion.

In this experiment I wanted to examine if recombination initiated by *RTS1* mediated RFBs can drive ectopic recombination within the genome. The strains having *RTS1* alleles and spacer control allele carrying one of the zzz alleles (*ade6-469*) were subjected to fluctuation analysis. Strains having *zzz7* (ectopic *ade6* allele located on chromosome II), had only a very mild stimulation of about 2-fold in recombination for the RFB-generating allele of *ade6::RTS1(2)* (Figure 5.9 A).

However, for *zzz15* (ectopic allele located on chromosome I), levels of *RTS1*-mediated recombination mirrored those observed for plasmid-by-chromosome recombination, with large elevations in recombination observed for *RTS1* in orientation 2 (the RFB active orientation) relative to the non-active orientation. Recombination frequency is elevated up to 100-fold in these strains (Figure 5.9 B), indicating that there is a strong positional influence on inter molecular recombination mediated by this RFB.

5.2.7 *sup3-e* does not stimulate chromosome-by-chromosome ectopic recombination.

Strains with spacer control *his3'*$_{283}$, *sup3-e(1) and sup3-e(2)* were similarly prepared to carry *zzz7* and *zzz15* alleles. Fluctuation analysis carried out on these shows that *sup3-e* does not stimulate recombination between the genomic *ade6* locus and the *zzz7* or *zzz15* alleles (Figure 5.10 A and B). This clearly demonstrates that *sup3-e* did not stimulate recombination in either allele, consistent with the results obtained for plasmid-by-chromosome events.

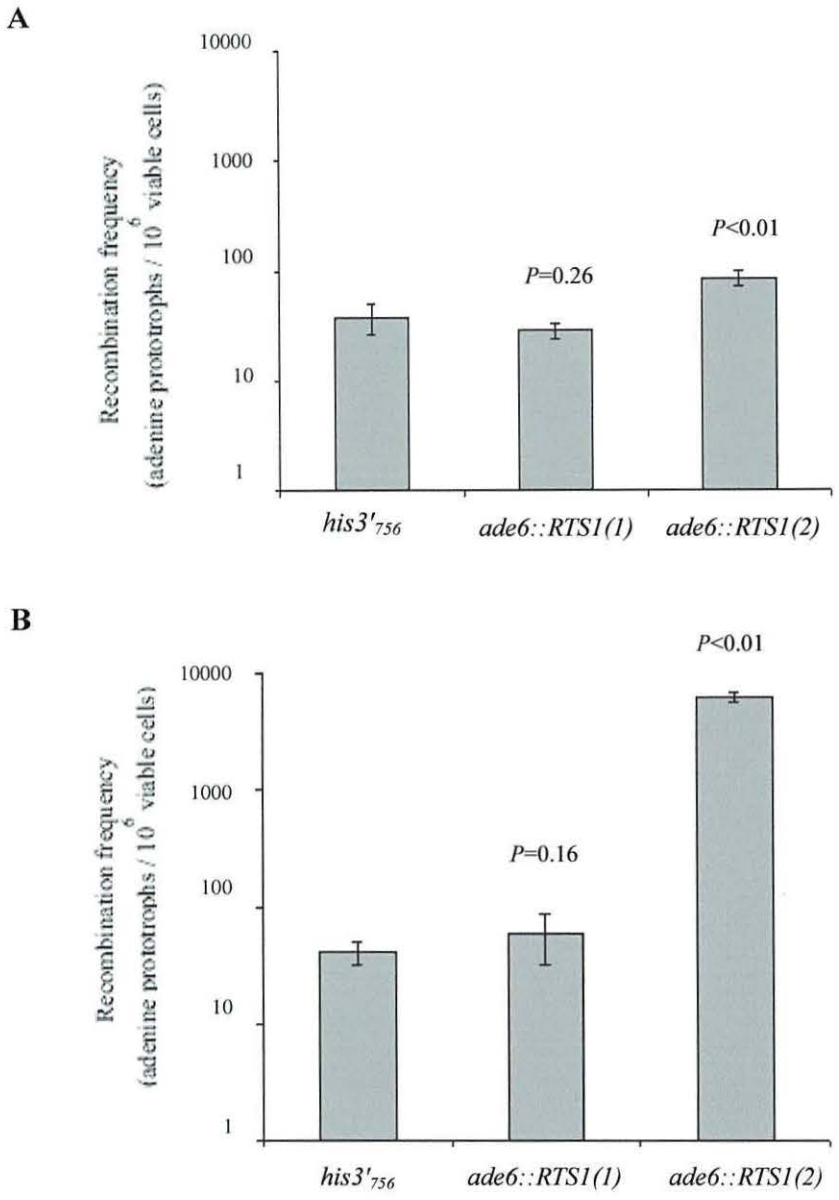


Figure 5.9 Recombination frequencies in chromosome-by-chromosome recombination assay in *S. pombe* with *RTS1* integrated in genomic *ade6* carrying *zzz7* and *zzz15* alleles.

Fluctuation analysis was carried out for strains with *RTS1* and spacer control *his3'*$_{785}$ that carried *zzz7* and *zzz15* alleles.

A: Recombination frequency for *RTS1* containing strain with ectopic *zzz7* allele. *RTS1*(2) produces significantly higher recombinants (approximately 2-fold) compared to *RTS1*(1). (P<0.01; Student's t-test).

B: Recombination frequency for *RTS1* containing strain with ectopic *zzz15* allele. *RTS1(2)* produces significantly higher number of recombinants compared to *RTS1(1)* (P<0.01;Student's t-test). However, the frequency of recombinants produced is higher in the strains carrying *zzz15* allele (approximately 100-fold) compared to that of the *zzz7* allele containing strains. P values are from pair wise comparison with spacer allele. Error bars shown are standard deviations.

(The data for chromosome-by-chromosome (*zzz7 and zzz15*) fluctuation analyses for *RTS1* and recombination frequency calculation is in supplementary materials 3 & 4).

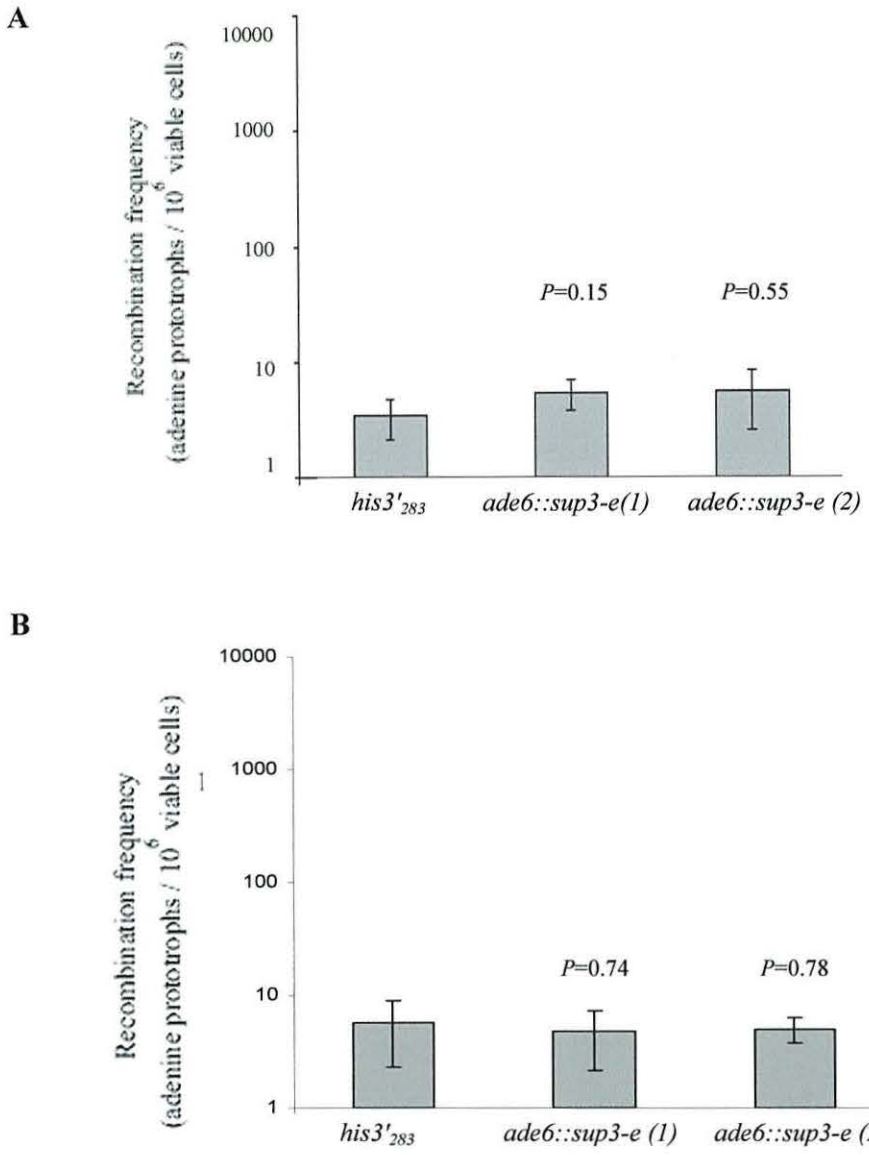


Figure 5.10 Recombination frequencies analysed using chromosome-by-chromosome recombination assay in spacer control *his3'$_{283}$* and *sup3-e* integrated in genomic *ade6.*

Fluctuation analysis was carried out for strains with *sup3-e* and spacer control that carried *zzz7* and *zzz15* alleles.

A: Recombination frequency for *sup3-e* containing strain with ectopic *zzz7* allele. *sup3-e(1)* and *sup3-e(2)* had no significant increase in recombination frequency compared to the control.

B: Recombination frequency for *sup3-e* containing strain with ectopic *zzz15* allele. Strains with *zzz15* had no significant difference in recombination frequency in both orientations of *sup3-e*.

