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Investigation into cis and trans acting factors in fission yeast genome stability

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Investigation into *cis* and *trans* acting factors in fission yeast genome stability

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Ph.D. thesis 2009

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Abstract

Genomic rearrangements give rise to a range of genetic diseases, including cancer. Translin was isolated as a DNA binding protein, which was found to associate with translocation breakpoint junctions in many human tumours. However the cellular function of Translin and its role in cancer progression is unknown. Translin has an interacting partner, TRAX (Translin-associated factor X). Translin and TRAX have been implicated in mRNA regulation, telomere stability, cell division, DNA repair and ribonuclease activity. Translin and TRAX are highly conserved and found in most eukaryotes, this suggests they may have a crucial function. We used the facile model system, *Schizosaccharomyces pombe,* to study the function of both TRAX and Translin. Results show that Translin and TRAX are not essential for growth, as deficient cells are viable. We found normal rates of mitotic proliferation and normal levels of intergenic and intragenic recombination, and we can also eliminate a key function in recovery from some types of DNA damage, but have identified a possible redundant role in the recovery from DNA damage.

Genome rearrangements can be caused by replication fork barriers (RFBs). These can occur by transcription by RNA polymerase II (RNA pol II) resulting in recombination, known as transcription-associated recombination (TAR). However it is not known if RNA pol III-induced DNA replication fork stalling leads to an increase in recombination which might generate deleterious genetic changes. A single $tRNA^{Glu}$ gene placed in an ectopic locus can act as an RFP, and becomes a mitotic hotspot upon the loss of Swi1 function. We have also identified an element of 5B-boxes which creates a strong orientation-dependent RFB with no associated recombination.

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Abbreviations:

ACT: activator of cremin testes
AKAP: kinase A anchoring protein
ALL: acute lymphoblastic leukeamia
AML: acute myeloid leukaemia
AP: apurinic or apyrimidinic
ARS: autonomous replication sequence
AT: Ataxia Telangiectasia
ATLD: Ataxia Telangiectasia-like disorder
BER: base excision repair
BIR: break induced replication
bp: base pair
C: circular uncut plasmid
CDK: cyclin-dependent kinase
CFS: common fragile site
cM: centimorgans
CML: chronic myelogenous leukaemia
COC: chromosome-organising clamp
CPD: cyclobutane prymidine dimer
CPT: camptothecin
CREM: cAMP-responsive element modulator
DN: double negative
DNA-PK: DNA-dependent protein kinase
DP: double positive
DSB: double strand break
DSBR: double strand break repair
dsDNA: double-stranded DNA
ssDNA: single-stranded DNA
EDTA: ethylenediaminetetraacetic acid
ERC: extra-chromosomal circle
FPC: fork protection complex
GADD: growth arrest and DNA damage
GGR: global genome repair
GRBP: glucose response element binding protein
GRE: glucose response element
HEK: human embryonic kidney
HJ: Holliday junction
HR: homologous recombination
HU: hydroxyurea
IDL: insertions/deletion loop
IR: inverted repeats
kb: kilobase
kDa: kilodalton
L: linearised plasmid
LB: Luria-Bertani media
L-PK: liver-type pyruvatekinase

LTR: long terminal repeat
Mb: megabase
MBC: methyl benzimidazol-2-yl carbamate
MEF: mouse embryonic fibroblast
MLTF: major late transcription factor
MMC: mitomycin C
MMR: mismatch repair
MMS: methyl methanesulphonate
mRNA: messenger RNA
mRNP: ribonucleoprotein
NB: nitrogen base
NBL: nitrogen base liquid
NER: nucleotide excision repair
NES: nuclear export signal
NHEJ: non-homologous end joining
NLS: nuclear localization signal
ORF: open reading fame
PCNA: proliferating cell nuclear antigen
PCR: polymerase chain reaction
PEG: polyethylene glycol
polI: polymerase I
polII: polymerase II
polIII: polymerase III
R: fraction of recombinant spores
RC: replication complex
rDNA: ribosomal DNA
RDR: recombination-dependent replication
RENT: nucleolar silencing and telophase exit
RIPA: radioimmunoprecipitation assay
RF: replication fork
RFB: replication fork barrier
RFC: replication factor C
RFP: replication fork pause
rRNA: ribosomal RNA
RPA: replication protein A
RPC: replication progression complex
r.p.m.: revolutions per minute
RSZ: replication slow zones
RTS: replication termination sequence
SCC: sister-chromatid cohesion
SCF: Skp, Cullin, and F-box protein complex
SDS: Sodium Dodecyl Sulfate
SDSA: synthesis-dependent strand annealing
SMC: structural maintenance of chromosome
SP: single positive
SPA: synthetic sporulation media

SSA: single-strand annealing
SSB: single-strand break
ssDNA: single-stranded DNA
TAR: transcription-associated recombination
TB-RBP: testes brain RNA binding protein
TBZ: thiabendazole
TCR: transcription-coupled repair
TLS: translesion synthesis
TF: transcription factor
TS: template switch
TTF: transcription terminator factor
TRAX: Translin asscociated factor-X
tRNA: transfer RNA
UTR: untranslated region
UV: ultra-violet
WCE: whole cell extract
YE: yeast extract
YEA: yeast extract agar
YEL: yeast extract liquid

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Chapter 1 Introduction

1.1 Genomic instability

Genomic instability is not only the cause of pathological disorders, but is also crucial for evolution by generating genetic diversity. The many processes that take place during cell proliferation must be tightly controlled and coordinated in order to preserve genetic integrity. The efficient and faithful replication of DNA, correct chromosome segregation during cell division, activation of checkpoints and DNA repair pathways are all key. Errors in these processes can be harmful for the cell and are often associated with pathological disorders, such as premature ageing, neurological disorders, predisposition to various types of cancer and inherited diseases.

The term genetic instability refers to a range of genetic alterations. These include chromosomal instability, which refers to changes in chromosome number that lead to chromsome loss or gain (Draviam *et al.*, 2004), and are caused by failures in either mitotic chromosome transmission or the spindle mitotic checkpoint. Micro and minisatellite instability leads to the expansion and contraction of repetitive DNA and can occur by DNA replication slippage, mismatch repair (MMR) impairment or by homologous recombination (HR). Base substitutions, micro-insertions and micro-deletions are mainly associated with replication errors, impairment of base excision repair (BER) and MMR, or error-prone translesion synthesis. Gross chromosomal rearrangements such as translocations, duplications, inversions or deletions are the result of increases in HR-mediated events, such as unequal sister-chromatid exchange and ectopic HR between non-allelic repeated DNA fragments, or end-joining between non-homologous DNA fragments. Replication stress is also associated with breakpoints and rearrangements. There are two types of elements that have a key role in instability; the first are factors that act *in trans* to prevent instability such as S-phase checkpoint factors which respond to replication fork (RF) stalling and intra-S-phase damage by preventing RF collapse and breakdown, they are crucial for

maintaining genome integrity (see later). The second key factors act *in cis*, such as highly transcribed DNA regions and chromosomal fragile sites.

Chomosomal fragile sites are DNA sequences that show gaps or breaks after partial inhibition of DNA synthesis (Sutherland, 1977), this makes them hotspots for translocations, gene amplifications, integration of exogenous DNA and other rearrangements (Durkin and Glover, 2007; and reviewed by Aguilera and Gomez-Gonzalez, 2008.) There are two main classes of fragile sites based on their population frequency and pattern of inheritance. Rare fragile sites are inherited in a Mendelian manner (Kremer *et al.*, 1991; Sutherland *et al.*, 1998), and are found in less than 5% of the population. Breakage at these sites is most often due to the expansion of trinucleotide repeats, such as is seen in the mental retardation disease Fragile X syndrome (Handt *et al.*, 2000; Hansen *et al.*, 1993). Common fragile sites (CFSs) are found in all individuals and are a normal component of the chrosomal structure. Both rare and common fragile sites have been found to be late replicating (Gacy *et al.*, 1995; Hewett *et al.*, 1998; Le Beau *et al.*, 1998). "Replication slow zones" (RSZs) have also been identified in *Saccaromyces cerevisiae* temperature sensitive *mec1* mutants, where double-strand breaks (DSBs) are found in specific genomic regions where there is slow moving replication (Cha and Kleckner, 2002). *mec1* is the ortholog of ATR, which is required for stability at CFSs in mammalian cells. It is thought that yeast RSZs are analogous to metazoan CFSs. Also in yeast, recombination between retrotransposons, especially Ty elements are a common source of genomic rearrangements, including deletions, translocations and inversions (Dunham *et al.*, 2002; Roeder and Fink, 1980). Ty elements arranged in a head-to-head configuration may mimic CFS regions as they are sites of DSBs under replicative stress. Chromosome translocations and deletions, probably mediated by HR, frequently occur between Ty elements (Lemoine *et al.*, 2005).

CFSs are also sites of frequent chromosome breakage and rearrangements in cancer cells. The fragile site-specific rearrangement most frequently observed are one or more large deletions of tens of hundreds of kilo bases directly within the

CFS region, resulting in inactivation of associated genes; interestingly few translocations involving CFSs have been reported. Translocations primarily reflect the misjoining of ends from two or more DSBs. The exposure of cells to agents that induce DNA DSBs typically results in both reciprocal and nonreciprocal chromosome translocations, in an event that appears to only involve the non-homologous end joining (NHEJ) pathway and not HR. Cancer is probably the most prevalent genetic disease. Two types of initiating genetic events for the cancerous state have been identified; the inactivation of genes by deletion, mutation or epigenetic mechanisms, and the activation or deregulation of genes as a consequence of point mutations, amplifications or cytogenetic abnormalities. Balanced rearrangements include reciprocal translocations, inversions and insertions and result in structurally altered chromosomes without the gain or loss of genetic information. Balanced chromosome abnormalities can lead to the formation of gene fusions, possibly resulting in the overexpression of a gene when a promoter and gene from opposite breakpoints become fused, or result in the creation of a hybrid gene through the fusion of two genes. Recurrent balanced rearrangements have been detected in almost every tumour type (Mitelman *et al.*, 2007). There are 267 known balanced rearrangements in acute myeloid leukaemia (AML) and 155 in acute lymphoblastic leukeamia (ALL). Many balanced rearrangements are associated with distinct tumour types and clinical features (Mandahl, 1996; Harrison and Foroni, 2002; Borden *et al.*, 2003; Johansson *et al.*, 2004). DNA DSBs are required for most chromosome aberrations that result in gene fusions (Aplan, 2006; Novo and Vizmanos, 2006; Povirk, 2006). Many external agents can cause DSBs, but it has also been shown that there are many endogenous genetic elements that can cause replication stress and ultimately DSBs. Exactly how this happens and how the resulting damage is processed is still unclear. Using the simple and facile model organism *Schizosaccharomyces pombe*, this work looks at both the potential of a single tRNA gene in the production of replication-induced recombination and also investigates the function of two closely associated proteins, Translin and TRAX, thought to possibly function at translocation breakpoint junctions and in DNA repair.

1.2 Replication fork blocks and their consequences

There are several naturally occurring elements that act as replication fork barriers (RFBs) where the progression of a fork is blocked (Brewer and Fangman, 1988; Little, *et al.*, 1993; Wiesendanger, *et al.*, 1994), or replication fork pauses (RFPs) where an elongating replication fork is transiently stalled (Greenfeder and Newlon, 1992; Deshpande and Newlon, 1996). Stalled forks are susceptible to breakage (Michel *et al.*, 1997; Ivessa *et al.*, 2000, 2002; Cha and Kleckner, 2002), which can lead to genome instability.

1.2.1 *Ter*-Tus barrier complex in bacteria

In *Escherichia coli*, replication of the circular chromosomes is bidirectional. Replication is initiated at the origin and is stopped in the diametrically opposed terminus region. The terminus region contains six specific nucleotide sequences known as the *ter* sites, which the protein Tus will bind to (Hill *et al.*, 1988). The *ter*-Tus complex blocks the progression of replication forks in a polar manner by physically inhibiting the unwinding by the replicative helicases DnaB (Sahoo *et al.*, 1995). The complex forms a replicative fork trap, which forks can enter but cannot escape. The stalled fork is resolved when a fork arrives from the permissive direction and triggers termination. *ter* sites however are not an absolute barrier, but act as pause sites, as fork movement from these sites can be detected under certain circumstances (reviewed by Rothstein *et al.*, 2000). Interestingly, it has been found that replication pauses at *ter* sites can induce homologous recombination (Horiuchi *et al.*, 1994), and up to 50% of forks from the *E. coli* origin are thought to collapse before arriving at the chromosomal terminus (Cox *et al.*, 2000).

1.2.2 Site-specific replication termination in *S. pombe*

In *S. pombe* there are three mating-type cassettes but only one is actively expressed and the others are backup copies for interconversion (reviewed by Egel, 2004). Mating-type switching starts with an imprinting event at the *mat1* locus on the newly synthesized lagging strand during S-phase of one cell cycle and it is shielded from repair until the S-phase of the following cycle. The imprint is only made when replication is in the centromere-proximal direction, this is regulated by *RTS1* which is a polar RFB (Dalgaard and Klar, 1999). In the second round of replication the imprint inhibits the progression of the replication fork and this initiates repair by homologous recombination (Arcongioli, 1998). One of the two transcriptionally silent gene cassettes, *mat2P* (containing plus information) or *mat3M* (containing minus information) both located distal to *mat1*, are used as donors for the recombination repair event (Kelly *et al.*, 1988). This can result in the change of mating-type specific information at the actively expressed *mat1* locus. The Swi1-Swi3 complex is essential for *RTS1* function in arresting replication forks travelling in the incorrect direction, and they are also involved in imprinting where they pause the replication fork in the vicinity of the imprint (Dalgaard and Klar, 2000).

The structure of *RTS1* consists of two types of *cis*-acting elements. There is a region of approximately 450 bp, termed region B, which contains four repeats of a 55 bp barrier motif which interacts with replication termination factor Rtf1, and a purine-rich region A, which is approximately 60 bp and has no intrinsic barrier activity, but acts cooperatively with region B to increase its termination activity 4-fold. Region A activity is dependent on the *trans*-acting factor Rtf2 (Refer to Fig. 1.1; Codlin and Dalgaard, 2003). Interestingly, Rtf1 has 32% identity with Reb1, which is involved in the transcription termination of the RNA polymerase I-transcribed rDNA genes (see below) (Melekhovets *et al.*, 1997; Zhao *et al.*, 1997; Codlin and Dalgaard, 2003).

It has been shown that *RTS1* located in an ectopic site can impede the replication fork in a polar fashion and cause an increase in recombination, and the nature of

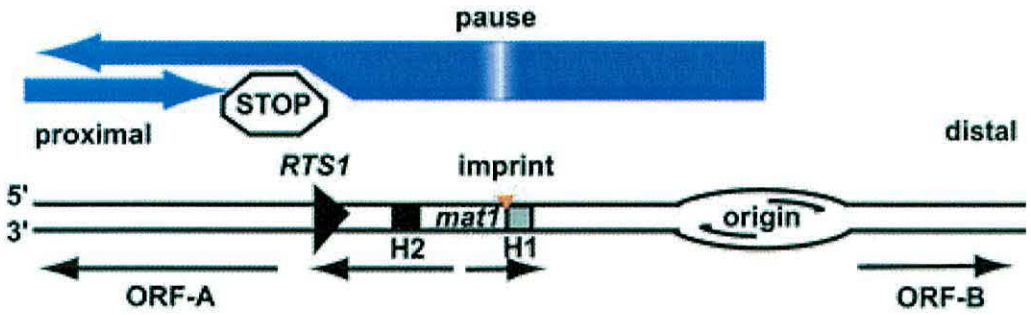


Figure 1.1 The *S.pombe mat1* region. The genetic elements and transcriptional units (thin horizontal arrows) located by *mat1* are shown. The orientation of *mat1* relative to the centromere is given. Large blue arrows display the direction of replication in the region and termination at the *RTS1* element. The position of the imprint that marks switchable cells is shown. *swi1*- and *swi3*-dependent pausing of the replication fork at the site of imprinting is shown as a gradient within the blue arrow, depicting replication. *H1* and *H2* are homology domains thought to be important during mating-type switching. ORF-A and -B are open reading frames of unknown function. (Figure and legend adapted from Codlin and Dalgaard 2003.)

the impedance influences the type of recombinant generated, from inter or intra-chromatid recombination in mitotic cells (Ahn *et al.*, 2005). *RTS1* induced recombination is due to its normal RFB function and recombination is dependent on Rhp51 and Rad22. The Rad22-dependent pathway is required for most of the direct repeat recombination in *S. pombe*, with a subpathway dependent on Rhp51, generating conversion types (Doe *et al.*, 2004). Interestingly the RecQ helicase homologue, Rqh1, seems to limit the recombination induced by an *RTS1* RFB, by possibly preventing replication fork breakage. Moreover, there is no evidence of fork reversal and Holliday Junction (HJ) formation in an *rqh1*Δ mutant (Ahn *et al.*, 2005). Rqh1 could help to protect the blocked fork from nucleolytic attack by promoting the stability of replisome components at the blocked fork (Cobb *et al.*, 2003) or by exposing ssDNA at the blocked fork to enable recombinases to load which might protect the fork (Courcelle and Hanawalt, 1999). Alternatively, it may work by unwinding DNA structures such as HJs formed by fork reversal that might otherwise be cleaved by an endonuclease (Doe *et al.*, 2000; Ahn *et al.*, 2005).

1.2.3 Replication fork barriers induced by RNA polymerase transcription

In both eukaryotes and prokaryotes, the rate of DNA synthesis is at least five times higher than the rate of RNA synthesis (Matsuzaki *et al.*, 1994). It is possible for DNA and RNA synthesis to occur on the same part of a DNA molecule simultaneously and collisions between the DNA replication and RNA transcription machineries are unavoidable. In *E. coli* it has been shown that replication forks pause when they meet a transcription complex (Liu *et al.*, 1993; Liu and Alberts, 1995).There are three nuclear RNA polymerases present in the cell. RNA polymerase I (polI) transcribes the rDNA, RNA polymerase II (polII) transcribes mRNAs and RNA polymerase III (polIII) functions to transcribe the tRNAs and 5S rRNAs (Roeder and Rutter, 1969).

1.2.3i) Pausing in the rDNA

The ribosomal DNA (rDNA) locus in *Saccaromyces cerevisiae* contains genes encoding the rRNAs within several hundred repeats of a 9 kb unit. Fork arrest within the intergenic spaces of rDNA has been conserved from yeast to humans (Brewer *et al.*, 1992; Hernandez *et al.*, 1993; Little *et al.*, 1993; Wiesendanger *et al.*, 1994; Sanchez *et al.*, 1998; Lopez-Estrano *et al.*, 1999). Recombination within this region is highly regulated, as recombination between repeats is required to maintain the copy number of the array, but must also be restrained to prevent the excision of extra-chromosomal circles (ERCs), as the accumulation of these has been implicated in aging (Defossez *et al.*, 1999).

Within the rDNA of budding yeast is the HOT1 sequence (Keil and Roeder, 1984). It consists of two key elements; a promoter for RNA pol I (I element) which transcribes the rRNA genes; recombination at HOT1 is only stimulated when RNA pol I transcribes into the recombining sequences (Voelkel-Meiman *et al.*, 1987; Huang and Keil, 1995), and an enhancer element of RNA pol I transcription (E element; Voelkel-Meiman *et al.*, 1987). The enhancer element is not essential for RNA pol I transcription in the rDNA (Wai *et al.*, 2001; Burkhalter and Sogo, 2004), but does contain a polar RFB that ensures unidirectional replication through the rDNA locus (Brewer and Fangman, 1988; Linsken and Huberman, 1988). The protein Fob1 (fork blocking 1) binds directly with the RFB sequence and surrounding sites (Huang and Moazed, 2003; Kobayashi, 2003; Mohanty and Bastia, 2004), and is required for HOT1 activity and for fork pausing at the RFB (Refer to Fig. 1.2; Kobayashi and Horiuchi, 1996). Fob1 has also been implicated in Sir2-mediated rDNA silencing by its association with the regulator of nucleolar silencing and telophase exit (RENT) complex (Huang and Moazed, 2003). Interestingly, the HOT1 sequence can stimulate recombination when placed at other loci (reviewed by Labib and Hodgson, 2007).