P values are from pair-wise comparison of *sup3-e* alleles with spacer control allele using Student's *t*-test. Error bars are standard deviations.
(The data for chromosome-by-chromosome (*zzz7 and zzz15*) fluctuation analyses for *sup3-e* and recombination frequency calculation is in supplementary materials 5 & 6).

5.3 Discussion

I have established an assay to induce replication fork stalling in the *ade6* gene using the *RTS1* replication fork barrier and tRNA genes (*sup3-e)*. I measured recombination associated with insertion of *RTS1* and *sup3-e*, the genetic elements that would act as RFB in the fission yeast. A plasmid-by-chromosome recombination assay was used to measure inter molecular mitotic recombination and chromosome-by-chromosome recombination system was used to measure ectopic inter molecular recombination.

I find that both RFBs give different results showing that recombination potential for each RFB can vary. In cells where the *RTS1* was integrated in an orientation that blocks replication fork progression (*RTS1* orientation 2), the stalled replication forks correlates with elevated inter molecular recombination; up to 20-fold compared to the control (Figure 5.4B). I confirmed that the recombination is related to replication fork stalling by 2D gel analysis. Previous studies by Ahn and co-workers (2005) have established that the *RTS1* element acts to stimulate recombination in an orientation-specific manner, when placed within an *ade6* direct repeat intra molecular recombination assay system. A stalled replication fork by *RTS1* was also shown to elevate intrachromosomal and ectopic recombination in fission yeast and a single *RTS1* site recruited recombination factors when ectopically located (Lambert *et al.*, 2005b).

On the contrary, I could not see high recombinations frequencies occuring in strains integrated with *sup3-e*. Unlike *RTS1*, *sup3-e* generated raised levels of recombination in both orientations, however, only at orientation 1 [*(sup3-e(1)*], the raised levels were just significant (P= 0.03; Student's t-test) compared to the spacer control. Previous studies have shown that DNA replication-associated fragile sites can contain tRNA genes, implicating these elements in the generation of recombinogenic lesions (Admire *et al.*, 2006). Besides, tRNA genes are known to use gene conversion mechanisms to maintain their genome wide sequence uniformity, suggesting they have recombination initiating potential (Amstutz *et al.*, 1985, Kohli *et al.*, 1984).

However, 2D gel analysis of DNA from these strains showed presence RFBs in both orientations, suggesting that *sup3-e* generates a RFB but it does not show any polarity. I

demonstrated that tRNA genes generate RFBs as reported previously, but did do not display polarity as previously reported (Deshpande and Newlon, 1996). This leads us to conclude that although RFB effect is present in both orientations, a recombination hotspot at *sup3-e,* if at all present, is limited. These findings suggest that the tRNA genes (*sup3-e*) are not highly recombinogenic although detectable RFB was present and it does not show any polarity effect. Thus I conclude that the recombinogenic potential demonstrated for the *RTS1* RFB is not universal for all RFBs.

I further confirmed these findings in strains carrying *zzz* alleles (*ade6-469*) located in different chromosomes. The ectopic recombination results obtained for strains having *RTS1* integrated in the genomic *ade6* gene mirrored the results obtained from the plasmid-by-chromosome system, with a high recombination frequency for the RFB orientation of *RTS1* in the *zzz15* allele compared to *zzz7*. Meanwhile, the *sup3-e* strains carrying *zzz* alleles again showed no recombinogenic potential in both *zzz7* and *zzz15* alleles (Figure 5.10 A; B). I conclude that recombination does not occur randomly between homologous alleles but possibly there are other factors that influence it. In the genome it could be possible that there are genes/elements that are recombinogenic, with the potential of causing harm to the cell if they are positioned away from each other, or in unfavourable areas for recombination to occur, in order to prevent detrimental recombination events. I could extend this in this model, by changing the location of the *ade6-469* alleles along the length of the same chromosome and repeat the fluctuation analysis to verify the recombinogenicity at various locations. In fact, the phenomena of replication fork arrest by various factors, inducing chromosomal rearrangements have been reported in other models studied. In bacteria, replication forks stalled at ectopically positioned *Ter* sites result in chromosomal rearrangements (Michel *et al.*, 2004).

My findings, are not consistent with the work by Admire and co workers (2006). They found that a particular site in the budding yeast genome having multiple tRNA genes contributed to genomic instability. They proposed that the genomic instability could be caused by in part the tRNA genes. Our experiments prove that not all replication fork barriers in the genome produce recombination hotspots. We see that a polar replication fork barrier, *RTS1,* generates a polar mitotic inter molecular recombination hotspot. The

tRNA gene cassette on the other hand, does not generate a mitotic recombination hotspot, although a non-polar replication pause occurs at the locus. This indicates that two distinct RFBs have a different potential for genomic disruption.

5.4 Summary of the main findings

1. *RTS1* integrated strains produce higher levels of recombinants in orientation-specific manner as shown in chromosome-by-plasmid and chromosome-by-chromosome analysis. Orientation 2, which causes the replication fork pausing, produces significantly higher recombinants compared to the spacer control.

2. Recombination initiated by a RFB can drive ectopic recombination within a genome in a region-specific fashion.

3. The *RTS1(2)* functioning as a RFB drives recombination.

4. In *sup3-e* integrated strains, the plasmid-by-chromosome and chromosome-by-chromosome recombination assay showed that *sup3-e* does not drive recombination.

5. The *sup3-e* does not generate a mitotic recombination hotspot although replication fork pausing occurs.

6. RFBs are not universally recombinogenic.

CHAPTER 6

DIFFERENTIAL REGULATION OF ELEMENT-SPECIFIC MITOTIC DNA REPLICATION-ASSOCIATED RECOMBINATION.

6.1 Introduction

The progression of DNA replication forks is regulated in diverse ways during the normal process of chromosome replication. This allows eukaryotic cells to couple chromosome replication to other cellular processes. Replication forks arrest at specific sequences to facilitate a variety of genetic events. For example, the pausing of a replication fork by the *RTS1* element in the mating type region of the fission yeast is linked to the establishment of a genomic imprint, contributing to mating type switching (Dalgaard and Klar, 2000). An arrested replication fork is characterised by the presence of persistent single-stranded DNA regions, which may be more sensitive to recombination processes and which are responsible for DNA rearrangements (Bierne and Michel, 1994). When an active replication fork is perturbed, multiple cellular pathways are recruited to stabilize the replication apparatus (Figure 1.7 and text) and help to bypass or correct the causative problem (Lambert and Carr, 2005a).

6.1.1 The functions of Swi1 and Swi3 their homologues

The *S. pombe* homologue of human TIMELESS, Swi1 is a component of the replication progression complex (RPC) (Aguilera and Gomez-Gonzales, 2008). It has previously been demonstrated that loss of Swi1 function results in loss of RTS1-dependent intra chromatid recombination (Ahn *et al.*, 2005). Sommariva and co-workers (2005) demonstrated that on loss of Swi1, intra chromatid recombination was elevated, although their system was devoid of a specific RFB. *RTS1* RFB activity in mating type switching requires several *trans* acting factors including *swi1* (for switch), *swi3, swi7,rtf1,rtf2* and *sap1* (Lee *et al.*, 2004; Vengrova *et al.*, 2002). Swi1 and Swi3 (the homologue of human TIPIN) proteins are required to pause the replication fork at the imprinting site and terminate replication at *RTS1* (Dalgaard and Klar, 2000). Swi1 and Swi3 are also defined as the fork protection complex that coordinates leading and lagging-strand synthesis and stabilizes stalled replication forks (Noguchi *et al.*, 2004).

During *S. pombe* mating type switching, Swi1 and Swi3 proteins have been shown to physically bind to a region around the imprint site where pausing of replication occurs and also interacts with *RTS1* (Lee *et al.*, 2004). Thus, the Swi1 and Swi3 proteins are proposed to produce a stimulatory effect on switching and imprinting through interaction with *mat1* regions. The Swi1-Swi3 complex is required for the replication checkpoint that arrests the cell cycle in response to a DNA replication fork arrest, it is unknown how this is accomplished (Noguchi *et al.*, 2003; Noguchi *et al.*, 2004). Swi1 has been shown to be required for proficient activation of Cds1 (Noguchi *et al.*, 2003); but Cds1 is not required for mating type switching. Thus, Swi1 has both Cds1-dependent and -independent activities. Cds1 is a critical effector of the replication checkpoint in *S. pombe* (Lindsay *et al.*, 1998). One of the major functions of Cds1 is to prevent the onset of mitosis by regulating proteins which stabilize replication forks (Boddy and Russel, 2001).

A similar situation exists in budding yeast. Tof1 (a Swi1 homolog in budding yeast), is involved in control of Rad53 (Foss, 2001). Tof1 travels with the replication fork and is needed to restrain fork progression when DNA synthesis is inhibited by HU (Katao *et al.*, 2003). This function is shared with Mrc1, another protein involved in Rad53 activation (Katao *et al.*, 2003; Osborn and Elledge, 2003). The Mrc1 and Tof1 proteins are conserved throughout evolution and in budding yeast they are known to associate with the MCM helicase and regulate the progression of DNA replication forks (Hodgson *et al.*, 2007).

6.1.2 Role Rqh1 in Replication Fork Progression

The RecQ family of DNA helicases includes Sgs1 and Rqh1 in *S. cerevisiae* and *S. pombe* respectively and BLM and WRN in humans. In *S. pombe*, Rqh1 is required to prevent recombination and suppression of inappropriate recombination (Stewart *et al.*, 1997). DNA helicases are ubiquitous enzymes that unwind DNA in an ATP-dependent and directionally specific manner. Helicases, separate the complementary strands of double-stranded DNA or RNA into single-stranded molecules, for use in biological processes such as replication, recombination, repair and transcription (Sharma *et al.*, 2007). RecQ helicase-deficient cells exhibit aberrant genetic recombination and/or DNA

replication, which result in chromosomal instability and a decreased potential for proliferation (Sharma *et al.*, 2007). The *rqh1* autolog gene is *recQ* in *E. coli* and *SGS1* in *S. cerevisiae.* In humans, the gene is related to the Bloom and Werner syndrome genes. Bloom syndrome is a rare inherited disorder characterized by a high frequency of DNA breaks and rearrangements in an affected person's chromosomes leading to cancer, mental retardation, smaller than average body size abnormal facial features and many other symptoms. Werner syndrome is characterised by accelerated aging, and is also known as the progeroid syndrome.

I wanted to further investigate the roles of Swi1 / Rqh1 in regulating the recombinogenic potential of distinct RFBs.

6.2 Results

6.2.1 Construction and PCR checking of *swi1* mutated *S. pombe* with *RTS1* and *sup3-e* and spacer control.

I wanted to construct *swi1Δ* strains with RFB elements at *ade6* (*RTS1*/*sup3-e* with appropriated controls). I obtained a strain, which was reported to have the *swi1* gene deleted (replaced with the *ura4* marker gene) and proceeded with the experiment. On PCR sequence checking of the strains for deletion of the *swi1* gene, we discovered that the *swi1* gene in the strain we obtained was mutated by *ura4* insertion at 2022 nt (Figure 6.1), so, the full open reading frame was *not* deleted. This strain had previously been tested using DNA 2D gel electrophoresis and found to have lost replication fork barrier activity at *RTS1,* suggesting that it was a *swi1* null mutant (personal communication D. Pryce). Moreover, this strain exhibits similar phenotypes to other independently generated *swi1Δ* mutants. However, one cannot rule out the possibility that this allele codes for a truncated Swi1 protein which retains some function. We will continue to refer to this allele as *swi1::ura4*.

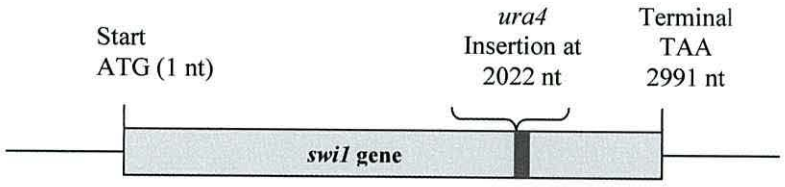


Figure 6.1 Diagrammatic presentation of the mutated *swi1* gene employed in these experiments.

Diagram showing the insertion point of the *ura4* allele at 2022 nt of the *swi1* gene which was confirmed by sequencing. 'A' of the ATG is nt =1 and the last 'A' of the TAA terminal is = 2991 nt of the *swi1* gene (unspliced).

6.2.2 Loss of *swi1* function eliminates the polar RFB activity of *RTS1*.

To determine whether Swi1 is required for RFB-associated inter molecular recombination, I tested plasmid-by-chromosome recombination levels in a Swi1 deficient strain. Similary, I analysed recombination frequency in wild-type *RTS1* integrated strains in Chapter 5 and discovered that polar replication fork barrier *RTS1* generates a polar mitotic inter molecular recombination hot spot resulting in up to a 20-fold increase in recombination frequency at *RTS1(2)* compared to the control (refer Figure 5.4B). Swi1 is known as an important *trans* regulator which controls *RTS1* for its function in mating type switching at the *mat1* locus (Dalgaard and Klar, 2000).

Increased recombination frequency was observed in all *RTS1(1)*, *RTS1(2)* and spacer control *swi1*Δ strains, although the polar RFB-dependent recombination was lost. The recombination frequency in spacer control in the *swi1* mutant was also elevated compared to the equivalent wild-type spacer control. It was reported that Swi1 and Swi3 mutants have have increased mitotic recombination, due to increased chromosomal instability attributed to increased ssDNA found at replication forks (Sommariva *et al.*, 2005). I found that when Swi1 function is lost, the orientation-dependent stimulation of inter molecular recombination by *RTS1* is almost totally lost, consistent with data for intra molecular recombination as demonstrated by Ahn and co-workers (2005). The recombination induced by *RTS1* in orientation 2 is abolished by the *swi1* mutation. Instead, a slight but significant increase in recombination frequency is seen at *RTS1(1)*, which is only about 1.6-fold (Figure 6.2). Thus, loss of Swi1 causes slight increase in recombination at *RTS1(1)* compared to *RTS1(2)*.

We also carried out further 2D gel analysis to check if the lack of recombinogenocity in the non functional Swi1 strains correlated with lack of RFB effect at the *RTS1* element. We found that the *RTS1* element lacked RFB activity in either orientation in the *swi1*Δ; strains (Figure 6.3B). This suggests that Swi1 is required for stimulation of recombination by *RTS1* and it is the normal RFB function of *RTS1* that is responsible for stimulating recombination. These findings are consistent with what has been observed for intra chromatid *RTS1*-mediated recombination (Ahn *et al.*, 2005; Codlin and Dalgaard, 2003).

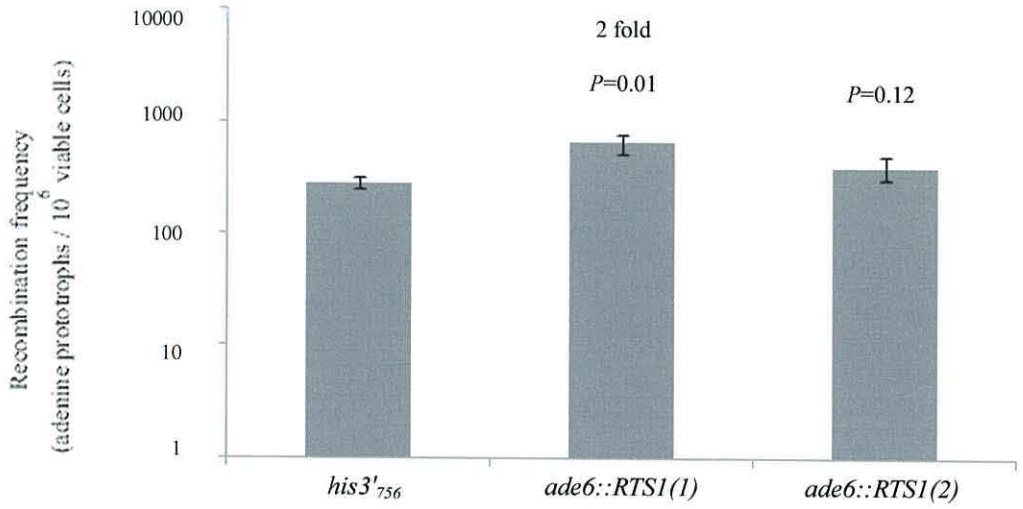


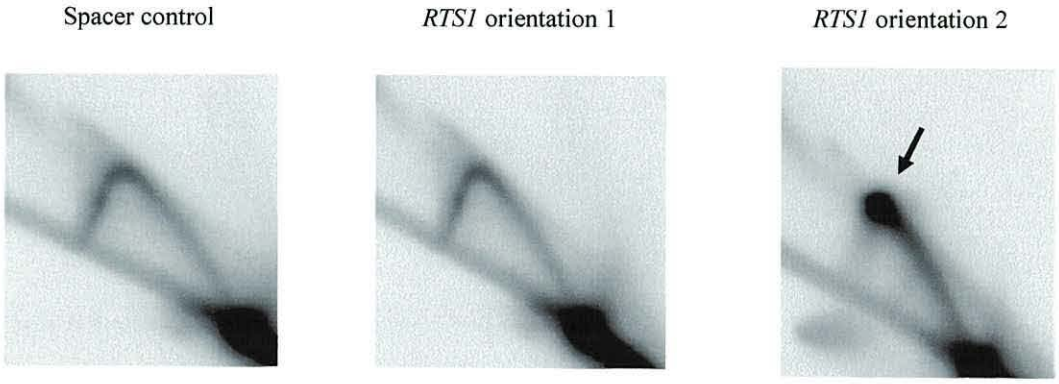
Figure 6.2 Effect of *swi1* mutation in production of recombination in *S. pombe* strains having *RTS1* in different orientations.

The recombination frequency in *RTS1* orientation 1 is slightly but significantly elevated in *swi1* mutated strains (P=0.01; Student's t-test, n ≥ 3 n all cases. This shows that the polar recombination hotspot activity of *RTS1* is dependent upon *swi1* (refer to text).

P values are from pair wise comparison of *RTS1* alleles with spacer control allele using Student's t-test. n ≥3 in all cases. Error bars are standard deviations.

(The data for plasmid (pSRS5)-by-chromosome fluctuation analyses for *RTS1* and recombination frequency calculation in *swi1*$^-$ strains is in supplementary material 7).

A. 2D gel analysis of wild type *RTS1*



B. 2D gel analysis of *RTS1- swi1* mutant

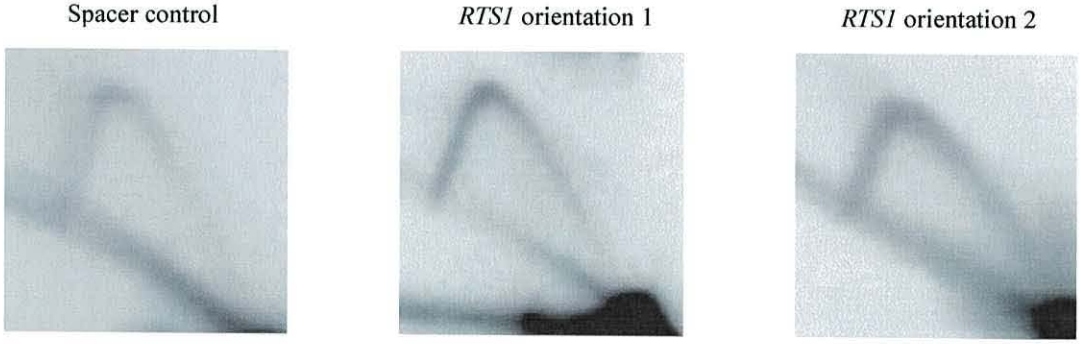


Figure 6.3 The polar RFB activity of *RTS1* is dependent upon *swi1*.