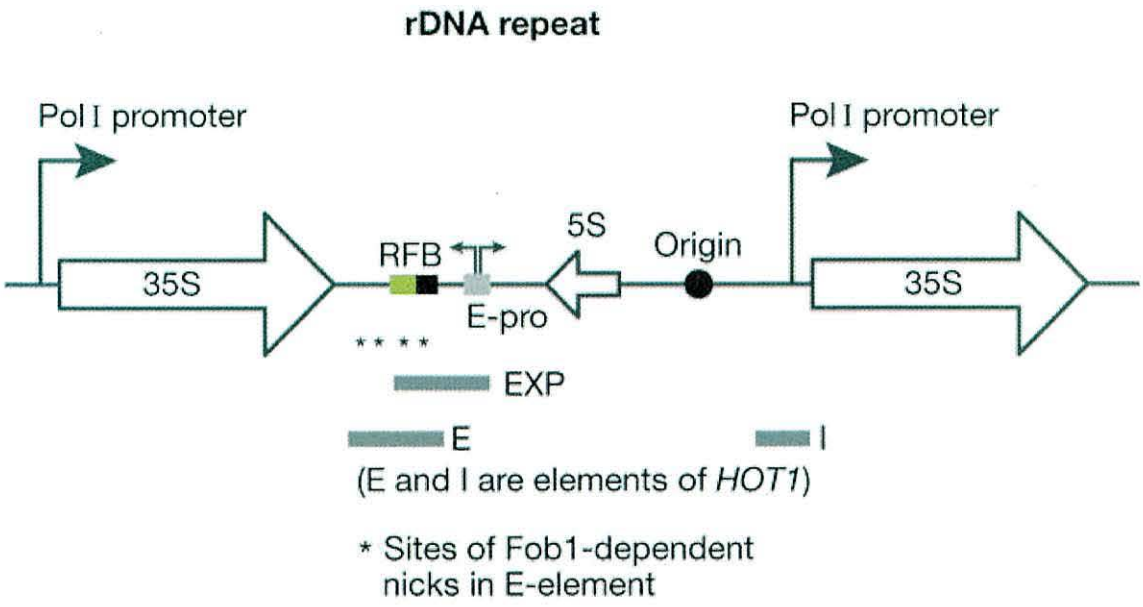


Figure 1.2 The budding yeast ribosomal DNA locus. The replication fork barrier (RFB) ensures that the 35S RNA gene is replicated in the same direction as RNA polymerase I (Pol I) transcription. Maintenance of the copy number of the rDNA repeats is dependent on the EXP sequence (expansion of rDNA repeats) that comprises the RFB and the bi-directional Pol II promoter E-pro (expressing two non-coding mRNAs). The enhancer of rDNA transcription (E) element (containing the RFB and associated 5′ sequences) and the initiation of rDNA transcription (I) element (containing the Pol I promoter) of HOT1 stimulate recombination when placed at other chromosomal loci. (Figure and legend taken from Labib and Hodgson, 2007.)

In mouse (and humans) the Sal box acts as the terminator of RNA pol I transcription in the rDNA (Little *et al.*, 1993). It is an 18 bp sequence motif that is repeated ten times downstream of the pre-rRNA coding region. This is recognised by the transcription terminator factor TTF-1 which mediates the stop of the elongation reaction of RNA pol I (Grummt *et al.*, 1985). This can stall replication forks in both directions but limits DNA replication to the same direction as transcription (Little *et al.*, 1993). In *S. pombe*, Reb1 acts as an RNA Pol I transcription terminator, causing fork arrest within the rDNA region (Morrow *et al.*, 1993; Sanchez-Gorostiaga *et al.*, 2004).

1.2.3ii) Pausing at tRNA genes

A RFP was identified in *S. cerevisiae* downstream of the Ty element Ty1-17 located near *CEN3* on chromosome III. The replication fork paused throughout a region of roughly 500 bp rather than at a specific point. This region includes the downstream long terminal repeat (3′ LTR) of Ty1-17 and the *SUP53* tRNA gene (see Fig. 1.3). Results suggested that forks moving leftward from *ARS307* were stalling. Inactivation of *ARS307* resulted in the region being predominately replicated by forks moving rightward from *ARS306*, which interestingly resulted in a reduction in the RFP activity of this region (Desphande and Newlon, 1996). It was found that the RFP does not depend on an intact Ty element and that *SUP53* alone could create an RFP but the LTR alone could not. Also a pause only resulted when Ty1-17 and *SUP53* opposed the direction of replication. It would seem the common features of the RFP include an LTR and a tRNA gene whose transcription opposes the approaching replication fork (Desphande and Newlon, 1996). An RFP was also mapped to a solo LTR and a $tRNA^{Glu}$ gene located roughly 2 kb left from Ty1-17. This RFP was lost when *ARS306* was inactivated, so again only forks moving in the opposing direction stall (Desphande and Newlon, 1996). An LTR and a $tRNA^{Arg}$ gene on ChrX (Rothstein *et al.*, 1987)

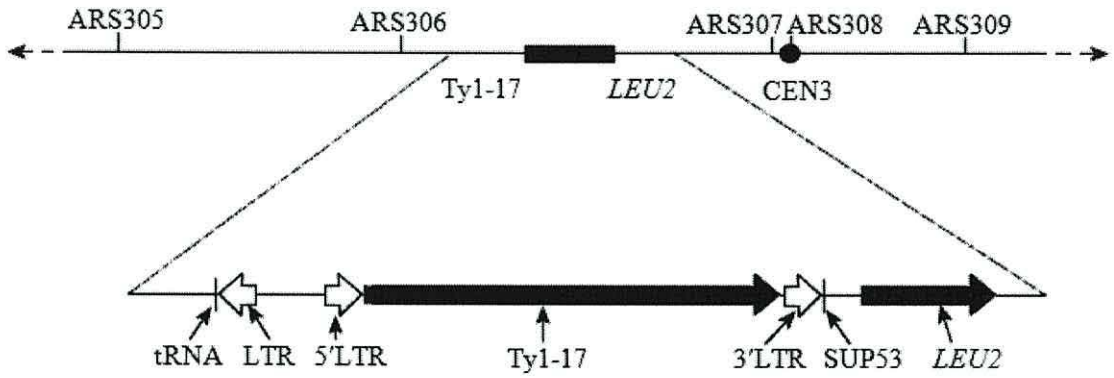


Figure 1.3 The central region of *S. cerevisiae* chromosome III. This region contains LTRs and a tRNA gene that act as polar RFB sites. *ARS* elements, genes and the centromere, *CEN3* within this region are shown. *ARS308* is an inefficient chromosomal origin of replication whereas the other *ARS* elements indicated are efficient. The region used to examine chromosomal RFP sites has been enlarged. Filled arrows indicate direction of transcription and open arrows show orientations of LTR elements. (Figure and legend adapted from Desphande and Newlon, 1996).

were also associated with an RFP site. Polar RFPs were also mapped to *SUP11-1* $tRNA^{Tyr}$ and a $tRNA^{Ile}$ gene on ChrV (Brewer and Fangman, 1993). A fagile site (the 403 site) was identified on ChrVII in *S. cerevisiae* and contains two tRNA genes. The site also contains four LTRs and three mitochondrial sequence fragments. It is thought that the tRNA genes found in the 403 site may be capable of replication fork stalling (Admire *et al.*, 2005), and that possibly any tRNA gene can cause a RFP, provided the orientation of the tRNA gene opposes the direction of DNA replication. Ty retrotransposons appear to preferentially insert near tRNA genes (Kim *et al.*, 1998; Bolton and Boeke, 2003).

1.2.3iii) Heterochromatin barrier function of tRNA genes

The DNA around the *S. pombe* centromeres takes a heterochromatic form so that the DNA within this region is transcriptionally silent. In many eukaryotes, including *S. pombe,* heterochromatin formation is promoted by hypoacetylation and hypermethylation of histone H3 at lysine 9 (Grewal and Elgin, 2002). Centromeric chromatin contains blocks of canonical nucleosomes methylated on H3 lysine 4, interspersed with blocks of nucleosomes containing the histone H3 variant, CENP-A (*S. pombe cen1*), which provides the structural and functional foundation of all active kinetochores (Sullivan and Karpen, 2004). Heterochromatin can spread *in cis*, causing the epigenetic silencing of flanking euchromatic regions (Talbert and Henikoff, 2006), this spreading is limited by chromatin barriers (Sun and Elgin, 1999). Interestingly, barrier activity at *S. pombe cen1* is dependent upon an intact $tRNA^{Ala}$ gene (*cen1* $tRNA^{Ala}$) (Scott *et al.*, 2006). Scott *et al.* demonstrated that *cen1* $tRNA^{Ala}$ blocks the spread of heterochromatin and permits the expression of an *ura4*$^+$ reporter gene placed proximal to the centromere. Replacement of this gene with a normally non-centromeric $tRNA^{Glu}$ or $tRNA^{Ile}$ gene also restored the expression of the reporter gene, suggesting that the ability to block the spread of centromeric heterochromatin is a general property of *S. pombe* tRNA genes. RNA pol III and

TFIIIC were found to associate with the barrier and barrier activity is independent of the orientation of the tRNA gene (Scott *et al.*, 2007).

In *S. cerevisiae*, a unique $tRNA^{Thr}$ gene located at the right boundary of the silent mating-type locus (HMR) can efficiently block the spread of heterochomatin (Donze *et al.*, 1999; Donze and Kamakaka, 2001). Mutations in the tRNA gene promoter elements or in the factors that bind the promoter greatly reduces the barrier activity of the tRNA gene, demonstrating that transcription of the tRNA gene is essential (Oki and Kamakaka, 2005). Interestingly, in yeast it has been found that actively transcribed tRNA genes can suppress transcription of nearby genes transcribed by RNA pol II (Kinsey and Sandmeyer, 1991; Hull *et al.*, 1994); this is known as tRNA gene position effect (Bolton and Boeke, 2003) or tRNA gene-mediated (tgm) silencing (Kendall *et al.*, 2000).

1.2.4 Processing of replication fork blocks

In eukaryotic cells, stalled forks elicit the activation of checkpoint kinases in response to altered features of the fork, including the exposure of more ssDNA (McGowan and Russell, 2004; Branzei and Foiani, 2005; Li and Zou, 2005). The checkpoint kinases stabilize the replication fork and maintain the proper assembly of the replisome components and DNA structures, preventing irreversible collapse of the stalled forks (Lopes *et al.*, 2001; Tercero and Diffley, 2001). For example, the replication checkpoint kinase Cds1 in *S. pombe*, prevents fork collapse in response to dNTP depletion by hydroxyurea (Noguchi *et al.*, 2004). If a stalled RF can remain associated with a functional replisome, then DNA synthesis can resume after the obstacle has been removed or the fork has been repaired (Rudolph *et al.*, 2007). The Swi1-Swi3 complex activates Cds1 and is also thought to travel with the replication fork, forming a "replication fork protection complex" (FPC) which is thought to stabilize stalled forks (Noguchi *et al.*, 2004). Hsk1 in *S. pombe* is also required for Cds1 activation and recovery of replication after S-phase arrest (Takeda *et al.*, 1999; Snaith *et al.*, 2000; Takeda *et al.*, 2001).

Hsk1 regulates late origin firing during replication perturbation and could also be required for stalled fork stabilisation or some aspect of replication recovery (Lambert *et al.*, 2007).

S. cerevisiae Rrm3 is a DNA helicase thought to prevent replication fork stalling. It travels with the replisome and its catalytic activity is required for the efficient replication past specific, particularly stable chromatin-associated complexes, in particular through the rDNA (Ivessa *et al.*, 2000), through the telomeres and subtelomeric DNA (Ivessa *et al.*, 2000, 2002), at tRNA genes, inactive replication origins, centromeres and the silent mating-type loci (Ivessa *et al.*, 2003). At these sites *rrm3Δ* strains show replication fork pausing and DNA breaks which elicit a checkpoint response and increased recombination, with the mutant being viable but requiring checkpoint, replication and repair genes for normal growth (Keil and McWilliams, 1993; Ivessa *et al.*, 2000, 2002, 2003; Schmidt and Kolodner, 2004; Tong *et al.*, 2004; Torres *et al.*, 2004a). It is unlikely that Rrm3 is required to restart or to repair stalled or broken replication forks as *rrm3Δ* cells are not sensitive to DNA damaging agents (Torres *et al.*, 2004b). Rrm3 loads onto origins at the beginning of S-phase and migrates with Mcm4 and Pol2 through all regions, even those that do not contain Rrm3-dependent sites (Azvolinsky *et al.*, 2008). It is proposed that the function of Rrm3 helicase is to clear the way for the replication machinery, preventing forks from stalling and ultimately collapsing. As yet no *S. pombe* homologue of Rrm3 has been identified.

As already mentioned, RFB sites are associated with an increase in recombination, suggesting that stalled forks stimulate recombination upon collapse (Defossez *et al.*,1999; Admire *et al.*, 2006; Kobayashi *et al.*, 1998; Lambert *et al.*, 2005). Interestingly, when forks pause at RFBs they do not seem to stimulate a checkpoint response, perhaps due to a lack of substantial ssDNA, therefore the fork is perhaps more liable to collapse and this leads to recombination (reviewed by Labib and Hodgson, 2007). Recombination at a stalled fork permits the reassembly of a functional replisome at a collapsed fork allowing the re-establishment of unidirectional replication independently of the

origin of replication (Kuzminov, 1995; Kogoma, 1996; Kogoma, 1997.) In *E. coli*, *oriC*-independent replication in SOS induced cells was shown to depend on homologous recombination and is known as recombination-dependent replication (RDR) (reviewed by Kogoma, 1997.) RDR requires the assembly of the primosome onto a D-loop recombination intermediate formed by strand invasion; this allows the binding of the replicative helicase, DNA polymerase and primase, the three enzymes required for replication initiation (Marians, 1992). This process involves the PriA protein, and the poor viability of *priA* mutants suggests that bacteria frequently experience premature replication arrest and replisome dissociation (Masai *et al.*, 1994). One way a collapsed fork is processed is for the fork to unwind and the two newly synthesized strands anneal together forming a four way branched structure known as a regressed or reversed fork or a "chicken foot" structure, similar to a HJ. These have been identified in replication restart in bacteria (see Fig. 1.4). Once the initial RFB has been removed, branch migration in the opposite direction can occur. The regressed fork is then processed by the RecBCD nuclease/helicase to initiate RecA-dependent recombination in a similar manner to DSB processing (Flores *et al.*, 2001).

Since there is no known eukaryotic PriA homologue, stalled and collapsed replication forks must be dealt with by other methods. One important process to restart broken replication forks in eukaryotic cells is break induced replication (BIR) (Paques and Haber, 1999; Kraus *et al.,* 2001). It is a non-reciprocal recombination event and can result in the co-conversion of genetic markers along hundreds of kilo bases of DNA (Golin and Esposito, 1984). A possible model for restart by BIR is that the initial strand invasion creates a D-loop structure that is converted into a complete (unidirectional) replication fork (reviewed in Lambert *et al.*, 2007).

In both fission and budding yeast, all recombination requires Rad22 and Rad52 respectively. Rad22 acts as a mediator, exchanging RPA for Rhp51 (the budding yeast Rad51 homologue and the homologue of the bacterial RecA recombinase (Aboussekhra *et al.*, 1992; Shinohara *et al.*, 1992; Sung, 1997; Petukhova *et al.*,

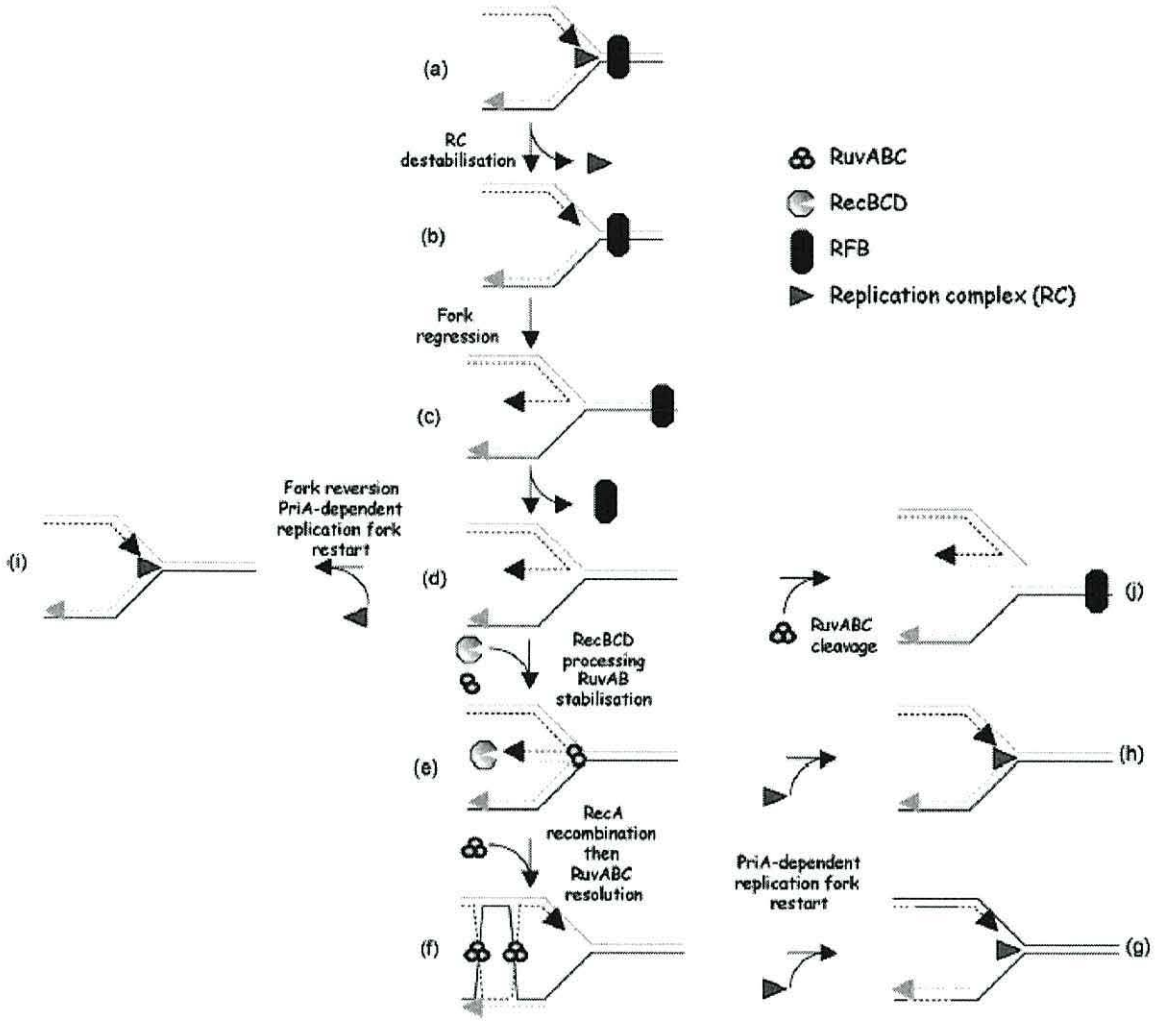


Figure 1.4 Replication fork restart in bacteria. Arrows indicate 3′end of each strand. Dashed lines indicate new synthesized strands. (a) Replication fork progression is compromised by a (RFB). (b) The replication complex collapses. (c) The two newly synthesized strands are unwound and anneal together resulting in a regressed fork. (d) The initial RFB is removed allowing replication progression to occur when the fork is restored. (e) The reversed fork is stabilized by RuvAB and processed by the nuclease complex RecBCD until the next Chi site allowing RecA-dependent recombination to occur between sister chromatids (f). After HJ resolution by RuvABC (f), the RC is reloaded by a PriA-dependent mechanism (g). (h) Alternatively, RecBCD-dependent degradation of the regressed fork generates a 3′-arm replication fork-like structure on which the replisome is loaded via the action of PriA. (i) The regressed fork can be processed by branch migration in the opposite direction and the RC is loaded via PriA activity. (j) Alternatively, the regressed fork, which resembles a HJ, can be cleaved by RuvABD leading to chromosome linearization. (Figure and legend taken from Lambert *et al.*, 2007).