A. Panel shows the 2D gel results carried out for wild type strains with control spacer *his3'*$_{756}$ (left), *RTS1(1)* (middle) and *RTS1(2)* (right). Strains having *RTS1(2)* shows a prominent spot (black arrow) at the arc indicating occurrence of a replication fork barrier.

B. 2D gel image shows that loss of *swi1* function eliminates the polar activity of *RTS1* at *RTS1(2)* (right image). Mean while, the spacer control (left) and *RTS1(1)* (middle) show no difference compared to the corresponding *swi1+* strains.

6.2.3 Loss of *swi1* function results in the de-repression of a DNA replication-associated recombination suppression pathway at *sup3-e.*

Contrary to the findings in the *RTS1* wild-type, the *sup3-e(1)* in the wild type, where the replication fork is expected to collide head-to-head with RNA polymerase III, showed no increase in recombination frequency (Figure 5.6). However, when *swi1* is mutated in *sup3-e* strains, a 100-fold increase was observed for the *sup3-e(1)* element (Student's *t*-test; $P<0.01$) compared to the control. On the other hand *sup3-e(2)* in *swi1* mutants results in only a 2-fold increase (Student's *t*-test; $P=0.03$) in recombination frequency compared to the control (Figure 6.4). It appears that the tRNA gene cassette (*sup3-e*) generates a polar mitotic recombination hotspot in the absence of a fully functional Swi1 protein.

2D gel analysis was carried out on these strains to determine whether the increase in recombination frequency is associated with a more intense RFP activity. I found that both *sup3-e(1)* and *sup3-e(2)* produce a distinct spot on the arc, confirming the presence of a replication pause in both orientations (Figure 6.5). These findings suggest that *swi1* mutation did not contribute to polar activity at *sup3-e.* It is also observed that although the replication pausing is seen in both orientations, the recombination frequency is not increased in both orientations in similar proportions.

I find that the RFB generated by both orientations of *sup3-e* are not lost on loss of Swi1 function, but the loss of Swi1 alters their morphology, becoming more focussed and less spread (Figure 6.5B).

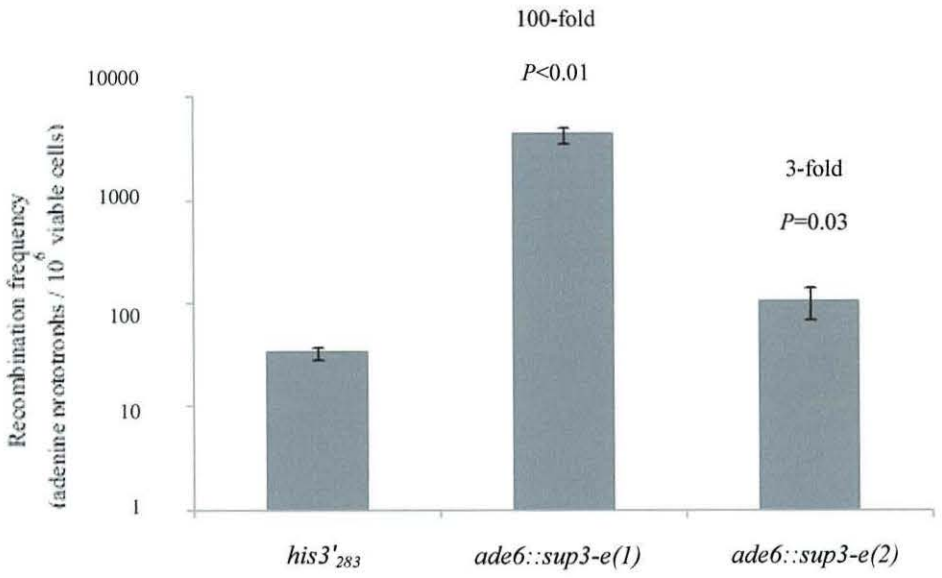


Figure 6.4 Loss of *swi1* function generates a polar recombination hotspot at *sup3-e*.

sup3-e integrated strains show recombination frequency elevated significantly in both orientations. However, the recombination frequency is 100-fold higher in *sup3-e(1)* compared to the control. Where as the *sup3-e(2)* recorded a 2-fold significant increase. This suggests that higher recombination activity is observed in *sup3-e(1)*. ($P<0.01$, $P\geq0.03$; Student's *t*-test); $n\geq3$ in all.

P values are from pair wise comparison of *sup3-e* alleles with spacer control allele using Student's *t*-test. Error bars are standard deviations; $n \geq 3$ in all cases.

(The data for plasmid (pSRS5)-by-chromosome fluctuation analyses for *sup3-e* and recombination frequency calculation in *swi1*$^-$ strains is in supplementary material 8).

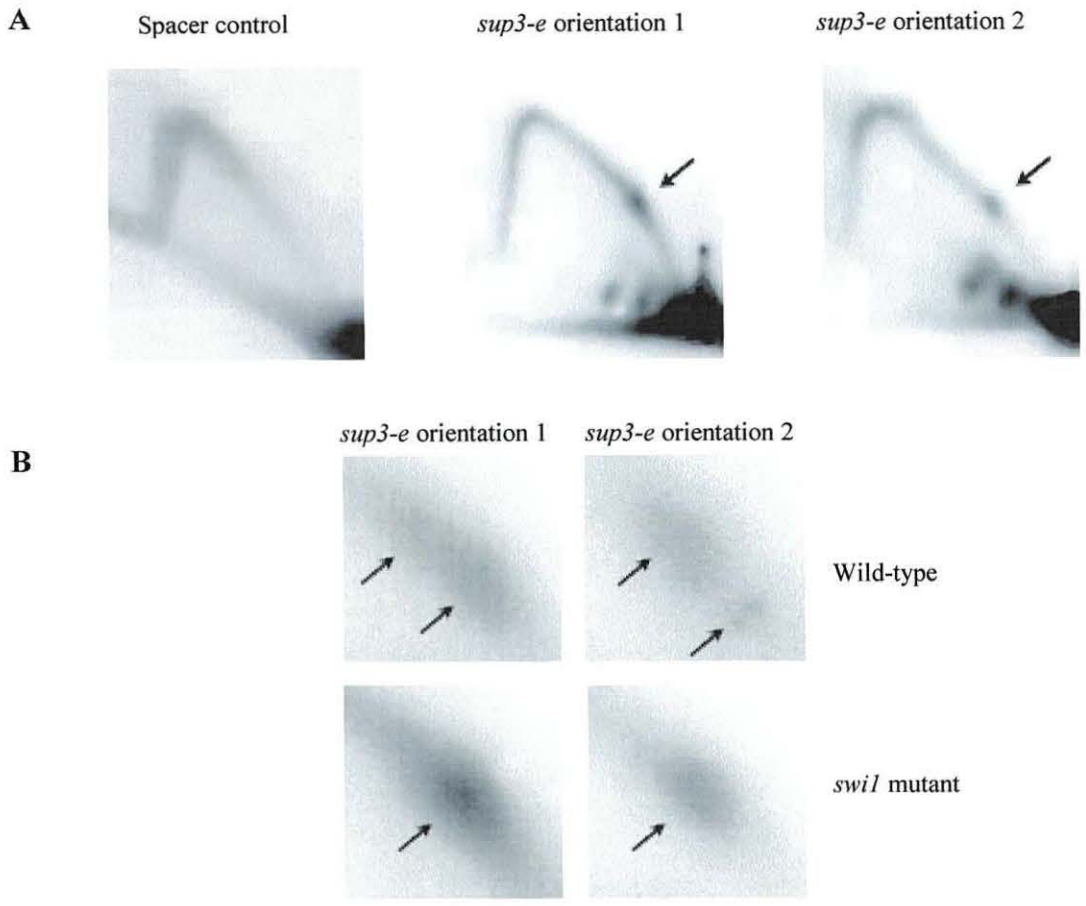


Figure 6.5 No polar activity seen in *sup3-e* with *swi1* mutation.

A. 2D gel images show that RFB activity (black arrow) is present at *sup3-e* in both orientation 1 (middle) and 2 (right) in strains where *swi1* function is loss. This is similar to the wild type strains with *sup3-e* (Figure 5.7).

Recombination occurs in both orientations, but a very high level (100-fold) of recombination frequency was observed in orientation 1 (refer to text).

B. The structure of the RFB created by *sup3-e* differs between the wild-type (top row) and *swi1* mutant (bottom row). In the wild-type two close but distinct RFBs, one weaker and one stronger are seen. The polarity of these two changes with a change in *sup3-e* orientation. In the *swi1* mutant only one RFB is detected (bottom row).

6.2.4 Loss of *swi1* function does not result in a DNA replication-associated increase in ectopic recombination between the *ade6* locus and the *zzz7* allele.

In previous experiments, I analyzed the recombination events in *swi1* mutated strains having *RTS1* or *sup3-e* integrated within the genomic *ade6*, using the plasmid-by-chromosome assay. I found that loss of *swi1* function resulted in up to a 100-fold increase in recombination at *sup3-e(1)*. I wanted to examine if *swi1* mutation / loss of function has any effect on ectopic recombination of DNA using chromosome-by-chromosome recombination assay.

I found that in the wild type strains with *sup3-e* carrying *zzz7* and *zzz15* there was no significant increase in recombination between the control and the *sup3-e* integrated strains (Figure 5.10 A; B), similar to results obtained with the plasmid-by-chromosome assay. When the cells with *sup3-e* carrying *zzz7* have no functional Swi1, there is a general increase of 2- to 8-fold in recombination frequency in all the strains including the control. This increase is significantly higher compared to the wild type (Figure 6.6). However, there is no difference in the recombination frequency between the spacer controls and the *sup3-e(1)* and *sup3-e(2)*, suggesting absence of functional *swi1* appears to increase recombination non-specifically. This is consistent with earlier work by others (Ahn *et al.*, 2005; Sommariva *et al.*, 2005). This shows that in the absence of *swi1* function recombination between *ade6* locus and the *zzz7* position is not significantly stimulated by *sup3-e* in an orientation specific manner.

I did not examine the *sup3-e, swi1* mutated *zzz15* allele carrying strain to see if the position of the *ade6-469* allele affected the result (recombination frequency).

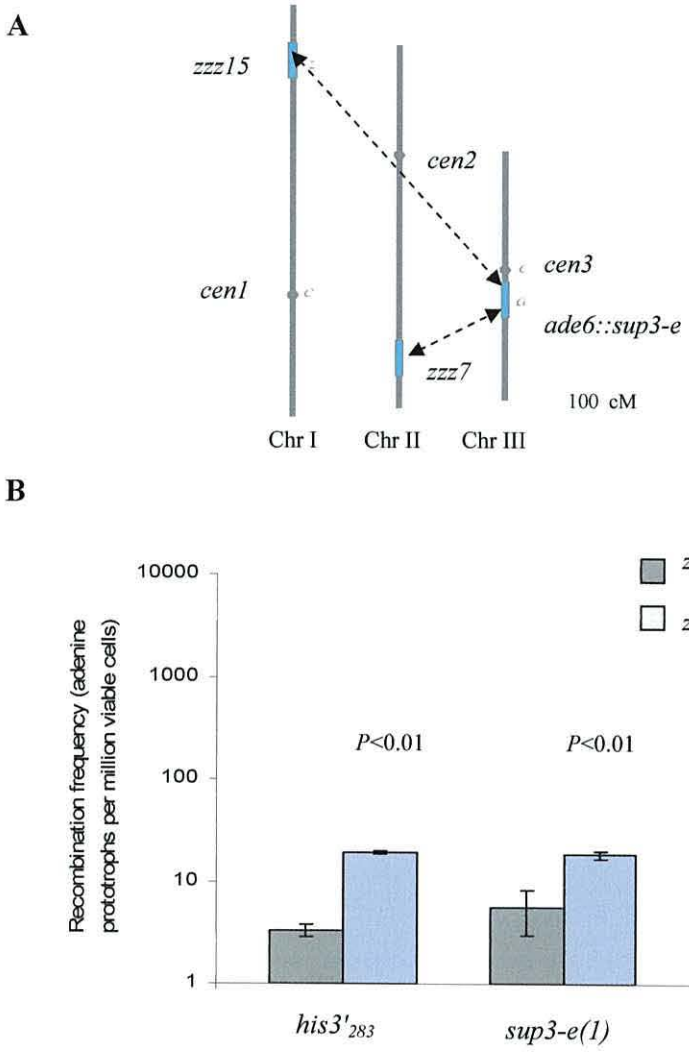


Figure 6.6 Comparison between the recombination frequencies in wild type *sup3-e* integrated strains against the *swi1* mutated strains.

A. A schematic representation of the ectopic recombination assay. The three fission yeast chromosomes are indicated by vertical lines, each with the centromere position approximately marked. The normal genomic *ade6* locus is shown on chromosome III and the two artificially introduced alleles (which will be in distinct strains) are shown on chromosomes I and II (*zzz15* and *zzz7* respectively). Gene conversion events (broken double-headed arrows) between different alleles located at distinct chromosomal positions (*ade6*, *zzz7*, *zzz15*) will generate adenine prototrophs. The frequency of prototroph production represents recombination frequency.

B. *swi1* mutated strains carrying *zzz7* (light grey coloured histograms) produce significantly higher adenine prototrophs in control and both orientations tested compared to the wild type. (P=<0.01,); Student's *t*-test; P values are from pair wise comparison of *sup3-e* alleles with spacer control allele between wild type and *swi1* mutant). However, there is no significant difference between the recombination frequencies between the *swi1* mutated spacer control and *sup3-e* orientation 1 and 2. Error bars are standard deviations. (The data for fluctuation analyses and recombination frequency calculation for the above strains is as in supplementary materials 5 & 9).

6.3 Effect of loss of Rqh1 on replication associated recombination.

6.3.1 Loss of *rqh1* function alters inter-molecular *RTS1*-mediated recombination.

Non-functional Rqh1 could cause replication fork reversal, leading to formation of reverse replication forks (chicken foot) at RFBs. This factor alone could cause an increase in recombination. I found that increased recombination occurs in *RTS1(1)* as well as *RTS1(2)* in *rqh1Δ* strains. The highest recombination increase of 10-fold was observed in the *RTS1(2)*, whereas in the strains having *RTS1(1)* the increase was 2.6-fold (Figure 6.7). This shows that in the absence of Rqh1, *RTS1* causes increased recombination in the RFB active orientation, [*(RTS1(2)*] suggesting that the RFB effect and the lack of Rqh1 together produced amplified elevation of recombination in a polar-specific manner.

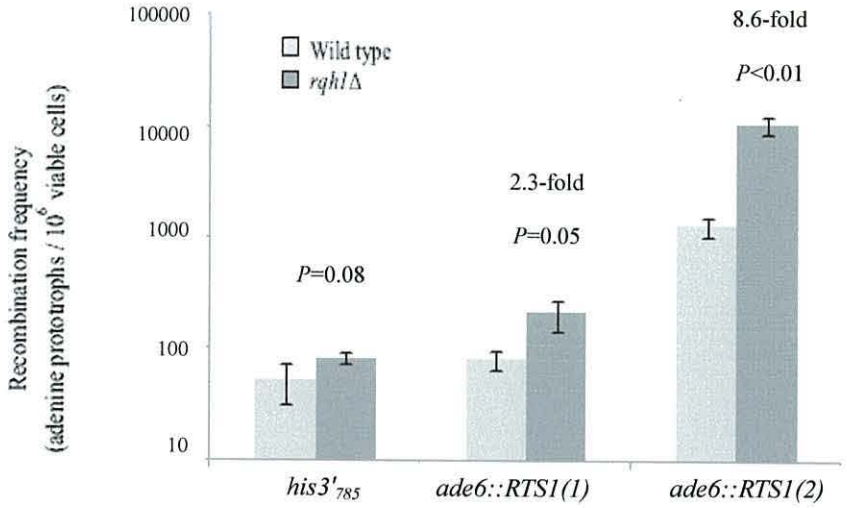


Figure 6.7 The recombination frequency in *RTS1* integrated *ade6* that are *rqh1Δ* mutants is significantly higher compared to the wild type.

Recombination frequency is significantly elevated in *rqh1Δ* strains carrying *RTS1(1)* and *RTS1*(2). However, the *RTS1(2)* has 8.6-fold higher recombination compared to the wild-type ($P<0.01$ Student's *t*-test). *RTS1(1)* has 2.3-fold in increase ($P=0.03$ Student's *t*-test). These finding suggests in absence of *rqh1* the recombination frequency is increased, particularly to the *RTS1* orientation 2, which causes the replication fork barrier in wild-type.

P values are from pair wise comparison of *RTS1* wild type with *rqh1Δ* strains for each element. Error bars are standard deviations.
(The data for plasmid (pSRS5)-by-chromosome fluctuation analyses for *RTS1* and recombination frequency calculation in *rqh*$^-$ strains is as in supplementary materials 1 & 10).

6.3.2 Loss of *rqh1* function does not de-regulate the DNA replication-associated recombination suppression pathway in strains with *sup3-e*.

The presence of *sup3-e* in the genomic *ade6* gene does not elevate the recombination frequency. Rqh1 functions in decatenation of the dsDNA ahead of the replication fork thus preventing replication fork reversal; a phenomena which could possibly lead to increased recombination activity. However, in the strains having the *sup3-e* without Rqh1, there is no difference in the recombination frequency seen, when compared with the wild type strain (Figure 6.8), indicating that Rqh1 is not suppressing recombination at RFB mediated by these tRNA genes.

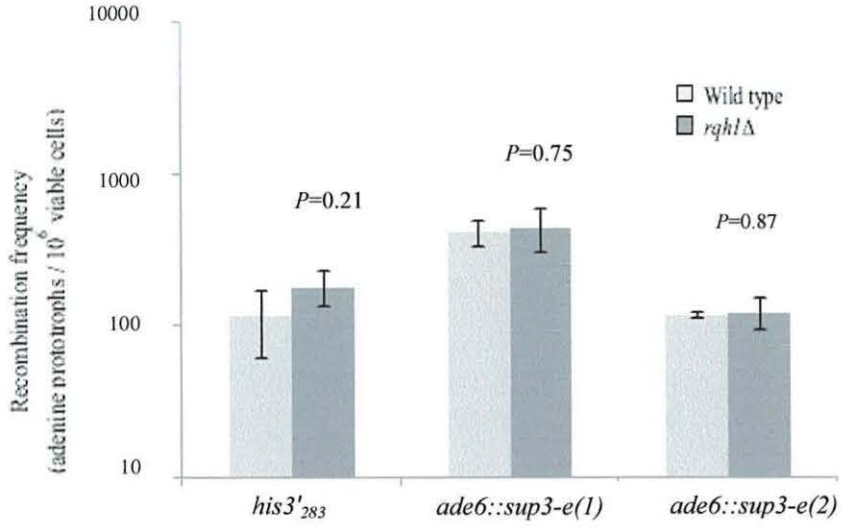


Figure: 6.8 The *rqh1Δ* strains with *sup3-e* integration (in both orientations) show no increased recombination.

No significant increase in recombination is seen in *rqh1Δ* strains with *sup3-e* compared to wild-type (in both orientations 1 and 2). (P=0.21, P=0.75; P=0.87; Student's t-test). P values are from pair-wise comparison of *sup3-e* alleles with wild type strains. Error bars are standard deviations. n ≥3 in all.

(The data for plasmid (pSRS5)-by-chromosome fluctuation analyses for *sup3-e* and recombination frequency calculation in *rqh*$^-$ strains is as in supplemenatary materials 2 & 11)

6.4 Discussion

6.4.1 RFB activity and recombination on loss of *swi1* function

Swi1 and Swi3 also are known to arrest replication forks in the rDNA locus (Krings and Bastia, 2004). Fork pausing is required to initiate a recombination event that switches mating type in *S. pombe*. Swi1 and Swi3 proteins are *trans* factors shown to pause or participate in replication fork arrest at the site of imprinting at the *mat1* mating type switching locus and by terminating replication at *RTS1* in *S. pombe* (Dalgaard and Klar, 2000; Codlin and Dalgaard, 2003). Swi1 and Swi3 are referred to as the replication fork protection complex that coordinate leading and lagging-strand synthesis and stabilize stalled replication forks in fission yeast. In the recombination reporter systems, *swi1* functions to stimulate recombination at *RTS1*.