1998; Shinohara and Ogawa, 1998; See later section on homologous recombination). HJ formation in *S. pombe* requires both Rad22 and Rhp51 (Segurado *et al.*, 2002), and both these proteins have been shown to be physically associated with arrested forks (Lambert *et al.*, 2005), and recombination appears to act at collapsed forks rather than stalled forks.

The RecQ family of 3′-5′ DNA helicases (Rqh1 in *S. pombe* and Sgs1 in *S. cerevisiae*), play a crucial role in the processing of blocked replication forks. It is known that defects in RecQ helicases lead to a genomic instability which is characterized by chromosome loss and hyper-recombination. Sgs1 in budding yeast travels with the replication fork and co-localises with the sites of nucleotide incorporation (Cobb *et al.*, 2003). It is required for full Rad53 (Chk2 in *S. pombe*) activation and possibly helps to recruit Rad53 to stalled forks (Bjergbaek *et al.*, 2005). Stable association of replicative polymerase Polε and Polα on stalled forks requires both Sgs1 and Mec1 (the budding yeast homologue of ATR), (Cobb *et al.*, 2003, 2005), suggesting that Sgs1 participates in preventing forks from collapsing during replication arrest. Sgs1 (and its orthologues) have been shown to interact with Top3 to form a complex which resolves recombination intermediates (such as double HJs) in a non-recombinogenic manner (Gangloff *et al.*, 1994b; Wu and Hickson, 2003). Sgs1 is thought to ultimately promote a non-recombinogenic stalled fork restart pathway.

The endonuclease Mus81 was identified as a Rad54 interacting protein in budding yeast and as a Cds1 interacting protein in fission yeast (Boddy *et al.*, 2000; Interthal and Heyer, 2000). It complexes with *Sc*Mms4/*Sp*Eme1 and is involved in DNA repair (Doe *et al.*, 2002). A *mus81*Δ mutant is synthetically lethal with *rqh1*Δ (Doe *et al.*, 2002), suggesting that Mus81 complexes act in an alternative pathway to Rqh1 (Sgs1) in the resolution of recombination intermediates and may have a direct role in arrested fork processing. It has been shown that the Mus81 complex can cleave various Y-like DNA structures that mimic modified arrested forks including leading strand gaps, regressed forks exhibiting 3′ or 5′ tails and D-

loop structures that would be created by a 3′ end strand invasion of the leading strand into the parental duplex (See Fig. 1.5 for a summary; Whitby *et al.*, 2003).

It is known that Holliday junctions can be resolved in many different ways, however, so far very little is generally known about eukaryotic proteins that carry out this process. Holliday junction resolvases that are only active in the mitochondria have been identified in budding yeast (Cce1) and fission yeast (Ydc2) (Kleff *et al.*, 1992; Whitby and Dixon, 1998). A mammalian resolvase is ResA, but this is in very low abundance in the cell and very little is know about it (Constantinou *et al.*, 2001; Constaninou *et al.*, 2002; Eldborough and West, 1990; Hyde *et al.*, 1994; Liu *et al.*, 2004). As well as cleavage by MUS81-EME1 (Constantinou *et al.*, 2002; Chen *et al.*, 2001), the Bloom's syndrome complex (BLM helicase-topoisomerase IIIα-RMI1/2) can 'dissolve' Holliday junctions (Wu and Hickson, 2003). Human GEN1 and its orthologue in *S. cerevisiae* Yen1 have recently been identified as highly specific Holliday junction resolvases, which cut symmetrically across junction points to produce nicked duplex products which can be readily ligated (similar to the RuvC model in bacteria) (Ip *et al.*, 2008).

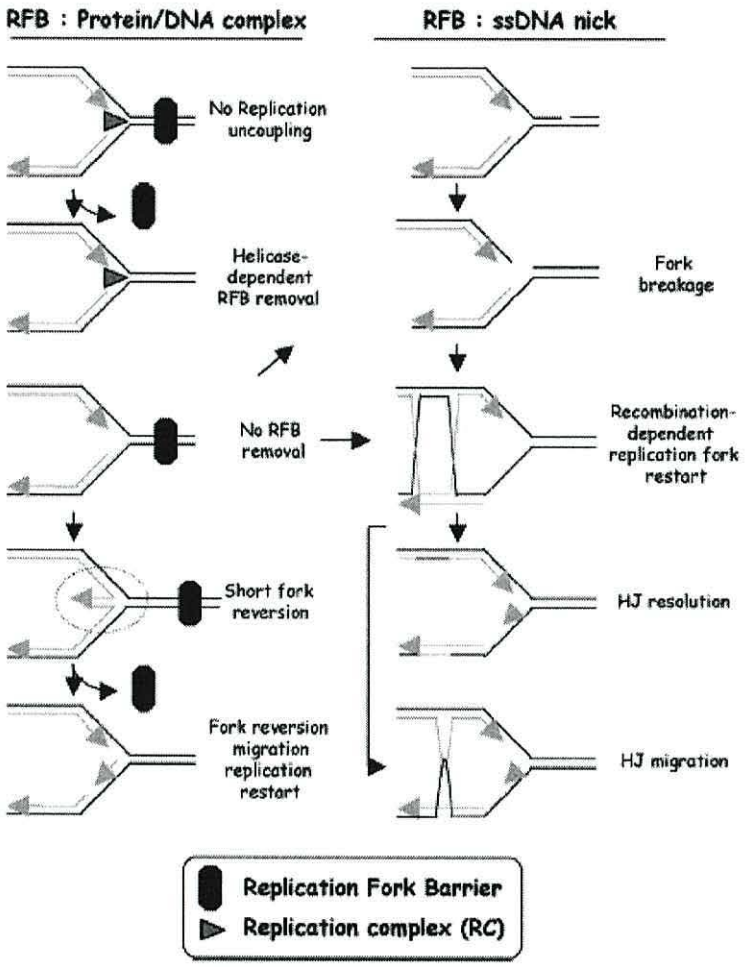


Figure 1.5 Replication completion models. Arrows indicate 3′ end of each strand. Grey lines and black lines indicate newly synthesized strands and template strands respectively. Replication fork stalled at protein/DNA complexes are substrates for specialized helicases that can remove the RFB to allow replication progression. In absence of RFB removal, two scenarios can be envisaged: first, short fork regression could occur (dashed circle). This could help stabilize and protect new synthesized strands from extended degradation. In addition, the displacement of the fork from the barrier would facilitate removal of the RFB and, after junction migration in the opposite direction, replication fork restart may be possible. Second, a strand invasion event involving the 3′end of the leading strand could occur, with or without fork breakage, to allow a recombination-dependent fork restart process to occur. A ssDNA nick on the leading strand template leads to fork breakage that can be repaired by a recombination-dependent fork restart process. The HJs formed can either be resolved by cleavage or migrate (via RecQ family helicase activity) to generate a hemicatenate structure between the new synthesized leading strand and the lagging strand's template. This, hemicatenate can be resolved by TopIII. (Figure and legend taken from Lambert *et al.*, 2007).

1.3.1 DNA repair pathways

The genome is constantly exposed to endogenous and exogenous factors that can cause DNA damage. It is important that this damage is repaired efficiently and accurately in order to prevent further damage and mutations that can lead to diseases such as cancer. There are several forms of damage including base modifications, mismatches, crosslinks and strand breaks. There are also multiple DNA repair pathways, each one designed to repair a different type of damage, but interestingly different types of damage can be targets of several DNA repair pathways. Which pathway ultimately processes the lesion depends partly on the stage of the cell cycle.

1.3.1 The base excision repair pathway

Base excision repair mainly repairs non-bulky lesions produced by alkylation, oxidation or deamination of bases. DNA glycosylases remove damaged, modified or even normal bases by hydrolyzing the N-glycosylic bond and releasing the base (Berdal *et al.*, 1998; Lindahl and Nyberg, 1972; Memisoglu and Samson, 2000a; Seeberg *et al.*, 2000). This leaves an apurinic or apyrimidinic (AP) site, which are one of the most frequently formed DNA lesions and are cytotoxic and premutagenic. AP sites can also be created by ionizing radiation, oxygen radical species and to some extent by alkylation (Lindahl, 1993). AP sites block DNA replication and transcription (Doetsch and Cunningham, 1990; Guillet and Boiteux, 2002), and bypass by translesion polymerases result in base substitutions and frameshift mutations (Haracska *et al.*, 2001; Loeb 1985; Yu *et al.*, 2003). AP sites are processed by AP endonuclease 1 (APE1 in humans) cleaving the sugar-phosphate backbone at the 5'-deoxyribose phosphate (dRP) group flanking a single nucleotide gap (Aspinwall *et al.*, 1997). DNA polymerase β (Pol β), inserts the first nucleotide into the gap, leaving a nicked DNA with a 5'-dRP flap (Srivastava *et al.*, 1998). Depending on the nature of the AP site, repair can then be completed by two different BER sub-pathways (Lindahl and Wood, 1999).

Short-patch BER processes regular AP sites, where Polβ removes the 5'-dRP group through its associated dRP-lyase activity (Matsumoto and Kim, 1995), and the resulting nick is sealed by the DNA ligase III/XRCC1 complex. If however, the sugar group is oxidized or reduced, Polβ cannot remove the 5'-dRP moiety and so repair proceeds through the alternate long-patch BER pathway, involving the removal and replacement of 2-10 nucleotides (Kim *et al.*, 1998; Prasad *et al.*, 2000). This is carried out by the action of replication factor C (RFC), proliferating cell nuclear antigen (PCNA) and the structure-specific nuclease FEN1 (Matsumoto and Kim 1995; Klungland and Lindahl 1997; Wu *et al.*, 1996). In humans the Rad9, Rad1, Hus1 complex has been shown to interact with APE1 and specifically stimulate the endonuclease activity (Gembka *et al.*, 2007). In *S. pombe* it has been shown that the nucleotide excision repair pathway (NER) also plays an important role in the repair of AP sites (Memisoglu and Samson, 2000b; Osman *et al.*, 2003a).

1.3.2 The nucleotide excision repair pathway

The nucleotide excision repair (NER) pathway targets a large variety of DNA damage, including bulky DNA adducts such as photoproducts and cyclobutane pyrimidine dimers induced by UV radiation, intrastrand cross-links produced by cisplatin and also, to a lower extent, non-bulky DNA lesions such as methylated bases and AP sites and even G/A and G/G base-base mismatches (Buschta-Hedayat *et al.*, 1999; Gunz *et al.*, 1996; Huang *et al.*, 1994). In fact, DNA that contains a lesion and some degree of helical distortion is processed more efficiently by NER (Hess *et al.*, 1997; Moggs *et al.*, 1997; Mu *et al.*, 1997). NER has two sub-pathways; transcription-coupled repair (TCR), which is generally 'fast', and global genome repair (GGR), which is generally 'slow'. TCR targets lesions found on the transcribed strand and is dependent upon ongoing transcription (Leadon and Lawrence, 1992; Sweder and Hanawalt, 1992). GGR targets lesions found in non-transcribed regions and removes lesions from the genome overall. The main difference between TCR and GGR is the requirement

for different factors in the initial recognition step. In GGR in humans, the complex UV-DDB, consisting of DDB1 and DDB2, along with XPC-hHR23B are involved in the recognition step. While TCR is thought to be initiated by RNA polII stalling at damaged sites (De Laat *et al.*, 1999). Then in both sub-pathways, transcription factor TFIIH is recruited to the damage site (Sugasawa *et al.*, 1998; Volker *et al.*, 2001; Yokoi *et al.*, 2000). The proteins XPB and XPD are subunits of the TFIIH complex and possess helicase activity in opposite polarity, which unwind the DNA around the lesion. The 3' endonuclease XPG and XPA-RPA are loaded onto the complex (Volker *et al.*, 2001). XPA binds to DNA distortions and RPA binds single-stranded regions. XPA-RPA possibly function to detect bent DNA and in verifying whether the NER complex is correctly assembled on the damaged substrate before incision (Missura *et al.*, 2001). After binding by XPF-ERCC1, a dual incision is made 3' to the lesion by XPG and 5' to the lesion by ERCC1-XPF, releasing a 24-32 nucleotide long oligomer (Huang *et al.*, 1992). Repair is completed by re-synthesis by polymerases δ or ε and the accessory factors RPA, PCNA and RFC and the remaining nick is ligated by ligase I (De Laat *et al.*, 1999; Petit and Sancar 1999). Besides the core NER proteins essential for all NER, specific proteins have been identified that are exclusively devoted to either TCR or GGR, indicating that both sub-pathways of NER are genetically distinct. For example, *S. cerevisiae* Rad26 and the human CSA and CSB proteins function specifically in TCR (van Gool *et al.*, 1994; Venema *et al.*, 1990), and *S. cerevisiae* Rad7 and Rad16 and human XPC proteins are all essential in GGR (Verhage *et al.*, 1994; Venema *et al.*, 1991).

The UV damage repair pathway (UVDR) is another DNA repair pathway that is targeted to UV damage. It has been identified in fission yeast and some other organisms and is dependent on the function of the endonuclease Uve1 which incises 5' to the lesion (Bowman *et al.*, 1994; Mansour *et al.*, 2001).

1.3.3 The non-homologous end joining repair pathway

Non-homologous end joining (NHEJ) repairs DSBs by joining two broken ends of DNA together, irrespective of sequence and sometimes with very little processing; this is 'quick' but is an error-prone method of repair (Lieber *et al.*, 2004). Ku70-Ku80 dimers form a ring-like structure and bind to the DNA ends (Doherty and Jackson, 2001; Smith and Jackson, 1999; Walker *et al.*, 2001). In higher eukaryotes the DNA protein kinase catalytic subunit (DNA-PK_{cs}) is then recruited and this activates its kinase function so that it can phosphorylate itself and other proteins. They possibly also function to tether the broken ends to facilitate rejoining (Cary *et al.*, 1997). In many organisms it has been found that the MRE11-RAD50-NBS1 complex involved in homologous recombination (see later), may be used along with other nucleases such as FEN1 to process the ends before ligation, and a DNA polymerase may also be required. The DNA ends are rejoined by the action of XRCC4-DNA ligase IV (Martin and MacNeill, 2002). They exist as a tight complex and XRCC4 acts to stimulate the ligase activity of the complex (Leber *et al.*, 1998; Matsumoto *et al.*, 2000). Xlf1 has been found to interact with XRCC4 and shows weak sequence homology and structural similarity to XRCC4 (Ahnesorg *et al.*, 2006; Buck *et al.*, 2006). In *S. pombe* Ku70 (Pku70), Ku80 (Pku80), and Ligase IV (Lig4) homologs have been identified (Baumann and Cech, 2000; Goedecke *et al.*, 1994; Manolis *et al.*, 2001), but no definite DNA-PK_{cs} and XRCC4 homologs have yet been identified. Interestingly Xlf1 has been conserved in *S. pombe* and is involved in NHEJ (Callebaut *et al.*, 2006).

1.3.4 The mismatch repair pathway

Mismatch repair (MMR) removes base mismatches and small insertions/deletion loops (IDLs) introduced during replication. In *E. coli* this repair is carried out by the mutHLS group of proteins (Modrich, 1991) and also maintains genome stability by preventing recombination between homologous sequences

(Rayssiguier *et al.*, 1989). The MutS protein recognizes and binds to mismatches. MutH is a site specific endonuclease that binds to hemimethylated *dam* (GATC) sequences. MutL connects both complexes by binding to MutS and MutH. The endonuclease activity of MutH is activated upon complex formation. It excises the non-methylated strand enabling discrimination between the newly synthesized strand and the template, this is followed by resynthesis, resulting in intact duplex DNA (Kolodner, 1996; Modrich, 1991, 1996). In mammalian MMR, the MutS-homologous heterodimer MSH2-MSH6 bind base mismatches and both MSH2-MSH6 and MSH2-MSH3 complexes bind IDLs. The MutL-homologous heterodimer MLH1-PMS2 is recruited. No MutH homolog has yet been found and it is unclear how the newly synthesized stand is distinguished from the template. In *S. pombe*, Msh2 and Swi4 have been identified as the MSH2 and MSH3 homologs respectively (Rudolph *et al.*, 99; Fleck *et al.*, 1992). Interestingly *msh2* is allelic with the mating-type switching gene *swi8*, and is also involved in mating-type switching along with *swi4* (Rudolph *et al.*, 1999; Egel *et al.*, 1984; Fleck *et al.*, 1990; Fleck *et al.*, 1992). *S. pombe pms1* is the *mutL* homolog (Schär *et al.*, 1997). There is evidence for two MMR pathways in *S. pombe*; a major repair pathway able to repair most base mismatches except C/C and a minor pathway able to correct C/C base-base mismatches and other mismatches that may occur during DNA replication (Schär and Kohli, 1993; Schär *et al.*, 1993).

1.3.5 The homologous recombination repair pathway

Homologous recombination (HR) is essential in both mitosis and meiosis. In meiosis HR mediates the exchange of information between maternal and paternal alleles, generating diversity. This process also ensures the correct segregation of homologous chromosome pairs at meiosis I through the formation of chiasmata in crossovers. HR also maintains genetic stability by promoting the accurate repair of DSBs induced by ionizing radiation and other agents, repair of incomplete telomeres, repair of interstrand crosslinks and the repair of damaged replication

forks (RFs). There are several DSB repair (DSBR) models in *S. pombe* (see Fig. 1.6), and they all have common key features. Firstly, HR is initiated by a DSB; this can be caused by an exogenous DNA damaging agent or by an endogenous event such as breaks made in mating-type switching or DSBs made by Spo11 (*S. pombe* Rec12) during meiosis (Krogh and Symington, 2004; Neale and Keeney, 2006; Paques and Haber, 1999). The DSB is processed by a complex of proteins known as the MRN complex (MRX in *S. cerevisiae*), consisting of Mre11 (*S. pombe* Rad32), Rad50 and Nbs1. MRN has both a structural function in capturing and aligning broken ends with each other or with the homologous stretch on the sister chromatid, and also an enzymatic activity in the nucleolytic resection of the DSB. However, the exonuclease activity of Mre11 is 3' → 5', and the DSB is resected 5' → 3'. It is thought in *S. pombe* that the exonuclease *exo1* is also partly involved (Szankasi and Smith, 1995). The resulting single-stranded DNA overhangs are then coated with the heterotrimeric single-stranded binding protein RPA, which is thought to aid in the removal of secondary structures before the binding of Rad51 (Krogh and Symington, 2004). *S. pombe rad11* has been identified to encode a homologous subunit of RPA (Parker *et al.*, 1997). The formation of a recombinase filament on the ssDNA is achieved by Rad51 (*S. pombe* Rhp51, and *E. coli* RecA). Rad51 utilizes ATP to assemble onto ssDNA or dsDNA, forming a right-handed helical polymer that can span thousands of bases and is known as the presynaptic filament, which catalyses the strand exchange reaction with homologous duplex DNA (Jang *et al.*, 1994; Muris *et al.*, 1993; Shinohara *et al.*, 1993; Sauvageau *et al.*, 2005). RPA competes with Rad51 for ssDNA binding, recombination mediator proteins assist Rad51 to overcome RPA and bind ssDNA. Rad52 is one of these mediators and will anneal DNA strands that are naked or are coated with RPA (Shinohara *et al.*, 1998; Sugiyama *et al.*, 1998). Loading of the Rad52-Rad51 complex onto ssDNA nucleates the formation of the Rad51 filament (Ostermann *et al.*, 1993). *S. pombe* has two Rad52 homologs, Rad22 and Rti1 (Schmidt *et al.*, 1989; Suto *et al.*, 1999). Rad22 has been shown to bind to DSBs and is involved in both mating-type switching and DNA repair (Kim *et al.*, 2000; Muris *et al.*, 1997; Stewart *et al.*, 1997). Rti1

mutant was identified as a suppressor of Rad22 (Suto *et al.*, 1999) and both homologs have been found to interact with each other and with Rhp51 and Rpa (Kim *et al.*, 2000; Tsutsui *et al.*, 2001; van den Bosch *et al.*, 2002).