I find that *S. pombe* strains with non-functional *swi1*, having *RTS1* incorporated in the genomic *ade6,* had neither RFB nor increased recombination at the *RTS1(2)* compared to the control strain, suggesting that Swi1 is vital for RFB activity to occur at *RTS1(2)*, which correlates with an increase in recombination. In the wild type strain I found very high recombination and a strong pause at the *RTS1(2)*. This is in agreement with previous findings (Ahn *et al.*, 2005; Lambert *et al.*, 2005b) which demonstrated that stalled replication forks are targeted by recombination proteins and that this leads to recombination events and gross chromosomal rearrangements.

Ahn and co-workers (2005) and Lambert and co-workers (2005b) used *RTS1* to demonstrate this and proved that RFBs can also become a recombination hotspot. In our findings the *RTS1* in wild type is recombinogenic but not when *swi1* is dysfunctional. Thus, I conclude that *swi1* is essential for the *RTS1* to function as a RFB and have recombinogenic potential. My results show that when there is no replication fork pause, then there is no recombination. Thus, the *RTS1* RFB function appears to play an important role in recombination.

In *sup3-e* incorporated strains I find the opposite results. *swi1* mutants are very recombinogenic producing up to 100-fold higher recombination at *sup3-e(1)* and 2-fold to the *sup3-e(2)* compared control (Figure 6.4). In these strains there was pausing of

replication (seen using 2D gel), in both wild type as well as *swi1* mutant (Figure 5.8; Figure 6.5). On careful examination it is found that the RFBs seen in *sup3-e* on loss of Swi1 functions has altered morphology, becoming more focused and less spread. However, orientation-dependent recombination activity was seen in *sup3-e(1)* on *swi1* mutation. In the wild-type two close but distinct RFBs, one weaker and one stronger were seen (Figure 6.5B). These results suggest that at *sup3-e*, Swi1 is not essential for pausing but removal of *swi1* function increases the recombinogenic potential of the element in an orientation-dependent fashion. Thus, we can say that Swi1 functions in a different manner at different RFBs. These findings are consistent with studies by Krings and Bastia, where they reported that Swi1 and Swi3 can function outside the mating type region in fission yeast with some site specificity and they act differently at different RFBs (Krings and Bastia, 2004). More over, Sommariva and co-workers (Sommariva *et al.*, 2005) demonstrated that loss of *swi1* function increases intra chromatid recombination in the absence of a defined RFB.

However, using *zzz7* strains, I found that *swi1* mutation / loss of function does not result in a DNA replication-associated increase in ectopic recombination between the *ade6* locus and the *zzz7* allele. Although there was a general elevation in frequency of recombination observed in both orientations of *sup3-e* as well as in controls (insert with *his3'*$_{283}$), compared to the wild-type strains (*swi*$^{+}$ strains) (see Figure 6.6), there was no significant difference between them, suggesting that absence of functional *swi1* appears to increase recombination non-specifically. This is consistent with earlier work by others (Ahn *et al.*, 2005; Sommariva *et al.*, 2005). This shows that in the absence of *swi1* function recombination between *ade6* locus and the *zzz7* position is not significantly stimulated by *sup3-e* in an orientation specific manner. I did not attempt to examine if similar results occured using *zzz15* strains to verify if this phenomena is associated with the location of the *ade6-469* allele.

In summary, I find that loss of Swi1 function alters the RFBs generated by both *RTS1* and *sup3-e*. The strong orientation-dependent RFB generated by *RTS1* in orientation 2 is lost in the *swi1Δ* background. Whereas, the RFBs generated by both orientations of *sup3-e* are not lost on loss of Swi1 function but the RFB morphology is altered on loss of Swi1

function. I find that Swi1, the TIMELESS homolog and component of the repliosome progression complex, functions differently at distinct RFBs. It both stimulates and suppresses replication fork barrier-associated mitotic recombination in a site-specific fashion.

6.4.2 Recombination in *rqh1Δ* strains

A similar study using *rqh1Δ* strains was carried out using strains of *S. pombe* with *RTS1* and *sup3-e* integrated in the *ade6* gene. Again, I find different results for the two RFBs examined. I find that *rqh1Δ* strains with *RTS1(1)* and *RTS1(2)* produced 2.6-fold and 10-fold increase in recombination frequency respectively. Rqh1 with Topoisomerase I is essential for decatenation of dsDNA a head of the replication fork. Absence of Rqh1 is known to cause fork reversal, which has potential for increased recombination activity. However, *sup3-e* integrated strains having *rqh1Δ* did not show any increase in recombinant compared to wild type. *sup3-e* does not function as a recombinogenic RFB even in *rqh1Δ* strains. It has been suggested that in the absence of Rqh1, stalled/blocked replication forks might be more susceptible to being cleaved by endonucleases like Mus81-EmeI leading to increased recombination events (Doe *et al.*, 2002)

6.5 Summary of the main findings

1. Loss of *swi1* eliminates the polar RFB and recombination activity of *RTS1*.
2. Loss of *swi1* activity confers polar mitotic recombination hotspot activity at the tRNA [*sup3-e(1)*] but not in *zzz7* alleles in ectopic recombination.
3. Loss of *swi1* did not eliminate the RFB activity of tRNA but alters the morphology of the RFB.
4. Loss of Rqh1 function increases recombination in *RTS1* but no increase was seen in *sup3-e*.

CHAPTER 7

FINAL DISCUSSION

7.1 Introduction

DNA replication processes are complex and important in the preservation and segregation of genomes. Chromosomes do not have uniform levels of stability along their length. There are many obstacles such as fragile sites and replication slow zones that are susceptible to initiating genetic changes. However, one uniform feature of genomes which directly impringes upon their stability is that they must be replicated correctly to ensure there is an equal distribution of genetic material during cell division. To ensure this, the replication machinery must be capable of responding to different features of the genomic landscape.

In this research project, I studied *cis* and *trans* factors that could cause chromosomal / genetic instability; factors which could be important in causation of cancers and other genetic diseases. I looked at Translin and its partner protein Trax, as factors that could be associated with genetic instability and genomic rearrangements. There are many studies that associate these proteins with genetic instability and translocation leading to genomic rearrangements.

My findings did not positively confirm the association of Translin or Trax protein with DNA damage repair, cell proliferation or GT repeats instability. It is important for further research to be carried out since these proteins are highly conserved from fission yeasts to mammals and some role in DNA damage recovery has been seen in *traxΔrhp14Δ, traxΔrad32Δ* and *translinΔ rhp14Δ; translinΔrad32Δ* double mutants (Jaendling personal communication). This suggests that the function of *trax* and *translin* genes may not be in a primary DNA repair pathway.

The role played by Translin protein in regulation of Trax protein is also conserved from fission yeast to mammals, suggesting an existence of an important unknown function / functions that requires the activity of these proteins.

A growing number of programmed RFBs or pause sites have been identified (Rothstein *et al.*, 2000). It is believed that RFBs function to ensure successful merging of opposing replication forks and promoting avoidance of collision between replication forks and transcription complexes (Reviewed in Labib & Hodgson 2007). RFBs have been suggested to be associated with recombination, which may also lead to genetic instability (Lambert *et al.*, 2007). I also demonstrated that Swi1, the fission yeast homologue for TIMELESS functions differently to regulate genome stability at RFBs, switching between a stimulatory and inhibitory role for replication-associated recombination in a site-specific fashion. I demonstrated that replication perturbation is not the only factor that contributes to DNA replication-associated fragile sites.

7.2 Translin and Trax have conserved function in lower eukaryotes

Trax and Translin are highly conserved suggesting that they may play an important biological role which provides a selective advantage. These proteins are seen in fission yeast, *S. pombe* (but not in budding yeast) and in higher eukaryotes.

We found that Translin is required for stable expression of Trax in *S. pombe*. This function is also conserved in MEFs and *Drosophila* genes (Chennathukuzhi *et al.*, 2003a; Clauβen *et al.*, 2006). It has been suggested that the regulation of Trax levels by Translin is by direct Translin-Trax interaction controlling the levels of ubiquitin-mediated Trax proteolysis (Yang *et al.*, 2004a; Clauβen *et al.*, 2006). In MEFs chemical inhibition of the proteasome results in a full restoration of Trax levels to levels seen in Translin-proficient cells (Yang *et al.*, 2004a). Consistent with this finding, Jaendling and co-workers (2008) reported that by mutating an *S. pombe* proteasome sub-unit, some Trax was restored. However, Trax levels were not fully restored. This might be due to the fact that the *mts1* conditional mutant used was functionally leaky and residual proteasome activity is sufficient to mediate some Trax degradation in absence of Translin. Alternatively, it is possible that there is a second role for Translin in positively regulating the translation of *trax* mRNA. It seems that this role of Translin in mediating Trax levels is important both in unicellular organisms and metazoans, thus I can postulate that the conserved function does not relate to tissue-specific biological processes in metazoans. This suggests that

Trax and Translin can confer some advantage to unicellular eukaryotes but the nature of this remains unknown.

In this study, I over expressed *trax*$^{+}$ mRNA, but this did not result in a measurable increase in the Trax protein. This suggests that the levels of Trax protein in the cells are determined by post transcriptional regulation, consistent with the proposal that Translin is required for efficient translation of the *trax*$^{+}$ mRNA. If Translin is responsible for Trax stabilization, then this regulation is more complex as co-over expression of Translin in the presence of higher levels of *trax*$^{+}$ mRNA did not increase the measurable Trax in the cell. It might be possible that I have failed to clone untranslated regions (UTRs) associated with the *trax*$^{+}$ orf which are essential for translation and that the control of Trax translation is regulated by as yet unidentified, UTR-dependent mechanisms, possibly requiring Translin. This needs to be explored further.

7.3 Biological function of Translin and Trax

I could not find any fundamental role played by Translin and Trax in the maintenance of genome integrity, meiotic/mitotic recombination and the cell growth process. There could be two ways of explaining this; one could be that, neither gene plays a prominent role in any biological pathway important to laboratory cultured cells; secondly, the pathways these proteins function in may be redundant and other pathways can substitute for Trax and Translin function or they have no function; but their conservation suggests that this latter proposal is not the case.

It has been suggested that neurological defects in mice and *Drosophila* defective in Translin (Chennathukuzhi *et al.*, 2003a; Stein *et al.*, 2006; Suseendranathan *et al.*, 2007) and the binding of Translin to neuronal mRNA, implicates Translin function in neuronal mRNA metabolism; moreover, it has been postulated that the conservation of function is important as loss of neural function would confer a serious selective disadvantage to flies and mammals. However, this does not account for conservation of sequence and function in the fission yeast. It may be that in *S. pombe* Trax and Translin may function in the regulation of other subsets of mRNAs and the higher eukaryotes have evolved tissue specificity for thus function. If *S. pombe* Trax and Translin are involved in mRNA

metabolism, loss of their function appears to have resulted in little overt phenotype in laboratory cultured strains; however, it might be the case that they function in a condition-dependent fashion which has yet to be evaluated. The finding by Laufman and co-workers (2005), that Translin has greater affinity for RNA repeat sequences than DNA supports a role in RNA metabolism.

No evidence of Trax and Translin involvement in DNA damage recovery was noted in our study. In HeLa cells mild increases in nuclear Translin were observed in response to the chemotherapeutic agents mitomycin C and cisplastin (Kasai *et al.*, 1997), but we demonstrated that in *S. pombe*, *traxΔ* and *translinΔ* (Jaendling personal communication) cells are as resistant as the wild-type cells, indicating that any increase in nuclear amount of Translin is not essential for recovery from the DNA damages these agents generate. This finding is also consistent with later findings that both MEFs and *Drosophila* embryo defective in Translin exhibit no increase in sensitivity to some DNA damaging agents relative to their Translin- proficient controls (Yang *et al.*, 2004a; Clauβen *et al.*, 2006). Furthermore, it has been suggested that because Trax interacts with C1D, a protein required for mitotic recombination, that Trax might have a role in the regulation of mitotic recombination (Erdemir *et al.*, 2002a; Erdemir *et al.*, 2002b); however we did not have any evidence to substantiate that proposal.

Further studies by other member of this group have found that double deletions of *trax* or *translin* with other DNA repair genes, *rhp14* and *rad32,* have shown some sensitivity to DNA damage recovery suggesting, Trax and Translin may function in a redundant repair pathway. In a recent report it was demonstrated that Translin-deficiency resulted in delayed hematopoietic regeneration after exposure to sub-lethal dose of ionizing radiation (Fukuda *et al.*, 2008). This suggests that Translin might have other pathways or methods of protecting cells from damage or help in DNA damage recovery that need to be explored (Fukuda *et al.*, 2008).

7.4 Role of RFBs in genome rearrangements.

Genomic rearrangement/instability is associated with pathological disorders and in humans it is often associated with premature aging, predisposition to cancer and genetic/inherited diseases and yet is crucial for genetic variation and evolution (Reviewed in Aguilera and Gomez-Gonzales, 2008). Replication arrests are associated with genome rearrangements, which result from either homologous or non-homologous recombination (Michel, 2000). Recombination is intimately linked to replication in eukaryotic cells as recombination proteins are recruited to collapsed replication forks, when DNA lesions/breaks cause absolute blockages for DNA replication (Bierne and Michel, 1994).

Genomic instability leading to rearrangements is caused by DNA breaks (Aguilera and Gomez-Gonzales, 2008). Replication fork breakage and recombination leading to genomic change has been reported to be associated with specific sites in the genome. Mammalian cells have fragile sites on chromosomes that are prone to breakage when DNA replication is disrupted (Richards, 2001; Durkin and Glover, 2007). In budding yeast removal of Mec1, the homolog of *S. pombe* Rad3 and mammalian ATR and ATM, leads to genome wide fork stalling followed by chromosome breakage at gene coded specific regions called the replication slow zones (RSZ) (Cha and Kleckner, 2002). Stalled replication forks by replication fork barrier were also shown to lead to elevated intrachromosomal and ectopic recombination promoting site-specific gross chromosomal rearrangements (Lambert *et al.*, 2005b; Admire *et al.*, 2006).

Sites that cause replication fork pausing have been identified in many genomes and are known as replication fork-blocking sites. Natural RFBs help coordinate certain events with replication. These include replication termination, *Ter* sites in *E. coli*, (Hidaka *et al.*, 1989), replication fork-blocking sites at rRNA gene repeats (rDNA) from yeast *S. cerevisiae* to human cells (Kobayashi, 2003; Krings and Bastia, 2004) and the *RTS1*, in fission yeast mating type switching (Dalgaard and Klar, 2000). Although it appears that these sites are important for the regulation of replication termination and prevent collisions between the replication and transcription machinery, there is no definitive evidence as to their precise function (Rothstein *et al.*, 2000). However, recent studies

suggest that sites with tRNA genes have recombinogenic properties that contribute to genomic instability (Admire *et al.*, 2006).

7. 5 *RTS1* and *sup3-e* posses different recombinogenic characteristics.

The *RTS1* is a well studied RFB. It has a polar replication fork stalling ability, which require *trans*-acting factors Swi1, Swi3, Swi7 (DNA polymerase α), Rtf1(replication termination factor 1) and Rtf2 (replication termination factor 2) (Codlin and Dalgaard, 2003). In this study, I find that the *RTS1* acts as a RFB when artificially inserted in the *ade6,* in chromosome III of *S. pombe.* Using this system I could study the effect RFB has on genomic stability. Lambert and co-workers (2005b) reported that *RTS1*-induced stalled forks and increased level of recombination (Lambert *et al.*, 2005b). I also find that in wild type strains the *RTS1* integrated in orientation opposing the replication fork [*RTS1(2)*] causes replication pause, which is associated high recombination, suggesting that the *RTS1*, can induce a recombination hotspot. It is not known if all RFBs function like the *RTS1*.

I see opposite results in the RFB generated by tRNA genes *(sup3-e),* when similar tests were carried out. Although fork pausing was observed a corresponding increase in recombination was not seen suggesting that pausing at *sup3-e* does not induce a recombination hotspot, even for the orientation when RNA polymerase III is opposing the replisome. Previous studies have shown that DNA-replication-associated fragile sites can contain tRNA genes, implicating these elements in the generation of recombinogenic lesion (Admire *et al.*, 2006). Deshpande and Newlon, (1996) reported that tRNA genes arrest replication forks causing RFP in a polar manner by stalling replication forks only when they oppose the direction of tRNA transcription. Hence there is the possibility of recombination activity being initiated. tRNA genes appear to use gene conversion mechanism to maintain their genome wide sequence uniformity, suggesting they have recombination initiating potential (Amstutz *et al.,* 1985; Kohli *et al.*, 1984).

Previously, tRNA genes are reported to be widely distributed throughout the genome and appear to be in multicopies that could possibly have importance in maintenance of genomic stability. In the fission yeast centromeric regions, there are a, large number of

symmetrically arranged tRNA coding sequences, located in the inner inverted regions and which are thought to be essential for centromere function (Takahashi *et al.*, 1992). This high-density distribution of tRNA genes in the centromere regions which are considered transcriptionally inactive perhaps has a role in the maintenance of genomic stability (Takahashi *et al.*, 1991). Our findings points towards the importance of the tRNA genes as factors for maintenance of genomic stability, especially in centromeric regions, which are transcriptionally inactive.

In summary, these findings indicate that *RTS1* and *sup3-e* as RFBs have very different recombinogenic potential, with very high levels of recombination seen in the *RTS1* element compared to no stimulation for recombination in *sup3*-e. Thus, I can conclude that different RFBs have different recombinogenic potentials.

7.6 Swi1 as a regulator of the genomic fragility of a genomic locus

Here I have demonstrated fundamentally opposing activities of the repliosome progression complex (RPC) in response to two distinct RFBs. My result also suggests that tRNA genes lead to increased levels of recombination when the RPC is compromised. RFB activity was observed in both orientations of *sup3-e* in Swi1 mutants; but higher levels of recombinants were seen in *sup3-e* orientation 1 compared to orientation 2. In the wild type strains no significant increase in recombination frequency was observed although RFB activity was detectable. This indicates that the tRNA gene cassette generates a non-polar RFB in both *swi1* mutant and wild type.

Previously, it has been shown that tRNA genes can generate strong polar RFBs when in an orientation predominantly opposing the replication fork (Deshpande and Newlon, 1996) and tRNA genes have been implicated in driving gross chromosomal rearrangements in a DNA replication-dependent fashion (Admire *et al.*, 2006). However, in the case of *sup3-e* I did not observe any stimulation of recombination in the despite a clearly measurable RFB activity. Given that RNA pol III is responsible for the transcription of many different species of RNA from the genome (Dieci *et al.*, 2007), my findings are consistent with a model that proposes that pol III does not generally present a significant recombination initiating factor during S-phase.

However, in *RTS1*, when Swi1 is not functional, no recombination or pause of the replication fork was observed, suggesting Swi1 is needed for the replication fork pausing and to activate recombination at the *RTS1* induced replication pause site. This observation is in stark contrast to the high levels of recombination observed in the wild type. These findings are in agreement with previous studies where Swi1 and Swi3 were shown to be essential for *RTS1* function as a RFB (Dalgaard and Klar, 2000). Therefore, in absence of *swi1*, the *RTS1* does not act as a RFB thus, no recombination is initiated.