The F-box DNA helicase Fbh1 prevents Rhp51-dependent recombination in the absence of Rad22 (Osman *et al.*, 2005) and is conserved in humans but is absent in budding yeast and several other model eukaryotic organisms (Kim *et al.*, 2002). Fbh1 contains the seven conserved motifs of a superfamily 1 DNA helicase and unwinds DNA with a 3' → 5' directionality; its close relatives include UvrD, Rep and Srs2. It is thought to function in a similar way as Srs2, in dissociating Rhp51 nucleofilaments (Osman *et al.*, 2005). It is the only known DNA helicase that contains an F-box motif, which are substrate recognition components of SCF (Skp, Cullin, F-box), ubiquitin-ligase complexes which catalyze the polyubiquitination of proteins to target them for degradation (Deshaies, 1999).

Other mediator proteins include budding yeast Rad54 (*S. pombe* Rhp54; Muris *et al.*, 1996; Tsutsui *et al.*, 2001) and Rdh54 proteins, which are members of the Sei2/Snf2 chromatin remodeling family of proteins, and has dsDNA-dependent ATPase, DNA translocase, DNA super-coiling and chromatin remodeling activities (Heyer *et al.*, 2006; Tan *et al.*, 2003). Rad54 interacts with Rad51 and is required in multiple stages of HR. In the early stages it promotes the search for DNA homology, chromatin remodeling, D-loop formation and dissociates Rad51-dsDNA filaments (Krogh and Symington, 2004). Removal of Rad51 is thought to allow access by DNA polymerases to initiate the repair DNA synthesis reaction (Tan *et al.*, 2003). Rad54 also mediates the ATP hydrolysis-driven migration of branched DNAs including the HJ and acts with Rad51 to promote the DNA strand exchange reaction (Bugreev *et al.*, 2006). *S. cerevisiae* recombination mediator proteins Rad55/Rad57, are thought to mediate Rad51 binding to ssDNA (Sung *et al.*, 2003; *rhp55* and *rhp57* are the *S. pombe* homologs; Khasanov *et al.*, 1999; Tsutsui *et al.*, 2000). Other Rad51-like proteins have also been identified in *S. pombe*, these include Rlp1 and Rdl1 (Khasanov *et al.*, 2004; Martin *et al.*, 2006). They have been found to interact with each other and also with other proteins of

the HR repair pathway. The *S. pombe* Swi5-Swi6 complex functions in mating-type switching (Akamatsu *et al.*, 2003). The Sfr1-Swi5 complex is needed for mitotic and meiotic HR (Haruta *et al.*, 2006; Akamatsu *et al.*, 2003) The function of the Sfr1-Swi5 complex in HR appears to be similar to that of the Rhp55-Rhp57 complex, by regulating the assembly and/or maintenance of the Rhp51 presynaptic filament and acts independently of Rhp55-Rhp57 (Akamatsu *et al.*, 2007). Sfr1-Swi5 physically interacts with both Rhp51 and Dmc1 (which is the meiosis-specific recombinase), through Sfr1 (Haruta *et al.*, 2006; Akamatsu *et al.*, 2003) and stimulates the homologous DNA pairing and strand exchange potential of the two recombinases.

After Rhp51-promotes strand invasion by the ssDNA into homologous duplex DNA, a D-loop is formed. This structure can then be processed in different ways resulting in crossover or noncrossover events. In the double-strand break repair (DSBR) model, capture of a second 3' overhang results in the formation of a second Holliday junction (HJ). Resolution of this HJ results in a gene conversion, with or without an associated crossover (Szostak *et al.*, 1983). In mitotic recombination however, most DSB repair is most frequently unassociated with crossovers. Here the migrating D-loop never leads to the capture of the second 3' overhang (Ferguson and Holloman, 1996; Nassif *et al.*, 1994; Strathern *et al.*, 1982). The invading strand is displaced and anneals to the second resected DSB end. Since no HJ is formed, only non-crossover products are made, this is known as synthesis-dependent strand annealing (SDSA). Sometimes a DSB is closely flanked by direct repeats, this DNA organization gives the opportunity to repair the DSB by a deletion process using the repeated DNA sequences, this is known as single-strand annealing (SSA). The resected ends are annealed to each other and the protruding tails are removed by nucleases. This results in the deletion of the sequences between the direct repeats. This process is independent of strand invasion and HJ resolution factors (Symington, 2002).

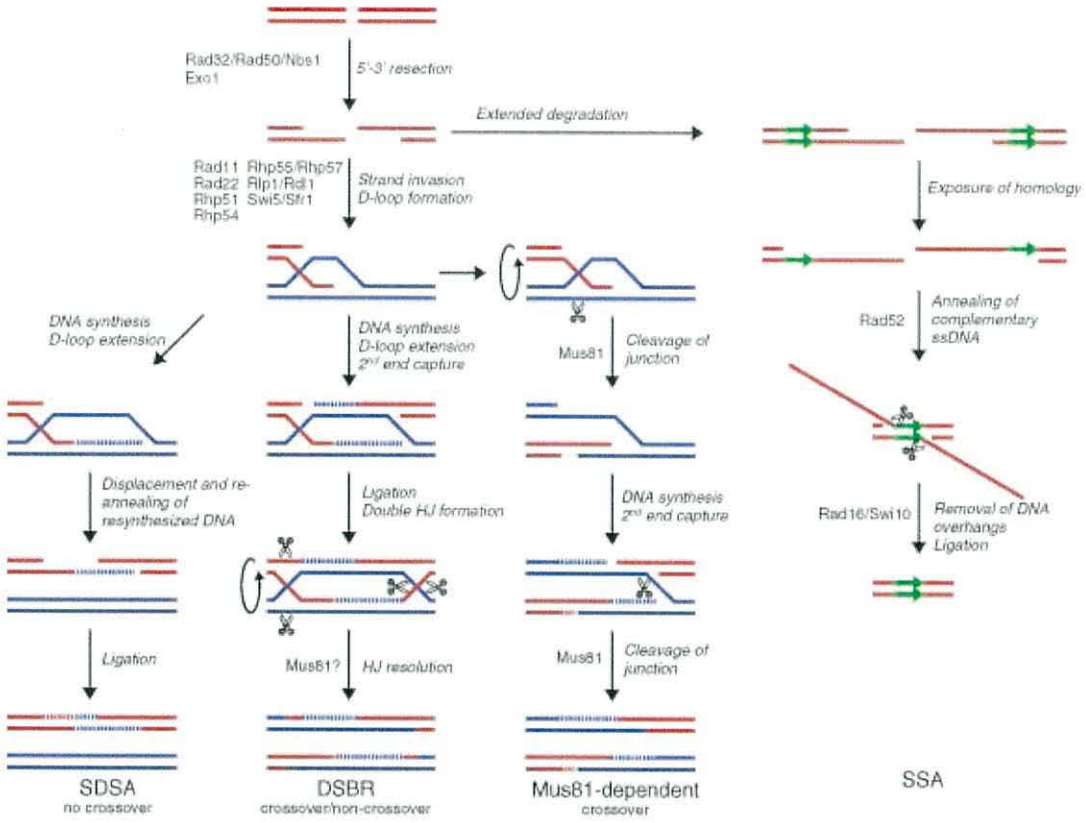


Figure 1.6 Overview of homology-mediated DSB repair pathway in *S. pombe*. SDSA, synthesis-dependent strand annealing; DSBR, double-strand break repair model; SSA, single-strand annealing; HJ, Holliday junction (see text for more details; taken from Raji and Hartsuiker, 2006).

1.3.6 Regulation of DNA repair through the cell cycle

During G1 damaged DNA needs to be repaired before the cell enters S phase. DNA damage can also change the conformation of the DNA and prevent or limit access by transcription and replication machineries. Different repair pathways are active at different times in the cell cycle. During G1 base excision repair (BER) acts to remove mutations such as OxoG which can result in G:C to T:A transversions (Russo *et al.*, 2004; Shibutani *et al.*, 1991). Pyrimidine dimers caused by UV radiation can block the function of DNA polymerases. They are removed by nucleotide excision repair (NER). NER is not restricted to G1. There are two branches of NER; global repair, which repairs lesions irrespective of the genome location and cell cycle phase (Sancar *et al.*, 2004), and transcription-coupled repair, which deals with bulky lesion of transcribed genes. Due to the high compaction of chromatin and the absence of sister chromatids, non-homologous end joining (NHEJ) is the predominant form of DSB repair during G1.

Common replication errors during DNA synthesis in S phase include nucleotide misincorporation, accumulation of nicks and gaps, slippage at repetitive sequence and fork collapse at DNA lesions (Branzei and Foiani, 2005). All of the above elicit a DNA repair response. Base-base mismatches and small insertions and/or deletion loops, which are generated by faulty replication are corrected by the mismatch repair (MMR) pathway, which functions mainly during S phase (Jiricny, 2006). As in G1, BER removes chemically altered nucleotides and also removes misincorporated uracils during S phase (Sancar *et al.*, 2004). DSBs in S phase are mainly repaired by the HR repair pathway, using the sister chromatid as a template. Studies in budding yeast have shown that the CDK activity facilitates the resection stage of the HR reaction and actively prevents NHEJ (Ira *et al.*, 2004; Aylon and Kupiec, 2004), even though the Ku heterodimer of the NHEJ pathway can still bind to DSBs more rapidly than HR factors (Kim *et al.*, 2005). As well as DNA repair, the cell has two mechanisms that promote damage tolerance in S phase. Translesion synthesis (TLS) polymerases can replicate

across a lesion, often in an error-prone manner, and template switch (TS) which is an error-free mechanism that fills in gaps in the DNA template by repriming events downstream of the lesion using the undamaged sister duplex (Branzei and Foiani, 2007; Lehmann *et al.*, 2007).

Gaps and DSBs are repaired in G2 by HR using the sister chromatid as a template. For HR to occur, the sister chromatids must be in close proximity to one another, this is probably accomplished by cohesion, which will provide a physical linkage that connects the sister chromatids from S phase until their separation during anaphase. In fact DSBs have also been found to trigger cohesion through ATM (*S. pombe* Tel1) after DNA replication is complete and this is required for sister-chromatid repair in G2 phase cells (Strom *et al.*, 2007; Unal *et al.*, 2007). Cohesion in budding yeast depends on the cohesin protein complex, which consists of two structural maintenance of chromosomes (SMC) proteins, SMC1 and SMC3, held together by sister-chromatid cohesion-1 (SCC1) and SCC3 (Rad21 and Psc3 in *S. pombe* respectively; Hirano, 2006). The topological problems that arise when two replicons fuse together at termination also need to be resolved during S-G2 in order to prevent chromosome breakage during segregation (Wang, 2002; Bermejo *et al.*, 2007). If DSBs occur during chromosome segregation, chromosomes are already highly compact and the search for homology is difficult, in this case repair is more likely to be carried out by NHEJ in the subsequent G1 phase if cell cycle arrest had not occurred during the G2-M transition (Deming *et al.*, 2001; 2002; Franchitto *et al.*, 2003).

DNA repair also occurs in non-dividing cells. Most DNA damage responses are associated with replication and it is likely that cells that are fully differentiated or senescent cells may still have dedicated repair mechanisms that repair endogenous damage (Nouspikel *et al.*, 2007).

1.4 Translin and TRAX

Translin was identified in human cells as a DNA binding protein. It binds to consensus sequences in single-stranded DNA found at translocation breakpoint junctions and recombination hotspots (Aoki *et al.*, 1995). It has also been found to bind to other single-stranded regions of the genome and has been implicated in DNA repair (Kasai *et al.*, 1997), recombination (Aoki *et al.*, 1995) and telomere stability (Jacob *et al.*, 2004). TB-RBP (testes brain RNA binding protein) is the mouse homolog of Translin (Wu *et al.*, 1997) and was isolated in testes and brain tissues as an RNA binding protein and translational regulator (Kwon and Hecht, 1993). Translin has a binding partner known as translin-associated factor X (TRAX) (Aoki *et al.*, 1997b). Translin and TRAX are highly conserved proteins found in all cell types, but their core function and importance remains unknown. Mainly focusing on Translin, in this work we employ the simple model organism *S. pombe* to try and discover the elusive function of these two proteins. It is known that Translin has a number of different binding targets. The table below contains some of the known Translin targets. Each one will be discussed below.

Table 1.1 Binding targets of Translin

Protein	Binding target	Possible Function	Reference
Translin octamer	Translocation breakpoints	Stabilises translocating strands	Aoki *et al.*, 1995; Kasai *et al.*, 1997
Translin octamer	Telomeric microsatellites	Aids telomerase activity	Jacob *et al.*, 2004
Translin or TRAX	Spindle microtubules	Speeds up cytokinesis	Ishida *et al.*, 2002
Translin	GADD34	Involved in DNA repair	Hasegawa and Isobe, 1999
Translin/TRAX complex	mRNA and microtubules	Suppresses translation and transports mRNAs	Kwon and Hecht, 1993
Translin	GTP	May alter mRNA binding capacity	Chennathukuzhi *et al.*, 2001b
Translin octamer	ss and ds RNAs	Ribonuclease activity	Wang *et al.*, 2004

1.4.1 Association of Translin with DNA translocations

Genomic translocations will cause a number of genetic alterations commonly found in human neoplasms (see earlier). Alterations can include gene deletions, gene fusions, tumour suppressor down regulation, or oncogene activation. These alterations contribute to, or initiate the neoplastic state and neoplasms usually contain many genetic mutations. However, certain abnormalities are indicative of specific cancers (Rabitts, 1994). For example, chronic myelogenous leukaemia (CML) is associated with a specific translocation, t(9;22) resulting in the tumour-specific gene fusion of *BRC-ABL* (Shtivelman *et al.*, 1985).

Interestingly, Translin has been reported to bind to consensus sequence motifs ATGCAG and GCCC(A/T)(G/C)(G/C)(A/T) found at chromosomal translocation breakpoint junctions in many human lymphoid neoplasms, involving 1p32, 3q27, 5q31, 8q24, 9q34, 9q34.3, 10q24, 11p13, 14q11, 14q32, 14q32.1, 17q22, 18q21, 19p13, and 22q11 (Aoki *et al.*, 1995; Kasai *et al.*, 1992; Kasai *et al.*, 1994). These consensus sequences contain gaps or a few intervening nucleotides (Aoki *et al.*, 1995). They are also usually found at 5' flanking site of breakpoint junctions, in the vicinity of oncogenes or genes with other important functions (Kasai *et al.*, 1997). Translin binding sites have been found on both participating strands of the t(9;22)q(34;11) translocation of CML. Alu binding sites were also found near the breakpoints (Martinelli *et al.*, 2000). The role of Alu sites is not known but they are often found around breaks and may just function to keep chromosome regions close together to aid recombination (Chalk *et al.*, 1997; Martinelli *et al.*, 2000).

Translin binding sites have also been identified in solid tumours (Chalk *et al.*, 1997; Hosaka *et al.*, 2000). The TLS-CHOP fusion gene is found in myxoid and round-cell liposarcomas. It is created by the translocation t(12;16)q(13;p11). Translin binding sites were found in 10 out of 11 liposarcomas with TLS-CHOP fusion genes investigated (Hosaka *et al.*, 2000). The chromosome translocation t(2;13)q(35;14) is a characteristic of alveolar rhabdomyosarcomas; the most common form of soft-tissue sarcoma in children (Douglass *et al.*, 1987). The translocation results in a gene fusion of PAX3 and FKHR genes, causing the expression of a chimeric protein (Barr *et al.*, 1993; Galili *et al.*, 1993). Translin

was isolated from rhabdoid cell lines, but is thought to only enter the nucleus as a result of DNA damage (Chalk *et al.*, 1997). Interestingly a mutation in *Translin* was found in a breast cancer cell line, out of 137 samples of various tissue-types tested (COSMIC database, Wellcome Trust Sanger Institute). It is not known if a mutation in the *Translin* gene contributes to the cancer line tested or any other cancer, and the significance of Translin binding sites at breakpoint junctions is still unclear. Translin may function to stabilise strands participating in recombination, it may activate translocation or it may sequester other proteins to the site.

1.4.2 Microsattellite binding

In a study by Jacob *et al.*, (2004), human Translin has been shown to have higher affinities for single-stranded overhangs of microsattelite repeats (d(GT)n) and G-strand telomeric repeats (d(TTAGGG)n), than for lymphoid cancer associated translocation Bcl-CL1 consensus sequences. The minimum length of oligonucleotides that translin can bind to is 11 nucleotides. This study also showed that intramolecular DNA quadruplexes formed by the single-stranded oligonucleotides d(TTAGGG)5' inhibits Translin binding (Cohen *et al.*, 2004; Jacob *et al.*, 2004; Kasai *et al.*, 1997). Translin will only interact with DNA where a staggered break exists; moreover, it cannot bind to target sequences in the interior of single-stranded ends(Aoki *et al.*, 1995). Binding of toroidal Translin was also found to be anticooperative, suggesting that only one Translin molecule will bind to an overhang (Jacob *et al.*, 2004).

d(GT)n and d(AC)n are associated with recombination hotspots (Majewski and Ott, 2000). This suggests that in humans at least, Translin may have a function in recombination at microsatellites where single-stranded overhangs are intermediates of recombination. This may have implications to human health, as d(GT)n microsatellite repeats have been linked to susceptibility genes for several human diseases including allergies, schizophrenia and colon cancer (Tamura *et al.*, 2001; Itokawa *et al.*, 2003; Komarova *et al.*, 2002; Hirai *et al.*, 2003).

Translin may also function at the telomeres to enhance telomerase activity (Cohen *et al.*, 2004). Telomeres prevent degradation of chromosome ends by nucleases. During DNA synthesis telomeres shorten, telomerase acts to extend the GT rich strand using a reverse-transcriptase-like activity (Reviewed by Blackburn, 2001). Cohen *et al.*, (2004) used synthetic oligonucleotides as primers for telomerase extention. Translin was found to slightly stimulate primer extension at low Translin/primer molar ratios (roughly 1:2). At high ratios it inhibited the reaction by as much as 80% as it bound to the G-rich oligonucleotides preventing telomerase extension. Translin may stimulate the reaction by unwinding unusual structures and hairpin structures formed by G-residues bonding (Nadel *et al.*, 1995; Chen *et al.*, 1995), thus allowing the oligonucleotide to align correctly to the telomerase (Cohen *et al.*, 2004).

1.4.3 Possible Translin and TRAX functions in DNA repair

Translin is found in the cytoplasm of cell lines of various lineages, but found in the nucleus mainly of lymphoid cell lines (Aoki *et al.*, 1995). In mouse cell lines treated either with the DNA-damaging agents mitomycin C, etoposide, irradiation or hydogen peroxide, Translin was found to localize to the nucleus after 6 hrs of exposure, however on longer incubations the Translin protein levels decreased (Fukuda *et al.*, 2008; Kasai *et al.*, 1997). The hematopoietic colony formation after ionizing irradiation is severely delayed in Translin null mice compared to wild type, this suggests that Translin contributes to hematopoietic regeneration by acting as a sensor protein for radiation induced damage (Fukuda *et al.*, 2008). Translin lacks a nuclear localization signal so is perhaps transported to the nucleus as a result of interacting with other proteins carrying nuclear targeting signals (Aoki *et al.*, 1997a; 1997b).

The GADD (growth arrest and DNA damage inducible) genes were isolated in mammalian cells on the basis of rapid induction by a variety of DNA damaging agents [e.g. methyl methanesulphonate (MMS) and UV radiation] and growth arrest treatments (e.g medium depletion) (Fornace *et al.*, 1989). Murine GADD34 is comprised of 657 amino acids containing a large basic amino-terminal domain,

a 38 amino acid sequence repeated 4.5 times (PEST) (Rogers *et al.*, 1986) and a carboxyl terminus that is able to substitute for the herpes simplex virus 1 (HSV-1) γ,34.5 domain (He *et al.*, 1996). In differentiated myeloid leukaemia cells, GADD34 was found to prevent terminally differentiated cells from undergoing apoptosis (Chou and Roizman, 1994). In conjunction with other proteins, GADD34 suppresses cell division during DNA repair in an attempt to prevent apoptosis (Selvakumaran *et al.*, 1994; Zhan *et al.*, 1994).

GADD34 has been found to interact with a whole range of proteins. To identify interacting proteins, a mouse cDNA library was screened using the PEST and γ,34.5 homology regions of GADD34 as bait (Hasegawa *et al.*, 2000). One of the proteins isolated was Translin. GADD34 and Translin interact under *in vitro, in vivo* and in the yeast two-hybrid system. On exposure to MMS, GADD34 was demonstrated to be highly induced but Translin was not. It is thought that GADD34 may possibly be involved in the nuclear transport of Translin from the cytoplasm in response to DNA damage (Hasegawa and Isobe, 1999).

Also, GADD34, protein phosphatase 1α (PP1α) and the hSNF/INI1 component of the chromatin remodelling complex hSWI/SNH have been found to exist as a trimolecular complex *in vivo*. hSNF5/INI1 itself does not effect GADD34 activity, but GADD34-PPI association can be disrupted by proteins interacting with hSNF5/INI1 in the trimolecular complex (Wu *et al.*, 2002). GADD34 can also bind to PCNA (proliferating cell nuclear antigen), which is essential for the loading of polymerases and plays a role in DNA repair (Brown *et al.*, 1997).GADD34 also interacts with murine GAHSP40, a member of the Hsp40 family of heat shock proteins (Hasegawa *et al.*, 2000). GADD34 activity is regulated by a number of proteins e.g. Src kinase lyn (Grishin *et al.*, 2001) and BAG-1 (Hung *et al.*, 2003). Understanding the function of proteins that associate with Translin may help in determining the function of Translin.

Translin associated protein X (TRAX) was identified in a yeast two-hybrid screen as a protein that interacts with Translin (Aoki *et al.*, 1997b). Translin and TRAX form a DNA/RNA binding complex isolated from the testes (Wu *et al.*, 1999) and the brain, (known as GS1 complex in the brain) (Finkenstadt *et al.*, 2000; Taira *et*

al., 1998). TRAX is a 33 kDa protein which has a large amount of homology to Translin but no intrinsic DNA binding activity (Aoki *et al.*, 1997b). The actual function of TRAX is unknown but it is known to complex with Translin and is also an interacting partner of C1D. In response to DNA damage caused by γ-irradiation, C1D is induced and targets DNA-dependent protein kinase (DNA-PK) to specific nuclear regions (Yasui *et al.*, 1991; Yavuzer *et al.*, 1998). DNA-PK is involved in the repair of DNA DSBs and V(D)J recombination which is essential for the development of immune cells (Smith and Jackson, 1999). Association of TRAX with C1D or Translin is mutually exclusive because a C1D/TRAX interaction only requires the TRAX leucine zipper motif, whereas TRAX/Translin interaction requires the full-length of TRAX. Interaction in mammalian cells of C1D with TRAX through the TRAX leucine zipper motif only occurs in the TRAX dimeric form and only after γ-irradiation (UV radiation had no effect on interaction. Erdemir *et al.*, 2002). C1D was also found to associate with the transcriptional repressor RevErb and the nuclear coreceptor N-Cor and SMRT indicating a role in transcriptional repression (Zamir *et al.*, 1997).

In fluorescence studies in COS-7 cells the subcellular localisation of Translin was found to be mainly cytoplasmic (Chennathukuzhi *et al.*, 2001a; Erdemir *et al.*, 2002; Kasai *et al.*, 1997). γ-irradiation or treatment with any other DNA damaging agent that induces DNA DSBs did not result in any Translin relocation (Chen *et al.*, 2004; Erdemir *et al.*, 2002). TRAX localisation was nuclear and C1D showed diffuse mainly nuclear staining. Neither protein relocalised when γ-irradiated (Erdemir *et al.*, 2002). In double transfection studies of Translin and TRAX, they are seen to colocalize in both the nucleus and cytoplasm. Localisation does not change in response to γ-irradiation. C1D and TRAX were found to colocalise in the nucleus, with C1D showing again diffuse mainly nuclear staining pattern. A triple transfection was also carried out but it was not possible to determine whether TRAX co-localised with Translin or C1D (Erdemir *et al.*, 2002). C1D alone is found to usually localise in the nucleolar regions, however upon HU treatment C1D relocalised to chromatin regions and was enriched in the rRNA rich nuceolar region throughout the cell cycle (Chen *et al.*,

2004). In a recent study the yeast homolog to human C1D was found to have some sort of role in nuclear processing of pre-rRNA, snoRNAs and snRNAs. Yeast C1D may be an exosome cofactor required specifically for the 3' processing of stable RNAs (Mitchell *et al.*, 2003).

Cti1, the human homolog of C1D was found to interact with the Cut3 domain of fission yeast SMC (structural maintenance of chromosome) (Chen *et al.*, 2004). C1D has also been found to interact with transcription repressor-nuclear hormone receptor (Zamir *et al.*, 1997), and small GTPase Rac3 (Haataja *et al.*, 1998).

1.4.4 RNA Binding and the role of Translin and TRAX in developing cells

Translin/TRAX expression in peripheral tissues is comparable with the expression in the brain. It has been discovered that the Translin/TRAX complex in the kidneys is in fact masked by endogenous RNAs, preventing it from being detected by exogenous radiolabelled probes (Finkenstadt *et al.*, 2001). The Translin/TRAX complex has also been identified to be the previously characterised glucose response element binding protein (GRBP) found in liver (Wu *et al.*, 2003). GRBP / Translin/TRAX complex binds to the major late transcription factor (MLTF)-like site within the glucose response element (GRE) of the liver-type pyruvatekinase (L-PK) gene (Thompson and Towle, 1991). This suggests that the Translin/TRAX complex may have crucial functions in a much broader range of tissues than first thought.

Testes Brain-RNA binding protein (TB-RBP), the mouse homolog of human Translin, (Wu *et al.*, 1997) is 99% identical, only varying at three amino acids (Aoki *et al.*, 1997a; Wu *et al.*, 1997, 1998). It was first identified as an RNA binding protein in mouse testes (Kwon and Hecht, 1991). It was thought to only bind Y and H elements in the 3' untranslated regions of single-stranded mRNAs found in mouse testes and brain (involved in dendritic RNA processing) (Finkenstadt *et al.*, 2000; Han *et al.*, 1995a, 1995b; Wu *et al.*, 1997; Wu and Hecht, 2000). However these elements are highly G-rich and it was shown later that Translin binds almost any G-rich single-stranded sequence (Li *et al.*, 2003), suggesting that Y and H element binding is not specific.

Spermatogenesis is the differentiation and development of haploid spermatozoa, produced when diploid spermatogonia stem cells go through meiosis, a process known as spermiogenesis (Hecht, 1998). Early stages of gene expression in spermatogenesis are controlled at the level of transcription. However, half-way through spermiogenesis transcription stops and the required spermatid and spermatozoan proteins are encoded by mRNAs that are stored as ribonucleoproteins (mRNPs.) Gene expression at later stages of spermatogenesis is therefore controlled at the level of translation, these include the expression of protamines, transitional proteins and structural DNA-binding proteins (Yelick *et al.*, 1989). Translation of these mRNAs is suppressed by control proteins binding to specific sequences in the 3' and/or 5' untrantranslated regions. RNA-binding proteins are important in post-transcriptional regulation of gene expression, their functions include splicing and capping in the nucleus, mRNA transport and translation in the cytoplasm (Shyu and Wilkinson, 2000). Translin will bind to Y, H elements or G-rich sequences present in the 3' UTR of mRNAs in both testes and neurons and prevent their expression (Finkenstadt *et al.*, 2000; Kobayashi *et al.*, 1998; Kwon and Hecht, 1991, 1993; Wu *et al.*, 1997). Proteins that do this must contain appropriate sensors to allow the correct temporal and spatial release of mRNAs. In male germ cells, Translin localises in the nuclei of meiotic spermatocytes but is found in the cytoplasm in all subsequent stages (Morales *et al.*, 1998).

Male germ cells and neurons are two of a number of cell types that undergo major differentiation and polarization. This requires the transport and specific localization of vesicles, proteins, mRNAs and organelles. Transport processes are carried out by the kinesin and dynein superfamily of motor proteins (Zou *et al.*, 2002). Translin associates with a number of proteins in order to carry out mRNA sorting. In male germ cells Translin will transport specific mRNAs intracellulary and intercellularly in a mouse ribonucleoprotein complex containing Ter ATPase (Morales *et al.*, 2002). AKAP-4 (Kinase A anchoring protein) is an X-chromosome linked protein which forms part of the fibrous sheath in the sperm flagellum and so is synthesized towards the end of spermatogenesis (Carrera *et*

al., 1994; El-Alfy *et al.*, 1999). Both AKAP-4 and protamine 2 mRNA associate with Translin/TRAX in a complex containing Ter ATPase (Morales *et al.*, 2002). Ter ATPase functions as a hexameric protein complex involved in the ATP-dependent formation and movement of vesicles. Certain mRNAs will be transported in this ribonucleoprotein complex through nuclear pores and intercellular bridges in male germ cells (Morales *et al.*, 2002). Translationally delayed mRNAs have also been found to be linked to microtubules by Translin (Han *et al.*, 1995a). The kinesin KIF17b was coimmunoprecipitated with Translin and its binding partner TRAX from mice testes cytoplasmic extract (Chennathukuzhi *et al.*, 2003a). KIF17b is responsible for the nucleoplasmic localization and transcriptional coactivation of activator of cremin testes (ACT), the activator of cAMP-responsive element modulator (CREM) in postmeiotic germ cells (Macho *et al.*, 2002). The translationally delayed and X chromosome encoded mRNAs bound by Translin (Morales *et al.*, 2002), are dependent on CREM for their transcription. This suggests that KIF17b controls both the transcription and translation of specific male germ cell mRNAs. Translation does not occur during mRNA transport, but after the dissociation of KIF17b and the later dissociation of Translin (Chennathukuzhi *et al.*, 2003a).

Cho *et al.* (2004) used COS-1 cells and mouse embryonic fibroblasts (MEFs) from *tsn* null mice to investigate the localization of Translin/TRAX dependent on their protein levels in the cells. Both proteins can be present in both the nucleus and cytoplasm (Cho *et al.*, 2004). Translin contains a putative nuclear export signal (NES) located in the centre of the protein, which may be dependent on CRM1 cellular export receptor for subcellular movement (Chennathukuzhi *et al.*, 2001a). TRAX contains a putative bipartite nuclear localization signal (NLS) (Aoki *et al.*, 1997b; Chennathukuzhi *et al.*, 2001a). The localization of Translin/TRAX in meiotic male and female germ cells varies through development. At pachytene Translin/TRAX are found mainly in the nucleus, but in the diplotene/diakinesis stage they are found in the cytoplasm. Coexpression of both proteins in COS-1 cells and MEF cells showed that the ratio of TRAX to Translin determines their subcellular localization. Translin/TRAX are found to

localize in the nucleus when TRAX levels are higher than Translin, and they localize in the cytoplasm when Translin levels are higher than TRAX (Cho *et al.*, 2004). A suggested model is that Translin/TRAX mRNA complex in the nucleus binds with KIF17b and is exported to the cytoplasm through the nuclear pores using the NES present on Translin and the CRM1 receptor. In the cytoplasm, the complex localizes the mRNA while suppressing its translation. KIF17b dissociates, then Translin/TRAX re-cycle back to the nucleus (Cho *et al.*, 2004). Translin will also bind α-Ca^{2+} calmodulin-dependent protein kinase II mRNA and ligatin to microtubules in developing neuronal cells (Wu and Hecht, 2000; Burgin *et al.*, 1990; Miyashino *et al.*, 1994), and in the testes it will bind protamines 1 and 2 to microtubules (Wu and Hecht, 2000), which are used to translocate proteins across the cell (Bassell *et al.*, 1994). Translocation requires the action of motor proteins such as kinesins along microtubules (Knowles *et al.*, 1995). It may be possible that Translin is acting as a kinesin as it contains two amino acid domains (residues 9-35 and 14-47) which have 55-62% similarity to two segments of human kinesin heavy chain (Wu *et al.*, 1997). Suppression of the kinesin heavy chain alters only the localization of the α-CAMK II mRNA, not ligatin (Severt *et al.*, 1999; Wu and Hecht, 2000). This implies that the differential sorting of mRNAs involves multiple mRNPs and a range of different motor proteins resulting in a range of mRNA localization sites (Severt *et al.*, 1999). Other evidence also suggests that Translin acts as an anchoring protein for mRNAs to dock onto microtubules and only when it is associated with TRAX and Ter ATPase is translocation possible (Wu *et al.*, 1999).

Translin contains a putative GTP binding site, between residues 159-163 (VTAGD), which share substantial homology with the DTAGQ sequence present in many G-proteins (Chennathukuzhi *et al.*, 2001; Takai *et al.*, 2001). This GTP binding site is identical in all known sequences of Translin, except in *Drosophila* and *Xenopus* where it differs by one amino acid relative to the human sequence (Chennathukuzhi *et al.*, 2001). In the presence of GTP, protamine 1 and 2 mRNA binding to Translin is reduced by 50% and by 90% in the presence of GTPγS compared to Translin alone or in the presence of GDP in both recombinant and

endogenous forms of Translin. Site-directed mutagenesis of the site to VTNSD (TB-RBP_{GTP}) from VTAGD, resulted in the loss of GTP binding, DNA binding however was unaffected (Chennathukuzhi *et al.*, 2001). Loss of GTP binding activity did not result in the loss of the multimeric structure (Chennathukuzhi *et al.*, 2001). GTP binding may be required for microtubule association as seen with the *Drosophila* gene product Orbit, which binds directly to microtules in a GTP-dependent manner (Inoue *et al.*, 2000). Interestingly, transfection of NIH3T3 cells with TB-RBP_{GTP} caused cell death (Chennathukuzhi *et al.*, 2001).

In an attempt to identify protein components of RISC (involved in the RNAi pathway), Translin was found tightly bound to siRNA. In overexpression studies in *E. coli* Translin was found to possess both ss RNase and ds RNase activities (Wang *et al.*, 2004), although Translin does not show traditional RNase folding (Pascal *et al.*, 2002). Under almost physiological conditions Translin can process 500 bp dsRNA into roughly 25 bp fragments from both open ends (ATP/GTP was not required.) However, turnover rates for digestion are very low due to strong product inhibition. Only when the protein is proteolyzed by a protease can the product be released (Wang *et al.*, 2004).

P450c17 is a steroidogenic enzyme which has two major activities, and these are controlled by post translational modifications (Zhang *et al.*, 1995; Pandey *et al.*, 2003) and by allosteric factors (Auchus *et al.*, 1998; Geller *et al.*, 1999). Transcriptional regulation of P450c17 is both species-specific and tissue-specific, and is regulated by steroidogenic factor-1 (SF-1) (Givens *et al.*, 1994; Zhang and Mellon, 1996, 1997). Translin has been found to activate SF-1-mediated transcription in a DNA-specific manner. Using a mutant form of murine Translin which cannot bind to DNA (Aoki *et al.*, 1999), it was found that Translin increases SF-1-stimulated transcription without binding to DNA (Mellon *et al.*, 2007). Co-transfection of Translin and TRAX lead to an even greater stimulation of SF-1 suggesting that TRAX works with Translin to activate SF-1-stimulated transcription (Mellon *et al.*, 2007).

In a yeast two-hybrid library screen of mouse testes cDNA, the kinesin family member KIF2Aβ was identified as a TRAX-interacting protein. KIF2Aβ only

interacts with TRAX, no interaction was detected with Translin. In transfected cells, KIF2Aβ was found to colocalise with TRAX in the perinuclear region. As with TRAX, the expression of KIF2Aβ is enhanced in mouse male germ cells, suggesting a role in spermatogenesis (Bray *et al.*, 2004). In a previous study TRAX was found to interact with four proteins (one of which was Mea2), and all were found to localize to perinuclear structures in mouse male germ cells undergoing spermatogenesis (Bray *et al.*, 2002). Mea2 is essential for mouse spermatogenesis and is expressed at elevated levels in pachytene stage of development (Matsuda *et al.*, 2004). TRAX and Mea2 were found to accumulate in the Golgi complex of mid-late pachytene spermatocytes (Matsuda *et al.*, 2004). *In vitro* the Translin/TRAX complex can bind to Mea2 (Bray *et al.*, 2004). The *S. pombe* homolog of Mea2 is Spo15p which was found to localise at spindle pole bodies (SPBs) (Ikemoto *et al.*, 2000), where vesicles derived from the Golgi and ER accumulate to later form the forespore membrane (Tanaka and Hirata, 1982; Moens, 1971). In mutant Spo15p strains, neither forespore membranes nor spore walls are formed, this may suggest that aggregation at the SPBs of vesicles that form the forespore membrane is dependent on Spo15p (Ikemoto *et al.*, 2000). It is not known if TRAX interacts with Spo15p, it could be that Spo15p is located in Golgi but remains at the SPBs during the meiotic divisions, this would explain the fluorescence data of Ikemoto, *et al.* (2000), where GFP tagged Spo15p is seen at the ends of the spindle.

Retinal ganglion cells (RGCs) retain a limited ability for axonal growth after birth (Busch and Silver 2007; Goldberg and Barres, 2000). Injured rodent RGCs upregulated the growth-associated protein (GAP)-43 (Skene and Willard, 1981; Fishman, 1996), which in adult neurons enhances sprouting at axon terminals (Caroni *et al.*, 1997), whereas its loss impairs axonal growth in response to cell-adhesion molecules such as laminin (Meyer *et al.*, 1994). GAP-43 alone appears insufficient to trigger regeneration of dorsal-column fibers following spinal cord injury (Neumann and Woolf, 1999). Interestingly TRAX function appears to be related to GAP-43 regulation, by functioning as a transcriptional controller of the regulation of GAP-43 expression and hence effecting axonal regeneration.

Silencing of TRAX at different stages of retina development, regulates both GAP-43 transcript and protein (Schröer *et al.*, 2007).

The tumour suppressor protein p53 mediates cell cycle arrest through the induction of p21, which inhibits cyclin-dependent kinases (el-Deiry *et al.*, 1994; Xiong *et al.*, 1993). In the central nervous system p53 has been implicated in neuronal apoptosis caused by various stresses and disorders (Bae *et al.*, 2005; Gilman *et al.*, 2003; Herzog *et al.*, 1998; Jordan *et al.*, 2003). Adenosine has been shown to play an essential role in mediating neuronal function via four adenosine receptors (Daval *et al.*, 1991). The A_{2A} adenosine receptor (A_{2A}-R) is involved in regulating neuronal plasticity and development (Cheng *et al.*, 2002; Ribeiro, 1999; Weaver, 1993). In PC-12 cells (protein kinase A-deficient), stimulation of the A_{2A}-R activates at least two major signaling cascades (Chang *et al.*, 1997; Huang *et al.*, 2001; Sobreviela *et al.*, 1994). In two dominant-negative p53 mutants, stimulation of the A_{2A}-R suppresses proliferation and rescues the differentiation process impaired by p53 activation. Interstingly, TRAX was identified as a novel interacting protein of the A_{2A}-R and overexpression of TRAX recovered nerve growth factor (NGF) induced neurite outgrowth impaired by p53 inhbition in PC-12 cells. Down regulation of endogenous TRAX using an antisense construct obliterated the rescue effect of A_{2A}-R (Sun *et al.*, 2006).