RTS1 is a specific genetic element which serves to prevent DNA replication through a particular region within the *S. pombe* mating type locus from one direction. There is no known need for *RTS1* to initiate recombination at this site and so it is thought that the recombinogenic nature of an ectopically sited *RTS1* is not functionally relevant at the *mat* locus. The fact that Swi1 functions very differently at tRNA genes, suggests that the more wide-spread function of Swi1 is to suppress unwanted recombination at RFBs throughout the genome and that the function at *RTS1* is a unique specialist activity which has evolved to mediate events at particular sites. Alternatively, it could be due to the fact that *RTS1* generates such strong RFBs that a 'tipping point' has been reached, beyond which Swi1 no longer has the ability to maintain replication fork stability. A more general role for Swi1 in suppressing recombination at milder RFBs is supported by the fact that elevated intra molecular recombination is observed in the absence of specified RFBs in a *swi1Δ* mutant (Sommariva *et al.*, 2005).

Consistent with these findings, in *S. cerevisiae* Tof1, the homolog of Swi1 has been shown to be required to maintain an intact/stable repliosome during stalling of replication fork (Katao *et al.*, 2003; Calzada *et al.*, 2005). In fission yeast, *swi1* and *swi3* mutant strains show increased levels of genomic instability and evidence of chromosome fragmentation, increased levels of single-stranded DNA, increased recombination, and instability of replication forks stalled in the presence of hydroxyurea was observed, consistent with the possibility that the replication process is affected in these mutants (Sommariva *et al.*, 2005).

Here, I clearly demonstrated, opposing activity of the RPC in response to two distinct RFB. In *RTS1*, the RPC component Swi1 is absolutely required for RFB activity and the associated recombination. Meanwhile, for tRNA genes, *sup3-e*, Swi1 functions either to prevent the RFB being processed into recombination initiating lesions (stabilization of replication forks) or it serves to prevent any such lesions being processed into recombination products. It is unknown how and what function of Swi1 makes the distinction between different RFB, but this protein has been implicated in maintenance of replication fork stability and S phase checkpoint function. The fact that recombination is increased at *sup3-e* on loss of Swi1 function is similar to the observation that loss of Mec1/ATR checkpoint activity increases instability at fragile sites, might suggest that there is a link to Swi1 checkpoint functions (Cha and Kleckner, 2002).

7.7 RFB-mediated inter chromosomal recombination outcome is governed by region-specific factors.

Further to this, my work also suggests that not only the type of RFB, but the location of the RFBs in the genome also plays an important role in the maintenance of genomic stability. Chromosomes are organised in the nucleus into defined chromosomal territories which may influence the function of a specific region. This will create defined regional juxtapositioning of different chromosomal regions. This in turn will influence whether or not different regions of short homology come into proximity and restrict partner availability for recombination.

Here, we demonstrate higher recombination in *ade6-469* alleles at *zzz15,* (located in chromosome I) compared to *zzzz7* (located in chromosome II) in *RTS1* integrated strains. However, no difference in recombination between *ade6-469* alleles at *zzz15* or *zzz7* was observed in strains having *sup3-e*. Similar results were also observed in recombination between strains carrying *zzz7* having Swi1 mutation. These outcomes suggest that partner selection for the recombination mediated by recombinogenic RFBs is governed in a region-specific fashion. This further indicates that the degree to which aberrant inter non-homologous chromosome recombination can occur during S phase must be governed by factors regulating normal nuclear architecture. If these factors become deregulated then this could potentially increase the likely hood that harmful recombination events, such as

oncogenic exchanges could occur due to perturbations of DNA replication. To what extent the regional separation restricts inter non-homologue recombination remains unknown, but this system provides a powerful tool to explore this fundamental question further and probe the factors regulating regional governance which prevent unprogrammed ectopic recombination between non-homologous chromosomes.

7.8 Loss of *Rqh1* does not result in stimulation of RFB-dependent recombination for tRNA genes

It has been clearly shown that the *RTS1* RFB generates lesions in the DNA which can serve to initiate recombination. This was demonstrated as a onesided DSB (Ahn *et al.*, 2005) In *rqh1Δ* strains, one sided DSBs were detectable at the *RTS1* element and this is concomitant with hyper RFB-dependent intra-chromatid recombination. Taken together these findings suggest that in the absence of Rqh1 the recombination initiating lesions are generated at a higher frequency or become more stable. I observed a similar hyper recombination for the *RTS1* element in the absence of *rqh1*.

However, it is uncertain if the RFB generated by the tRNA genes (*sup3-e*) generates a similar lesion, which is subsequently prevented from generating mature recombination products or whether no lesion is being formed at this RFB. I find that the loss of Rqh1 does not stimulate RFB-dependent recombination at tRNA genes (*sup3-e*). It is possible that the recombinogenic lesions are not being generated, although this remains to be proven. From these findings we infer that Rqh1 is not required to suppress unprogrammed recombination at the majority of RFBs in the genome and that suppression of RFB-associated recombination is not a global function of Rqh1.

In conclusion, my findings suggest that not all RFBs can serve as mitotic recombination hotspots, although previously it has been reported that RFBs can serve as a mitotic recombination hotspots. Here, I demonstrate that this is not a universal feature of all eukaryotic RFBs. I propose that RFBs generated widely throughout the genome may not serve as mitotic recombination hotspots and that limited RFB activity is not sufficient to create a fragile site capable of mediating recombination events under normal cellular conditions. Natural fragile sites in budding yeast genome are complex and are not

generated by a simple RFB element alone. Furthermore, I demonstrate that the recombination-mediated outcomes from RFB-associated recombination are governed by genome regional organization, which is likely to be intimately linked to features controlling nuclear architecture.

Lastly, I demonstrated that a key component of the RPC, Swi1 (*Sc*Tof1/*Hs*TIMELESS), functions differently to regulate the outcome of an encounter between a RFB and the repliosome in a site-specific fashion. These finding expose a new level of complexity in the study of chromosomal fragile sites associated with DNA replication perturbation.

7.9 Summary of findings

In this thesis, I made the following main findings;

a. Translin and Trax have no primary role in the regulation of cell viability, recovery from genotoxic damages or GT repeat stability. Moreover, levels of Trax are post transcriptionally regulated by Translin, a function which is conserved in higher eukaryotes.

b. *RTS1* and *sup3-e* RFBs have a different recombination potential. In wild type strains, *RTS1* was recombinogenic in a polar specific manner and *sup3-e* was not recombinogenic although non-polar RFB activity was present.

c. Ectopic recombination between alleles depends on the location in the genome. *RTS1* was highly recombinogenic with *zzz15* alleles compared to *zzz7*. *sup3-e* was not recombinogenic at both *zzz7* and *zzz15* alleles.

d. *swi1* and *rqh1* mutations affect the *RTS1* and the *sup3-e* differently. *RTS1* replication fork pausing and recombinogenic potential is dependent on *swi1*. The non-polar *sup3-e* pausing was not affected by *swi1* but polar reombinogenic potential was shown by *sup3-e*. Similarly, no increased in ectopic recombination with *zzz7* allele in the absence of *swi1* function was observed.

7.10 Future studies

Trax and Translin do not primarily contribute to genomic stability or repair in *S. pombe*. However, multiple mutations involving other checkpoint proteins have shown that they have some role in cell recovery after DNA damage. Both these proteins are conserved from *S. pombe* to higher eukaryotes, suggesting crucial biological functions in these organisms. Recent studies also have associated Translin in cell recovery following sub lethal levels of radiation (Fukuda *et al.*, 2008). Considering that these proteins are conserved and have been associated with various diseases, more research needs to be carried out to establish their possible role and function in maintenance of genomic stability; specifically future work should focus on elucidating which DNA damage recovery pathway these proteins are associated with. .

RFBs function as both sites for recombination hotspots and stable sites without any recombination activity. RPC components such as Swi1 have been shown to regulate functions of RFBs in different ways depending on the RFB. There are various other RFBs and other groups of tRNA genes that are associated with RFB function. The genomic location and species of tRNA genes need to be identified for their roles in causing genomic instability.

LIST OF FILES IN ATTACHED CD

1. Supplemtary material 1
Data for plasmid (pSRS5)-by-chromosome fluctuation analyses for *RTS1* and recombination frequency calculation in *swi1*$^{+}$ strains.

2. Supplemtary material 2
Data for plasmid (pSRS5)-by-chromosome fluctuation analyses for *sup3-e* and recombination frequency calculation in *swi1*$^{+}$ strains

3. Supplemtary material 3
Data for chromosome-by-chromosome (*zzz7*) fluctuation analyses for *RTS1* and recombination frequency calculation in *swi1*$^{+}$ strains

4. Supplemtary material 4
Data for chromosome-by-chromosome (*zzz15*) fluctuation analyses for *RTS1* and recombination frequency calculation in *swi1*$^{+}$ strains

5. Supplemtary material 5
Data for chromosome-by-chromosome (*zzz7*) fluctuation analyses for *sup3-e* and recombination frequency calculation in *swi1*$^{+}$ strains

6. Supplemtary material 6
Data for chromosome-by-chromosome (*zzz15*) fluctuation analyses for *sup3-e* and recombination frequency calculation in *swi1*$^{+}$ strains

7. Supplemtary material 7
Data for plasmid (pSRS5)-by-chromosome fluctuation analyses for *RTS1* and recombination frequency calculation in *swi1*$^{-}$ strains

8. Supplemtary material 8
Data for plasmid (pSRS5)-by-chromosome fluctuation analyses for *sup3-e* and recombination frequency calculation in *swi1*$^{-}$ strains

9. Supplemtary material 9
Data for chromosome -by-chromosome (*zzz7*) fluctuation analyses for *sup3-e* and recombination frequency calculation in *swi1*$^{-}$ strains

10. Supplemtary material 10
Data for plasmid (pSRS5)-by-chromosome fluctuation analyses for *RTS1* and recombination frequency calculation in *rqh*$^{-}$ strains

11. Supplemtary material 11
Data for plasmid (pSRS5)-by-chromosome fluctuation analyses for *sup3-e* and recombination frequency calculation in *rqh*$^{-}$ strains

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