1.4.5 Role in mitosis

By comparing basal expression levels of various proteins during mitotic cell division, it has been discovered that the rate of cell proliferation correlates with the level of Translin in the cell (Ishida *et al.*, 2002). The expression of Translin was looked at in ATM-deficient mouse spleen cells exposed to ionising radiation. In wt ATM(+/+) spleen cells Translin expression was down-regulated, while Translin levels in ATM-null mice (no cell cycle arrest) did not change and in heterozygous cells Translin expression was at an intermediate level (Ishida *et al.*, 2002).When Translin expression was increased using controllable promoters the rate of cell proliferation increased. Translin expression was found to be periodic during the cell cycle in both wt and overexpressing cells, indicating that Translin

levels are strictly controlled during cell cycle progression; Translin levels being maximal at S phase and at G2/M phase, suggesting roles in DNA replication and accelerating cell division (Ishida *et al.*, 2002).

Confocal microscopic analysis (see Fig. 1.7) showed a dispersed distribution of Translin in the cytosol. At prophase Translin was detected on the centromeres, which has also been observed in *Xenopus* oocytes (Castro *et al.*, 2000). When the cells entered mitosis, Translin was seen on the astral microtubules which radiate from the centromeres at prometaphase/metaphase transition. This may imply that Translin accelerates microtubule organization and chromosome segregation during mitosis (Ishida *et al.*, 2002). It has also been seen that the reduction of TRAX in HeLa cells results in reduced levels of proliferation (Yang and Hecht, 2004). The slow proliferation of MEFs from TB-RBP null mice can be rescued with the reintroduction of TB-RBP into the null MEFs (Yang *et al.*, 2003). When TB-RBP is reintroduced, TRAX levels return to normal. Translin is required to stabilize TRAX by forming heteroligomers, in the absence of Translin, TRAX is ubiquinated and degraded (Yang *et al.*, 2004). In mice TRAX mRNA levels were the same in wild-type, heterozygous and homozygous TB-RBP-deficient progeny, but translated TRAX mRNA was decreased by 50% in heterozygotes and absent entirely in null mice. Interestingly, the overexpression of TRAX still only results in a basal level of protein being produced, thought to be due to Translin regulating the amount of TRAX translation (Chennathukuzhi *et al.*, 2003). This means that both Translin and TRAX can be directly linked with cellular proliferation.

Translin has been suggested to work together with Survivin, a member of the inhibitor of apoptosis (IAP) family (Li *et al.*, 1998). They share many similarities in cell cycle-dependent expression and spindle localization. Translin may work with Survivin or other chromosomal passenger proteins to allow efficient cytokinesis (Ishida *et al.*, 2002).

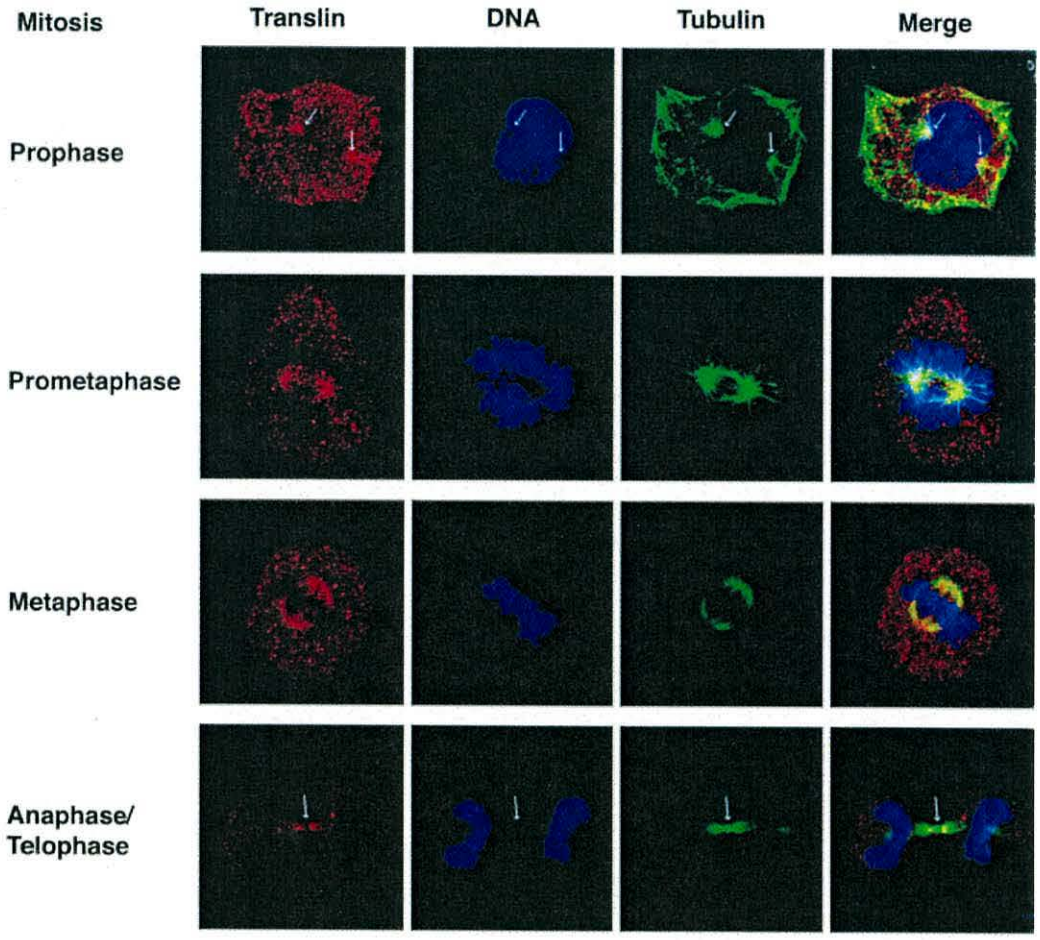


Figure 1.7 Localisation of Translin. Translin localizes to the centrosome, bipolar mitotic spindles and midzone. HeLa cells at prophase, prometaphase, metaphase and anaphase/telophase were stained Hoechst 33258 for the nucleus (blue), FITC anti-α-tubulin (green), and anti-Translin followed by goat anti-rabbit IgG-TRITC (red). Localization of tubulin and Translin was examined by confocal laser scanning microscopy. (Figure and legend taken from Ishida *et al.*, 2002).

1.4.6 Translin null mutants

Chennathukuzhi *et al.* (2003) studied the effects of *wt*, heterozygous and homozygous TB-RBP null mice. All progeny were viable from heterozygous crosses with normal Mendelian ratios. TB-RBP null mice were stunted by 10-30% compared to wild-type and heterozygous progeny. All internal organs were morphologically normal, and by 6 months of age the null mice were the same weight as the wt and heterozygotes. Null mice had reduced cell proliferation in the first few months of development and a disproportionate accumulation of fat mass in the abdominal region (Chennathukuzhi *et al.*, 2003). In the same study, TB-RBP null males were fertile but produced fewer spermatozoa and showed abnormal spermatogenesis, demonstrating that Translin plays an important role in sperm development. A similar phenotype was seen in null females, where smaller litters were produced (Chennathukuzhi *et al.*, 2003).

Translin is proposed to be required for early lymphocyte development, and is thought to be transported into the nucleus for processes such as immunoglobulin or TcR rearrangements (Aoki *et al.*, 1995). Early T and B cell precursors in the thymus lack the cell surface expression of the coreceptor molecules CD4 and CD8, they are $CD4^-$, $CD8^-$ double negative (DN). Production of $CD4^+$ and $CD8^+$ double positives (DP), requires a recombination event at the TcRβ locus in order to differentiate into DP thymocytes. Additional rearrangements in DP at the TcRα locus give rise to TcRα- TcRβ heterodimers which differentiate into $CD4^+$ $CD8^-$ or $CD4^-$ $CD8^+$ single positives (SP) T cells (Kruisbeek, 1993; von Boehmer and Fehling, 1997). If TB-RBP played a crucial role in T-cell development then in null mice T-cell development would be arrested at the DN stage as seen in V(D)J recombinase-activating gene (Rag) deficient mice (Mombaerts *et al.*, 1992; Shinkai *et al.*, 1992). However, both wild-type and Translin-null mouse T-cell development was normal with an equivalent percentage of DN, DP and SP cells. Similar rearrangements occur in B-cell development and this again was not found to require Translin (Chennathukuzhi *et al.*, 2003).

Microarray analysis of the brain mRNA showed altered gene expression in null mice brain extracts compared to wild-type. 14 genes showed up-regulation and 217 genes were down-regulated in a screen of 12,473 probe sets. Among those strongly down-regulated were numerous neurotransmitter receptors and ion channels, including γ-aminobutyric acid A receptor α 1 and glutamate receptor α 3. Behavioural abnormalities were also observed and TB-RBP null mice appeared docile with reduced Rota-Rod performance compared to wild-type mice (Chennathukuzhi *et al.*, 2003). Translin null mice also have reduced levels of the monoamine neurotransmitters, norepinephrine and serotonin in several forebrain regions in both males and females (Stein *et al.*, 2006). Interestingly many of the behavioral changes displayed by Translin-null mice resemble those reported for mice lacking FMRP, which has been implicated in regulating translation of dendritic transcripts and in fragile X mental retardation (Stein *et al.*, 2006). This evidence further links Translin/TRAX with the development of dendrites.
As in the mouse model, studies in *Drosophila* show that Translin-null mutants are viable. *Drosophila* Translin was also found to be essential for normal TRAX levels and the loss of Translin led to the loss of TRAX protein (Clauβen *et al.*, 2006). *Drosophila* Translin was found to be expressed in several tissues throughout development, and in the adult brain Translin was expressed in a large number of neuronal bodies and cytoplasmic expression in early spermatocytes (Suseendranathan *et al.*, 2007). In *Drosophila* Translin mutants, defects in locomotion were detected but no defect in fertility was found compared to wild-type (Clauβen *et al.*, 2006; Suseendranathan *et al.*, 2007). Interestingly *Drosophila* Translin was found to have almost no nucleic acid-binding propensity (Sengupta *et al.*, 2006), suggesting that *Drosophila* Translin has no chromosomal function. In embryonic stages 8-9, *Drosophila* Translin appears in ventral neuroblasts. During embryogenesis, neuroblasts delaminate from the ventral neuroectoderm and subsequently divide asymmetrically to form a neuroblast and a ganglion mother cell (GMC). Division of GMCs forms the neuronal and the glial components of the brain and the ventral nerve cord (Urbach *et al.*, 2003). *Drosophila* Translin is found to be enriched in neuroblasts after delamination

(Suseendranathan *et al.*, 2007). Much of this evidence strongly suggests a role for Translin and TRAX in neuronal development.

1.5 *Schizosaccharomyces pombe* as a model eukaryote

S. pombe is an African brewing yeast which was originally isolated from millet beer by Lindner and developed as an experimental model by Urs Leupold in the 1950s. It has a fully sequenced genome of 4979 genes and has 14 Mb of DNA distributed between only three chromosomes (Wood *et al.*, 2002). It has very few duplicated genes, making it an ideal model for functional genetics. Laboratory strains are believed to be predominantly isogenic, avoiding many of the problems encountered with studies in *S. cerevisiae*. Several groups of genes are conserved in *S. pombe* and humans, but missing in other model organisms.

Haploid or diploid strains can be easily propagated, having a doubling time of only 2-4 hrs and regularly shaped cells. Under starvation or nutritional depletion zygotes are formed. Haploid strains of opposite mating-type can be crossed, creating new strains of the desired genotype or for studying meiosis and recombination. Since *S. pombe* has only three chromosomes, a significant number of viable spores from mutants exhibiting random segregation at MI or MII can be obtained. It has also been commonly used to study cell cycle dynamics, mitosis, meiosis, chromosome dynamics, epigenetics, DNA repair and mRNA processing. It is genetically tractable and commonly used methods of mutagenesis, selection, suppressor and synthetic lethal analysis are all easily performed. It can be rapidly transformed with a wide selection of fission yeast-specific integrating or episomal plasmids. This system also lends itself to biochemical and proteomic approaches. With the sequencing of many other species underway, *S. pombe* is also a powerful tool for comparative genomics.

Thus far the actual function of Translin and TRAX has eluded researchers. *S. pombe* has both Translin and TRAX homologs (which *S. cerevisiae* does not). Understanding the function of Translin and TRAX in a simpler eukaryote like *S. pombe* may result in the breakthrough required to elucidate their function in higher organisms. *S. pombe* has also been used to study tRNA genes and non-sense suppression (Kohli *et al.*, 1989). Due to the fact that the *S. pombe* genome can be easily manipulated and studied, it makes it the perfect model to study the

effect a single tRNA gene may have on replication dynamics when placed in an ectopic site.

1.6 Overriding aims of this study:

- Investigate a possible role for Translin and TRAX in DNA damage repair, recombination and cell cycle progression
- Investigate any recombinogenic effect and RFP potential of a single tRNA gene and B-box repeats placed at an ectopic site

Chapter 2 Materials and Methods

2.1 Yeast crosses

Cells of the opposite mating type (h^+ or h^-) were grown in 5 ml YEL at optimum temperature until saturated. Equal volumes of cells (600 µl) were then mixed, washed and spotted onto fully supplemented synthetic sporulation media (SPA), and incubated at 30°C for a minimum of 2 days. The cells were then scrapped off the plates and resuspended in 1 ml water with 3 µl β-glucuronidase (Sigma) and incubated at 25°C for 16 hrs. Following incubation 0.5 ml 100% ethanol was added, the spores washed and plated out onto YEA media. Plates were incubated for 3 days at 33°C, and colonies were replica plated onto selective media as required.

2.2 Mating-type tests

Progeny from a meiotic cross were mixed with h^+ cells (BP1) and h^- cells (BP8) on fully supplemented synthetic sporulation media (SPA) and incubated for 2 days at 30°C. Plates were then stained with iodine and iodine positive stained spots indicate the original mating-type of the parental strains.

2.3 Storage of *S. pombe* strains

Single colonies were grown in 5 ml liquid media until saturation. Glycerol was added to a final concentration of 25% and cells stored at -80°C.

2.4 Targeted gene replacement

The kanamycin cassette used for transformation was amplified using plasmid pFA6a-kanMX6 (Bähler, *et al.*, 1998), as template DNA, with primer pairs Tsn-kan-F and Tsn-kan-R giving the kanamycin cassette for *tsn1* replacement. PCRs were carried out using FINNZYME polymerase and a PCR programme of: 94°C for 1 min, then 25 cycles of 94°C for 1 min, 55°C for 30 secs, 72°C for 90 secs, with a final extension at 72°C for 5 mins. Several PCRs were pooled and purified

using phenol/chloroform (see 2.5). The cassettes were transformed into *S. pombe* strains (BP90 and BP296) as described by Baehler, *et al.,* (1998).

2.5 DNA purification by phenol/chloroform

0.1 M NaCl and an equal volume of phenol/chloroform at a 1:1 ratio was added to a DNA solution, then spun at 13,000 r.p.m. for 15 mins, and the aqueous layer removed and 3 x volume 100% ethanol added and left at -80°C for 1 hr to precipitate the DNA. The DNA was spun down at 13,000 r.p.m. for 15 mins, washed with 70% ethanol, the pellet air-dried and dissolved in TE buffer (10 mM Tris-HCl (pH 7.5) and 1 mM EDTA (pH 8.0)).

2.6 Yeast lysis and DNA extraction

Single colonies were inoculated into 5 ml YEL and grown at optimum temperature (usually 33°C) until saturated. Cells were harvested by centrifugation at 4,000 r.p.m. on a bench top centrifuge and resuspended in 1 ml H_2O and washed. 200 µl of Lysis Buffer (1 ml Triton-X 100, 5 ml of 10% SDS, 0.5 ml of 1 M TRIS, 5 ml of 1 M NaCl and 100 µl of 0.5 M EDTA) was added along with 100 µl Phenol, 100 µl Chloroform and 0.3 g acid washed beads. The cells were vortexed for 3-4 mins and then centrifuged for 12 mins at 13,000 r.p.m.. The top aqueous layer was removed and added to 1 ml of 100% Ethanol. This was then left at -18°C for at least 1 hr and then centrifuged for 12 mins at 13,000 r.p.m.. The supernatant was removed and the pellet washed with 1 ml 70% Ethanol, then air-dried and resuspended in 100 µl TE buffer [10 mM Tris-HCl (pH 7.5) and 1 mM EDTA (pH 8.0)].

2.7 PCR screening

For the PCR screening of progeny from specific crosses, 1 µl of 10% dilution in water of extracted genomic DNA was used in a reaction using either 2 x ReddyMix (BioLine) (PCR program: 96°C for 1 mins, then 30 cycles of 96°C 1 mins, X°C for 30 secs, 72°C as necessary, then 72°C for 5 mins final extension),

or using Phusion High-Fidelity DNA Polymerase (FINNZYME) (PCR program: 98°C for 1 mins, then 30 cycles of 98°C 10 secs, X°C for 30 secs, 72°C as necessary, then 72°C for 5 mins final extension). The annealing temperature (X) varied with the primer sets, and the extension times were calculated as 1 mins per kb for 2 x ReddyMix and 15 secs per kb for Phusion High-Fidelity DNA Polymerase.

2.8 Sequencing

All sequencing of PCR products was done out of house by MWG Biotech.

2.9 Yeast transformation procedures

2.9i) Chemical transformation using lithium acetate (for transformation with plasmids)

Cultures were grown in 100 ml of YEL or selective media to a density of 0.5-1 x 10^7 cells per ml. Cells were harvested by centrifugation at 3,000 r.p.m. for 5 mins at room temperature, then washed in 40 ml of water. The cells were resuspended at 1 x 10^9 cells per ml in 0.1 M lithium acetate (adjusted to pH 4.9 with acetic acid) and dispensed into 100 µl aliquots and incubated at 30°C for 1-2 hrs. Sedimented cells were resuspended and 1 µg of plasmid DNA in 15 µl TE buffer (pH7.5) was added, along with 290 µl of 50% (w/v) PEG in LiAc pre-warmed to 30°C. The cells were gently mixed with a cut 1 ml tip and incubated at 30°C for 1 hr. After incubation the cells were heat shocked at 43°C for 5 mins and then allowed to cool to room temperature. After centrifugation at 5,000 r.p.m. for 2 mins, the supernatant was removed and the pellet resuspended in 1 ml of ½ YE5S (YE supplemented with adenine, uracil, leucine, histidine and arginine), and incubated at 32°C for 10 mins. The cells were then washed and dilutions plated out on selective media.

For the NHEJ assay; plated cells on NB supplemented with adenine and leucine to select for transformants and on NB fully supplemented with adenine leucine and uracil for the viable cell count.

2.9ii) Yeast transformation by electroporation

Cultures were grown to a density of 1 x 10^7 cells per ml in YEL media. 50 ml samples were then chilled on ice for 20 mins, and then harvested at 3,000 r.p.m. for 5 mins at 4°C. Each sample was then washed three times in ice-cold 1 M Sorbitol before being resuspended in 1 M Sorbitol to a density of 1 x 10^9 cells per ml. 200 µl of prepared cells were placed in chilled 0.2 cm cuvettes (Geneflow) with 1 µg of transforming DNA (with a volume of < 10 µl), then immediately shocked at 2 kV. After pulsing 1 ml of ice-cold 1 M Sorbitol was added, and the cells plated out onto selective media and incubated for 6 days at 30°C.

2.10 Plasmid by chromosome recombination assay, fluctuation analysis and determination of mutation rates

Both plasmid by chromosome recombination assays and fluctuation analysis were carried out following the same method. For one repeat five or seven, whole single colonies were picked off YE solid media or selective NB (for plasmid retention), and grown until stationary phase at 33°C with shaking in 5 ml YEL or NBL. Serial dilutions were then made in 0.85 % saline solution and plated out on YE solid media or on fully supplemented NB to give the viable cell count, and on YE + guanine (adjusted to pH 6.5 with 1 M HCl and containing 20 mg/ml of guanine dissolved in 0.35 M NaOH) or NB fully supplemented but lacking adenine for the adenine prototroph count. Mutation rates or recombination rates were calculated from the median Ade^+ colonies per total cell number of culture (Lea and Coulson, 1949).

2.11 Recombination assays

2.11i) Intragenic recombination assay

Serial diluted spore suspensions were plated out on YE solid media or NB fully supplemented media to give the viable spore count, and on YE + guanine (adjusted to pH 6.5 with 1 M HCl and containing 20 mg/ml of guanine dissolved in 0.35 M NaOH) or NB fully supplemented but lacking adenine for the adenine

prototroph count. Plates were incubated at 33°C for 5 days. Recombination frequencies were determined as prototrophs / 10^6 viable spores.

2.11ii) Intergenic recombination assay

Serial diluted spore suspensions were plated out onto YE to roughly 100 colonies per plate and the plates incubated at 33°C for 5 days. Colonies were then replica plated onto NB selective media to allow only recombinant prototroph growth. The intergenic recombination frequency is the summed values of double auxotrophs as a percentage of viable spores. Recombination frequencies were used to determine the genetic distance (cM) by employing Haldane's mapping function (genetic distance [cM] = -50ln[1-2R], where R = the total fraction of recombinant spores amongst all spores analysed) (Wells, *et al.*, 2006).

2.12 DNA damage sensitivity tests

Cultures were grown overnight in 5 ml YEL at 30°C and resuspended to roughly 5 x 10^6 cells/ml. Four serial dilutions were made and 5 µl of each dilution was spotted onto supplemented YEA containing various concentrations of DNA damaging agents, then incubated for approximately 4 days at 20°C, 30°C or 37°C.

2.13 Antibodies, whole cell protein extraction and Westerns blots

Anti-Tsn and anti-Trax polyclonal antibodies were raised out of house (Eurogentec, Liege, Belgiun). Synthetic peptides EQSRNENLQEKEHGL and LKNDSLRRHFDGLKY corresponding to residues 45-58 and 204-218 respectively of the translation of the *S. pombe* gene SPAC30.03c were inoculated into rabbit to produce N-terminal and C-terminal anti-Tsn antibodies. Synthetic peptides SDGFPLPKDFDRTSI and VDTATPPEEKRLRST corresponding to residues 46-60 and 217-231 respectively of the translation of the *S. pombe* gene SPCC736.09c were inoculated into guinea pig to produce N-terminal and C-terminal anti-Trax antibodies. Both serum sets were affinity purified prior to use. For whole cell protein extracts, 50-100 ml of culture was centrifuged at 4,000 r.p.m. for 5 mins for the cells to be harvested. The pellet was resuspended in 1.5

ml of ice cold STOP buffer (150 mM NaCl, 10 mM EDTA (pH 8.15) and 1 mM NaN_3), and transferred to a 1.5 ml centrifuge tube and spun at 5,000 r.p.m. for 5 mins. The supernatant was discarded and 50 µl RIPA buffer [10 mM Tris-HCl (pH 7.8), 1% Triton-X 100, 0.1% SDS, 2 mM EDTA (pH 8.15), 150 mM Sodium Ortho-Vanadate and complete protease inhibitor cocktail tablets (Roche)], was added and 0.3 g acid washed glass beads (Sigma). Cells were ruptured at maximum setting for 20 secs using a Bead Beater, then a further 400 µl RIPA buffer added and run in the Bead Beater for an additional 10 secs, then centrifuge at 20,000 g for 20 mins at 4°C. The lysate was collected and centrifuged at 20,000 g at 4°C for a further 10 mins. The lysate was collected again and stored at -80°C. The protein concentration was determined using Bradford Reagent (Sigma) by following the manufacturer's instructions. Roughly 30 µg samples of protein with the addition of reducing agent (Fermentas), were run on a 10-12% SDS-Page gel and then electroblotted onto PVDF transfer membrane (Amersham Biosciences UK Ltd). Membranes were blocked in 10% non-fat milk in 1 x PBS (Sigma) overnight, then washed in 1 x PBS and 0.5% TWEEN (Sigma) for 5 mins. All membranes were probed with primary and secondary antibody in 10% non-fat milk, 1 x PBS and 0.5% TWEEN for 1.5 hrs. Between probing the membrane was washed 2 x 5 mins, then 1 x 10 mins in 1 x PBS and 0.5% TWEEN. Donkey anti-rabbit IgG-HRP (1/5000) (Santa Cruz Biotechnology), donkey anti-guinea pig IgG-HRP (1/5000) (Jackson Immuno Research) were used as secondary antibodies to C-terminal anti-Tsn (1/4000) and C-terminal anti-Trax (1/5000). Blots were also probed with monoclonal anti-α-tubulin antibody (1/10000) (Sigma-Aldrich [T5168]) and goat anti-mouse IgG-HRP (1/5000) secondary antibody (Santa Cruz Biotechnology). The blots were visualized using ECL chemiluminescence technique as described by the manufacturer (Roche).

2.14 Genomic DNA extraction and Southern blots

Cultures of 100 ml were grown to late stationary phase and the cells harvested at 3,000 r.p.m. for 5 mins. The pellet was resuspended in 5 ml of lysis buffer [50 mM citrate/phosphate (pH 5.6), 40 mM EDTA (pH 8.0) and 1.2 M Sorbitol] and

15 mg Zymolyase-20T was added and then incubated at 37°C for 30-60 mins or until cells have been lysed. Then spun down at 3000 r.p.m. for 5 mins, and the pellet was resuspended in 15 ml 5 x TE buffer and 1.5 ml 10% SDS. 5 ml of 5 M potassium acetate was then added and incubated on ice for 30 mins, then centrifuged at 4000 r.p.m. at 4°C for 15 mins. The supernatant was collected and spun again. 20 ml ice cold isopropanol was then added and left for 5 mins at -20°C. This was then centrifuged at 10,000 r.p.m. for 10 mins and the pellet resuspended in 3 ml of 5 x TE and RNAse added to a final concentration of 20 µg/ ml and incubated for 2 hrs at 37°C. The DNA was then purified by adding 3 ml of phenol/chloroform (1:1) and the aqueous phase collected after centrifugation at 10,000 r.p.m. for 10 mins. DNA was precipitated using 0.3 ml of 3 M sodium acetate and 7.5 ml of ethanol, incubating on dry ice for 1 hr and finally spun down at 10,000 r.p.m. for 10 mins. The pellet was washed with cold 70% ethanol, dried and resuspended in 0.2 ml of TE.

For Southern blots probed with the *tsn1* and *kanamycin* probes (see chapter 3) genomic DNA was digested with *Bsp*HI, purified using phenol/chloroform and precipitated with ethanol before being run on an 0.8% agarose gel. DNA was then blotted onto Hybridization Transfer Membranes (GeneScreen) by following the manufacturers' instructions. Probes were radio labelled with ^{32}P using MegaPrime DNA Labelling System (Amersham Biosciences) following the manufacturer's instructions. The blot was probed and then visualised using a phosphor imager.

2.15 2-dimensional electrophoreses of *ade6* disrupted strains.

S. pombe cells were grown in 400 ml YEL with adenine to a cell count of 1 x 10^7 cells/ml, then decanted into pre-chilled centrifuge tubes and sodium azide added to a final concentration of 0.1% to stop the cells metabolising further. Ice flakes were added to the flasks and the flasks chilled on ice with swirling for 1 mins before the cells were harvested at 10,000 r.p.m. for 10 mins at 4°C. The supernatant was removed and the pellet washed with ice cold water and spun at 6,000 r.p.m. for 10 mins at 4°C. (At this stage the pellet of cells can be stored at -70°C.)

For cell lysis, the pellet was resuspended in 2 ml NIB Buffer [Nuclear isolation buffer; 17% glycerol, 50 mM MOPS (pH 7.2), 150 mM KAc, 2 mM $MgCl_2$, 0.5mM spermidine, 0.15 mM spermine, to a final pH7.2 with KOH], and transferred to cold 50 ml Falcon tubes, containing 2-3 ml of acid washed glass beads and then under went 15 cycles of 30 secs of vortexing and 30 secs on ice. The lysate was removed from the beads and transferred to cold Oakridge tubes, the beads were then washed x 3 with equal volumes of ice cold NIB and each time the solution pooled. The pooled suspension was centrifuged at 13,000 r.p.m. for 30 mins at 4°C. The supernatant was removed and 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 hrs, then spun at 5,000 r.p.m. for 5 mins at 4°C and the supernatant transferred to 15 ml Falcon tubes containing 4.2 g cesium chloride. Samples were then loaded into Quick-Seal centrifuge tubes (Beckman) and 0.025% Hoescht Dye 33258 (5 µl of a 5 mg/ml stock solution in water) was added and centrifuged at 55,000 r.p.m. for 18 hrs at 20°C. DNA was visualized on long wave UV and the middle band collected via syringe and washed 5 times with equal volume of 5:1 isopropanol:distilled water. DNA was mixed and spooled out with 2 volumes of ice cold 70% Ethanol (EtOH) and the precipitated DNA was washed in 3 ml 70% EtOH and air dried and resuspended in 400 µl TE buffer pH 8 at 4°C. DNA was digested with *Sac*I overnight. Digests were purified using Phenol/chloroform mix (see 2.5) and ethanol precipitated for a minimum of 2 hrs at -20°C, then washed in 70% EtOH and resuspended in 200 µl NET buffer (10 mM Tris, 1 mM EDTA and 1 M NaCl; pH 8) and left for 3 hrs. The DNA was purified using BND cellulose. 2 ml of prepared BND cellulose solution was added to a 3 ml syringe column (plugged with glass wool) and the liquid allowed to drip through to 0.5 ml off the column bottom. The column was then washed with 2 ml x 1 M NET buffer. The DNA samples were then loaded and the fractions collected. Columns were washed twice with 1 ml NET buffer. 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 mins to remove residual BND cellulose.

Samples were then transferred to fresh tubes containing 750 µl isopropanol and precipitated at 4°C for 2 hrs, then spun at 14,000 r.p.m. for 60 mins, and washed in 70% EtOH and resuspended in about 15 µl TBE /loading dye buffer.
Samples were run in the first dimension in 0.5% Agarose/ TBE for 16 hrs, 20 V and in the second dimension in 1.2% agarose / TBE 0.5 µg/ml ethidium for 1.5 hrs 200 V all at 4°C and in 0.5 µg/ ml Ethidium TBE buffer.
The DNA was transferred onto Gene Screen membrane (NEN Life Science Products) following the manufacturer's instructions for salt transfer. A probe homologous to the 3′ end of *ade6* was produced using primers Ade6A-F and Ade6B-R. The probe was labeled with ^{32}P using the MegaPrime DNA Labelling System (Amersham Biosciences). The membrane was probed and then scanned on a phosphor imager.

2.16 Production of competent *E. coli* cells

2.16i) Preparation of electro-competent cells and electroporation

E. coli DH5α cells were grown in 1 L LB at 37°C to an Abs_{600} 0.5-1.0. They were then harvested and washed with 100 ml chilled, distilled water at 4,000 r.p.m. at 4°C. This was repeated twice. The cells were then resuspended in 20 ml 10% glycerol and centrifuged at 4,000 r.p.m., 4°C for 15 mins. The pellet was resuspended in 2 ml 10% glycerol, aliquoted into Eppendorf tubes and stored at –80°C. Cells were used within 6 months.
For electorporation, 50 µl of electro-competent cells were placed in chilled 0.2 cm cuvettes (Geneflow) with 1-2 µl of ligated DNA. Cells were shocked at 2.5 kV, then 1 ml of ice cold LB liquid media was added and the cells plated out on LB + ampicillin plates and incubated overnight at 37°C.

2.16ii) Preparation of chemically-competent cells

A 200 ml culture of *E. coli* DH5α* cells [or HB101** cells (Promega)] were grown to Abs_{550} 0.5, then chilled on ice for 5 mins, then harvested at 4000 r.p.m. for 5 mins and resuspended in 80 ml TfbI solution [30 mM KOAc, 100 mM RbCl, 10 mM $CaCl_2$, 50 mM $MnCl_2$ and 15% (v/v) glycerol, adjusted to pH 5.8 with 0.2

M HOAc], and left on ice for 5 mins. Cells were harvested again and resuspended in 8 ml TfbII solution [10 mM MOPS, 75 mM $CaCl_2$, 10 mM RbCl, and 15% (v/v) glycerol, adjusted to pH 6.5 with 1 M KOH], and incubated on ice for 15 mins. Cells were aliqoted, rapid freezed in liquid nitrogen and stored at -80°C. For transformation, 1-50 ng of DNA was added to 100 µl of competent cells and incubated on ice for 10 mins. The cells were then heat-shocked at 42°C for 45-50 secs, and then placed on ice for 2 mins. Then 900 µl of cold LB media was added and the cells where then incubated at 30°C for 60 mins with shaking. Dilutions were then made and plated out on LB + ampicillin media and incubated overnight at 37°C.

* DH5α genotype: F^-, φ80d*lacZ*ΔM15, Δ(*lacZYA-argF*)U169, *deoR*, *recA*1, *endA*1, *hsdR*17(rk^-, mk^+), *phoA*, *supE*44, λ^-, *thi*-1, *gyrA*96, *relA*1

*HB101 (Promega) genotype:F–, *thi*-1, *hsd*S20 (r_B^-, m_B^-), *sup*E44, *rec*A13, *ara*-14, *leu*B6, *pro*A2, *lac*Y1, *gal*K2, *rps*L20 (str^r), *xyl*-5, *mtl*-1

2.17 Cloning

2.17i) Translin genomic clone

A *tsn1* genomic clone was made by inserting a 3.757 kb *Eco*RI fragment of cosmid SPAC30 (from the Sanger Institute) into the shuttle vector plasmid pFY20 (D. Pryce personal communication), giving plasmid pDP1. The vector pFY20 contains amp^R gene, *ura4* gene, autonomous replication sequence *ARS*, the fission yeast stability element *stb* and a poly-linker.

2.17ii) Translin ORF clones

The *tsn1* ORF was amplified using TsnBam++ primers, which include a *Bam*HI site at either end of the fragment and extra nucleotides to enhance digestion of the fragment by *Bam*HI. Using Phusion High-Fidelity DNA Polymerase (Finnzyme) the PCR programme was 98°C for 1 min, then 25 cycles of 98°C for 1 min, 57°C for 30 secs, 72°C for 20 secs, and a final extension at 72°C for 5 mins. This fragment was ligated into pREP3X and pREP81X digested with *Bam*HI and treated with Alkaline Phosphatase (Promega) using T4 ligase (Roche) at a ratio of

1:3 or 3:5. Ligated plasmid was then transformed into chemically competent *E. coli* cells and ampicillin resistant colonies were screened.

2.17iii)*ade6* genomic clones

PCR fragment of 2,488 bp containing the *ade6* ORF and flanking sequences was amplified using Phusion High Fidelity Polymerase (FINNZYME) and primers Ade6Clone-F and R which have an *Xma*I site incorporated at either end. The PCR programme used was as follows; 25 cycles of 98°C for 30 secs, 55°C for 30 secs, 72°C for 90 secs, using BP1 genomic DNA as template. PCR product was then purified using PCR Purification kit (Roche). The cut PCR fragment and CIAP treated digested pFY20 where ligated overnight at 4°C using T4 ligase (Roche) at a ratio of 3 insert to 1 plasmid and then transformed into competent *E. coli* cells.

2.17iv) *His3* control (pDP10), $tRNA^{Glu}$ (pAJ5 and pAJ7) and 5B-box (pAJ4 and pAJ8) plasmids

To construct these plasmids 283 bp of *his3* sequence, $tRNA^{Glu}$ (SPBTRANGLU.08) and 5B-box (*IR-R* ChrIII) sequence was cloned into the *Bst*XI site located in *ade6* on pDP9. Primers tRNA-his3-BstX1-F and tRNA-his3-BstX1-R, tRNAGlu1-F and tRNAGlu1-R, tRNAGlu2-F and tRNAGlu-R, 5B-box1-F and 5B-box1-R, and 5B-box2-F and 5B-box2 were used to amplify the *his3* fragment, $tRNA^{Glu}$ in orientation 1 and 2, and the 5B-box sequence in orientation 1 and 2 respectively. Phusion Fidelity High Polymerase (FINNZYME) was used in all with a PCR programme of 98°C for 1 mins, then 30 cycles of 98°C for 10 secs, 55°C for 30 secs, 72°C for 15 secs, and a final extension of 72°C for 5 mins. All the PCR products were digested with *Bst*XI and ligated into *Bst*XI digested, CIAP treated pDP9, using T4 ligase (Roche.) Ligated plasmid was transformed into electrocompetent *E. coli* cells and amp^r colonies selected. The orientation of the insert was determined by the production of PCR product using primer Ade6E-R with either the forward or reverse primers originally used to amplify the relevant insert. Orientation was further confirmed by sequencing.

2.17v) pSRS5 plasmid

The pSRS5 plasmid was constructed for plasmid by chromosome recombination assays in strains carrying an insertion in *ade6*. Using a two-step PCR, a G was deleted in the *ade6* sequence at position 1483 bp. Primer pairs Ade6-clone-PstI and Ade6-frameshift-F, and Ade6-clone-SacI and Ade6-Frameshift-R were used to ampify the 5′ region and 3′ region of *ade6* respectively, introducing a site directed deletion of a single G residue resulting in a frameshift and the creation of four stop codons. Then the two PCR products were used as template in a second step to give a full copy of the mutated *ade6*. Phusion High Fidelity Polymerase (FINNZYME) was used and the following PCR programme: 94°C for 2 mins, then 25 cycles; 94°C for 1 min, 55°C for 1 min and 72°C for 1.30 mins with a final extension of 72°C for 4 mins.

After digestion with *Pst*I and *Sac*I, the insert was ligated into *Pst*I and *Sac*I digested CIAP treated pREP42 plasmid. Digestion of the plasmid with these two enzymes removes the *nmt*-promoter, the multicloning site and the termination sequence, leaving a plasmid backbone containing the selective marker *LEU1* and *amp*^{r}. (Refer to fig 6.4A for a plasmid map; plasmid constructed by S. Ramayah).

2.18 Media recipes

For minimal media, appropriate amino acid and nucleotide supplements were added to a final concentration of 200 mg/l. For liquid media, agar was omitted.

YE (1 litre)

Yeast extract 5 g
Glucose 30 g
Agar 14 g

NB (1 litre)

NB 1.7 g
Ammonium sulphate 5 g
Glucose 5 g
Agar (Difco) 10 g

EMM2 (1 litre)

Potassium hydrogen phthalate 3 g
Di-sodium hydrogen phosphate 2.2 g
Ammonium chloride 5 g
Glucose 20 g
Vitamins (x1000) 1 ml
Minerals (x10,000) 0.1 ml
Salts (x50) 20 ml
Agar 14 g

SPA (1 litre)

Glucose 5 g
Potassium di-hydrogen orthophosphate 0.5 g
Agar 15 g
Vitamins (x1000) 0.5 ml

LB (1 litre)

Tryptone 10 g
Yeast extract 5 g
Sodium chloride 10 g
Agar 14 g
50 mg/ml Ampicilin 2 ml

Salts (x50) (1 litre)

Magnesium chloride hexa-hydrate 52.5 g
Calcium chloride di-hydrate 0.735 g
Potassium chloride 50 g
Di-sodium sulphate 2 g

Vitamins (x1000) (1 litre)

Pantothenic acid 1 g
Nicotinic acid 10 g
myo-inositol 10 g
Biotin 10 mg

Minerals (x10,000) (1 litre)

Boric acid 5 g
Manganese sulphate 4 g
Zinc sulphate septa-hydrate 4 g
Iron chloride hexa-hydrate 2 g
Potassium iodide 1 g
Molybdic acid 0.4 g
Copper sulphate 0.4 g
Citric acid 10 g

After autoclaving add a few drops of 1:1:2 Chlorobenzene/dichloroethane/ chlorobutane

Table 2.1 Strain list

Strain	Genotype	Source
BP1	*h⁻ 972 (wt)*	Lab collection
BP8	*h⁺ 975 (wt)*	Lab collection
BP11	*h⁻ ade6-M26*	Lab collection
BP88	*h⁻ ura4-D18 leu1-32*	Lab collection
BP89	*h⁺ ura4-D18 leu1-32*	Lab collection
BP90	*h⁻ ade6-m26 ura4-D18 leu1-32*	Lab collection
BP107	*h⁺ rad32::ura4 ade6-704 ura4-D18 leu1-32*	Lab collection
BP135	*h⁺ ade6-m216 ura4-D18 leu1-32 his3-D1*	Lab collection
BP296	*h⁺/h⁻ ade6-M216/ade6-M210 ura4-D18/ura4-D18 leu1-32/leu1-32*	Lab collection
BP420	*h⁻ pro1-1*	Lab collection
BP513	*h⁻ lig4::ura4 ade6-704 ura4-D18 leu1-32*	Lab collection
BP743	*h⁻ rad3-136*	
BP572	*h⁻ spc1::ura4 ade6-3006 ura4-D18 leu1-32*	Lab collection
BP621	*h⁺ ura1-61*	Lab collection
BP685	*h⁻ lig4::kanmx6 ade6-704 ura4-D18 leu1-32*	Lab collection
BP794	*h⁺ ade6-m375 ura4-D18 leu1-32*	Lab collection
BP816	*h⁺ ade6-469 ura4-D18 leu1-32*	Lab collection
BP1004	*h⁻ swi1::ura4 ade6-M210 ura4-D18*	J. Dalgaard
BP1022	*h⁻ ade6-52 ura4-D18 leu1-32*	Lab collection
BP1023	*h⁺ ade6-52 ura4-D18 leu1-32*	This study
BP1079	*h⁻ tsn1::kanmx6 ade6-m26 ura4-D18 leu1-32*	This study
BP1080	*h⁻ tsn1::kanmx6 ade6-m26 ura4-D18 leu1-32*	This study
BP1081	*h⁻ tsn1::kanmx6 ade6-m26 ura4-D18 leu1-32*	This study
BP1082	*h⁻ tsn1::kanmx6 ade6-m26 ura4-D18 leu1-32*	This study
BP1083	*h⁻ tsn1::kanmx6 ade6-m26 ura4-D18 leu1-32*	This study
BP1089	*h⁻ trax::kanmx6 ade6-m26 ura4-D18 leu1-32*	S. Ramayah
BP1121	*h⁺ ura4-D18 his3-D1lys1-37*	Lab collection
BP1162	*h⁺ bub1::kanmx6 ura4-D18 leu1-32*	Lab collection
BP1201	*h⁻ ade6-m26 ura4-D18 leu1-32* (pSRSB3)	S. Ramayah
BP1205	*h⁻ ade6-m26 ura4-D18 leu1-32* (pREP4X)	S. Ramayah
BP1220	*h⁻ ade6-$((GT)_8$-1397) trax::kanmx6 ura4-D18 leu1-32*	S. Ramayah
BP1243	*h⁻ ade6-$((GT)_8$-1397) tsn1::kanmx6 ura4-D18 leu1-32*	This study
BP1244	*h⁻ ade6-$((GT)_8$-1397) ura4-D18 leu1-32*	This study
BP1219	*h⁻ ade6-$((GT)_8$-1397) ura4-D18 leu1-32 his3-D1*	Mansour *et al.*, 2001
BP1267	*h⁻ ade6-m26 tsn1::kanmx6*	This study
BP1268	*h⁺ tsn1::kanmx6*	This study
BP1269	*h⁻ ade6-m26 ura4-D18 leu1-32* (pREP3X)	This study
BP1271	*h⁻ ade6-m26 ura4-D18 leu1-32* (pAJ1)	This study

BP1282	*h+ tsn1::kanmx6 ade6-52 ura4-D18 leu1-32*	This study
BP1288	*h+ arg1-14*	Lab collection
BP1304	*h- msh2::his3 his3-D1*	Oliver Fleck
BP1310	*h- rhp14::kanmx6 ura4-D18 leu1-32 his3-D1*	Oliver Fleck
BP1345	*h- pro1-1 tsn1::kanmx6*	This study
BP1346	*h+ ura1-61 tsn1::kanmx6*	This study
BP1348	*h+ arg1-14 tsn1::kanmx6*	This study
BP1351	*h+ tsn1::kanmx6 ade6-469 ura4-D18 leu1-32*	This study
BP1352	*h+ msh2::his3 ura4-D18 leu1-32 his3-D1*	This study
BP1354	*h+ tsn1::kanmx6 ade6-m26 ura4-D18 leu1-32 his3-D1*	This study
BP1356	*h+ trax::kanmx6 ade6-m26 ura4-D18 leu1-32 his3-D1*	This study
BP1357	*h- rhp14::kanmx6 ura4-D18 leu1-32*	This study
BP1358	*h+ rhp14::kanmx6 ura4-D18 leu1-32*	This study
BP1383	*h+ msh2::his3 tsn1::kanmx6 ade6-m26 ura4-D18 leu1-32 his3-D1*	This study
BP1385	*h+ msh2::his3 trax::kanmx6 ade6-m26 ura4-D18 leu1-32 his3-D1*	This study
BP1390	*h- tsn1::kanmx6 ade6-m26 ura4-D18 leu1-32* (pade6-469)	This study
BP1391	*h- trax::kanmx6 ade6-m26 ura4-D18 leu1-32* (pade6-469)	This study
BP1392	*h- rad32::ura4 tsn1::kanmx6 ade6-m26/704? Ura4-D18 leu1-32*	This study
BP1394	*h+ lig4::ura4 ade6-704/m375? ura4-D18 leu1-32*	This study
BP1395	*h- tsn1::kanmx6 trax::kanmx6 ade6-m26 ura4-D18 leu1-32*	This study
BP1397	*h- rhp14::kanmx6 tsn1::kanmx6 ade6-m26 ura4-D18 leu1-32*	This study
BP1413	*h- ade6-216 ura4-D18 leu1-32*	D. Pryce
BP1415	*h+ rad32::ura4 trax::kanmx6 ade6-m26/704? ura4-D18 leu1-32*	This study
BP1417	*h- lig4::ura4 tsn1::kanmx6 ade6-704 ura4-D18 leu1-32*	This study
BP1419	*h- lig4::ura4 trax::kanmx6 ade6-704 ura4-D18 leu1-32*	This study
BP1443	*h- ade6::his3 ura4-D18 his3-D1 lys1-37 leu1-32*	D. Pryce
BP1455	*h- ade6-m26 ura4-D18 leu1-32* (pREP3X, pREP4X)	This study
BP1456	*h- ade6-m26 ura4-D18 leu1-32* (pAJ1, pSRSB3)	This study
BP1461	*h- ade6-m26 ura4-D18 leu1-32* (pade6-469)	This study
BP1462	*h- rhp7::kanmx6 ura4-D18 leu1-32 his3-D1*	O. Fleck
BP1463	*h+ ade6::tRNA^{Glu} (or1) ura4-D18 his3-D1 lys1-37* (pFY20)	This study

BP1465	*h$^+$ ade6::5B-box (or1) ura4-D18 his3-D1 lys1-37* (pFY20)	This study
BP1467	*h$^+$ ade6::5B-box (or2) ura4-D18 his3-D1 lys1-37* (pFY20)	This study
BP1469	*h$^+$ ade6::tRNA^{Glu} (or1) ura4-D18 his3-D1 lys1-37*	This study
BP1471	*h$^+$ ade6::5B-box (or1) ura4-D18 his3-D1 lys1-37*	This study
BP1473	*h$^+$ ade6::5B-box (or2) ura4-D18 his3-D1 lys1-37*	This study
BP1478	*h$^-$ ade6::tRNA^{Glu} (or1) leu1-32 ura4-D18 his3-D1 lys1-37*	This study
BP1481	*h$^-$ ade6::5B-box (or1) leu1-32 ura4-D18 his3-D1 lys1-37*	This study
BP1485	*h$^-$ ade6::5B-box (or2) leu1-32 ura4-D18 his3-D1 lys1-37*	This study
BP1508	*h$^-$ ade6::tRNA^{Glu} (or2) leu1-32 ura4-D18 his3-D1 lys1-37*	This study
BP1512	*h$^-$ ade6::his3 ura4-D18 his3-D1lys1-37 leu1-32* (pade6-469)	This study
BP1513	*h$^-$ ade6::tRNA^{Glu} (or1) leu1-32 ura4-D18 his3-D1 lys1-37* (pade6-469)	This study
BP1514	*h$^-$ ade6::tRNA^{Glu} (or2) leu1-32 ura4-D18 his3-D1 lys1-37* (pade6-469)	This study
BP1515	*h$^-$ ade6::5B-box (or1) leu1-32 ura4-D18 his3-D1 lys1-37* (pade6-469)	This study
BP1516	*h$^-$ ade6::5B-box (or2) leu1-32 ura4-D18 his3-D1 lys1-37* (pade6-469)	This study
BP1527	*h$^+$ ade6::tRNA^{Glu} (or2) ura4-D18 his3-D1 lys1-37*	This study
BP1533	*h$^-$ ade6::his3 ura4-D18 his3-D1 lys1-37 leu1-32* (pSRS5)	This study
BP1534	*h$^-$ ade6::*tRNA^{Glu} *(or1) leu1-32 ura4-D18 his3-D1 lys1-37* (pSRS5)	This study
BP1535	*h$^-$ ade6::*tRNA^{Glu} *(or2) leu1-32 ura4-D18 his3-D1 lys1-37* (pSRS5)	This study
BP1537	*h$^+$ swi1::ura4 ura4-D18 leu1-32*	This study
BP1538	*h$^-$ rhp7::kanmx6 tsn1::kanmx6 ura4-D18 leu1-32 his3-D1*	This study
BP1540	*h$^-$ rhp7::kanmx6 trax::kanmx6 ura4-D18 leu1-32 his3-D1*	This study
BP1545	*h$^-$ smt0 rhp51::his3 ura4-D18 his3-D1*	O. Fleck
BP1561	*h$^-$ ade6::his3 swi1::ura4 ura4-D18 his3-D1 lys1-37 leu1-32*	This study
BP1563	*h$^-$ ade6::*tRNA^{Glu} *(or1) swi1::ura4 leu1-32 ura4-D18 his3-D1 lys1-37*	This study
BP1565	*h$^-$ ade6::*tRNA^{Glu} *(or2) swi1::ura4 leu1-32 ura4-D18 his3-D1 lys1-37*	This study
BP1596	*h$^-$ rad13::kanmx*	O. Fleck

BP1597	*h+ rad13::kanmx*	O. Fleck
BP1611	*h- smt0 nbs1::kanR ura4-D8*	E. Hartsuiker
BP1614	*h- rad50::ura4 ade6-216 ura4-D18 leu1-32*	E. Hartsuiker
BP1616	*h- rhp14::kanmx6 trax::kanmx6 ade6-m26 ura4-D18 leu1-32*	This study
BP1617	*h- rhp14::kanmx6 trax::kanmx6 ade6-m26 ura4-D18 leu1-32*	This study
BP1619	*h- rad13::kanmx trax::kanmx6 ade6-m26 ura4-D18 leu1-32*	This study
BP1627	*h- rad32::kanmx4 ura4-D18 leu1-32 his3-D1*	N. Rhind
BP1634	*h+ rad13::kanmx tsn1::kanmx6 ade6-m26 ura4-D18 leu1-32*	This study
BP1643	*h+ nbs1::kanR tsn1::kanmx6 ura4-D18 leu1-32*	This study
BP1645	*h- mts3-1 ade6-M210 arg3 his3 leu1-32 ura4-D18*	C. Gordon
BP1668	*h+ tsn1::kanmx6 mts3-1 ade6 arg3 his3 leu1-32 ura4-D18*	This study
BP1673	*h+ rad32::kanmx4 ade6-m26 ura4-D18 leu1-32 his3-D1*	This study
BP1674	*h+ rad32::kanmx4 ade6-m26 ura4-D18 leu1-32 his3-D1*	This study
BP1675	*h+ rad32::kanmx4 tsn1::kanmx6 ade6-m26 ura4-D18 leu1-32 his3-D1*	This study
BP1676	*h+ rad32::kanmx4 tsn1::kanmx6 ade6-m26 ura4-D18 leu1-32 his3-D1*	This study
BP1677	*h+ rad50::ura4 tsn1::kanmx6 ura4-D18 leu1-32*	This study
BP1678	*h+ rhp51::his3 tsn1::kanmx6 ura4-D18 leu1-32 his3-D1*	This study
BP1681	*h+ rad50::ura4 trax::kanmx6 ura4-D18 leu1-32*	This study
BP1683	*h+ rhp51::his3 trax::kanmx6 ura4-D18 leu1-32 his3-D1*	This study
BP1684	*h- ade6::his3 swi1::ura4 ura4-D18 leu1-32 his3-D1* (pSRS5)	This study
BP1685	*h- ade6::*$tRNA^{Glu}$ *(or1) swi1::ura4 leu1-32 ura4-D18 his3-D1 lys1-37* (pSRS5)	This study
BP1687	*h- ade6::*$tRNA^{Glu}$ *(or2) swi1::ura4 leu1-32 ura4-D18 his3-D1 lys1-37* (pSRS5)	This study
BP1694	*h- smt0 nbs1::kanR trax::kanmx6 ura4-D18 leu1-32 his3-D1*	This study
BP1707	*h+ rad32::kanmx4 trax::kanmx6 ade6-m26 ura4-D18 leu1-32 his3-D1*	This study
BP1708	*h+ rad32::kanmx4 trax::kanmx6 ade6-m26 ura4-D18 leu1-32 his3-D1*	This study
BP1710	*h+ rad32::kanmx4 trax::kanmx6 ade6-m26 ura4-D18 leu1-32 his3-D1*	This study
BP1856	*h- tsn1::kanmx6 ade6-m26 ura4-D18 leu1-32*	This study

	(pFY20)	
BP1857	*h⁻ tsn1::kanmx6 ade6-m26 ura4-D18 leu1-32* (pDP1)	This study
BP1858	*h⁻ tsn1::kanmx6 ade6-m26 ura4-D18 leu1-32* (pFY20)	This study
BP1859	*h⁻ tsn1::kanmx6 ade6-m26 ura4-D18 leu1-32* (pDP1)	This study
BP1860	*h⁻ tsn1::kanmx6 ade6-m26 ura4-D18 leu1-32* (pFY20)	This study
BP1861	*h⁻ tsn1::kanmx6 ade6-m26 ura4-D18 leu1-32* (pDP1)	This study
BP1862	*h⁻ tsn1::kanmx6 ade6-m26 ura4-D18 leu1-32* (pFY20)	This study
BP1863	*h⁻ tsn1::kanmx6 ade6-m26 ura4-D18 leu1-32* (pDP1)	This study
BP1864	*h⁻ tsn1::kanmx6 ade6-m26 ura4-D18 leu1-32* (pFY20)	This study
BP1865	*h⁻ tsn1::kanmx6 ade6-m26 ura4-D18 leu1-32* (pDP1)	This study
BP1893	*h⁻ rhp14::kanmx6 ade6-m26 ura4-D18 leu1-32*	L. Stanczuk
BP1900	*h⁻ rhp14::kanmx6 tsn1::kanmx6 ade6-m26 ura4-D18 leu1-32*	L. Stanczuk
BP1901	*h⁻ rhp14::kanmx6 trax::kanmx6 ade6-m26 ura4-D18 leu1-32*	L. Stanczuk
BP1922	*h⁻ rhp14::kanmx6 tsn1::kanmx6 ade6-m26 ura4-D18 leu1-32*	L. Stanczuk
BP1923	*h⁻ rhp14::kanmx6 tsn1::kanmx6 ade6-m26 ura4-D18 leu1-32*	L. Stanczuk
BP1924	*h⁻ rhp14::kanmx6 tsn1::kanmx6 ade6-m26 ura4-D18 leu1-32*	L. Stanczuk
BP1925	*h⁻ rhp14::kanmx6 trax::kanmx6 ade6-m26 ura4-D18 leu1-32*	L. Stanczuk
BP1926	*h⁻ rhp14::kanmx6 trax::kanmx6 ade6-m26 ura4-D18 leu1-32*	L. Stanczuk
BP1927	*h⁻ rhp14::kanmx6 trax::kanmx6 ade6-m26 ura4-D18 leu1-32*	L. Stanczuk
BP1928	*h⁻ rhp14::kanmx6 trax::kanmx6 ade6-m26 ura4-D18 leu1-32*	L. Stanczuk
BP1929	*h⁻ rhp14::kanmx6 trax::kanmx6 ade6-m26 ura4-D18 leu1-32*	L. Stanczuk
BP1933	*h⁻ rhp14::kanmx6 trax::kanmx6 ade6-m26 ura4-D18 leu1-32*	L. Stanczuk

Table 2.2 List of primers

Primer name	Sequence	Description
Tsn-kan-F	5′-TTA TTT GCA TAC TGA AAA CAT CAT TCG AAT ATC AAC ACT ACT CAA CAG CAT ACA TTA CAG ATT AAG TCG ACG GAT CCC CGG GTT AAT TAA-3′	Forward primer for the kanamycin cassette for *tsn1* replacement
Tsn-kan-R	5′-ATA TTA AAA AAG CAA TTT TAT CGG CTC AAT TTT AGT CAA GCG TAC AGC TGG CAA ATA AAT TGT TAG CAA TGA ATT CGA GCT CGT TTA AAC-3′	Reverse primer for the kanamycin cassette for *tsn1* replacement
Tsn-F	5′-GAT CTA AAC AAC CCA AGC G-3′	Upstream of *tsn1* ORF
Tsn-R	5′-GCA TTC ATC ATA GGA CTG CC-3′	Downstream of *tsn1* ORF
Tsn-intC-F	5′-TCA TAT TCC ATT TGA GGC CC-3′	Internal Translin primer
Screentag-F	5′-CAG TTC TCA CAT CAC ATC CG-3′	Located within kan cassette
Screencorrect-R	5′-CGG ATG TGA TGT GAG AAC TG-3′	Located within kan cassette
Scrkan 1458-R	5′-CGA CAG CAG TAT AGC GAC CA-3′	Located within kan cassette
Tsn-1-F	5′-TAT CAA CAC TAC TCA ACA GC-3′	Nested PCR primer
Tsn-2-R	5′-TTT GAA AAG ATC TGA GGA CG-3′	Nested PCR primer
Tsn-3-F	5′-AAA CTG ACT GCA GAG GTC G-3′	Nested PCR primer
Tsn-4-R	5′-CTA ACA AAT CGA CCT CTG C-3′	Nested PCR primer
Tsn-5-R	5′-GCA AAA TTT GAC CAA CTT CG-3′	Nested PCR primer
Tsn-6-F	5′-CGA AGT TGG TCA AAT TTT GC-3′	Nested PCR primer
Tsn-7-F	5′-CCC GGT TTT TCC AGA AGA G-3′	Nested PCR primer
Tsn-8-R	5′-GAA CAC AGA GAT AGT ACT GC-3′	Nested PCR primer
Tsn-9-R	5′-GAG TTT ACA GAT TGA CGA GC-3′	Nested PCR primer
Tsn-10-F	5′-GCT CGT CAA TCT GTA AAC TC-3′	Nested PCR primer
Tsn-11-R	5′-CGA AAT GTC TTC TTA AGG AG-3′	Nested PCR primer
Tsn-12-F	5′-TTT GAA AAG ATC TGA GGA CG-3′	Nested PCR primer
Tsn-13-F	5′-TCT TCG GAT ACA TAA ATT GG-3′	Nested PCR primer
Tsn-14-R	5′-GGC TCA ATT TTA GTC AAG CG-3′	Nested PCR primer
TsnBam++F	5′-CGC GGA TCC GCG ATG AAT AAA TCA ATA TTT ATT CAG C-3′	Forward primer for *tsn1* ORF with 5′ *Bam*HI site
TsnBam++R	5′-CGC GGA TCC GCG TTA AAC CAA TTT ATG TAT CCG-3′	Reverse primer for *tsn1* ORF with 5′ *Bam*HI site
pREPInt-F	5′-TCA TTC GGC AAT GTG CAG CG-3′	Located upstream of the polylinker
pREPInt-R	5′-CAC CCG TCT ACG TTT CTA CG-3′	Located in the terminsation sequence of pREP
Traxcheck-F	5′-CAA ATA GTC ATC TTG ATT TGC-3′	Upstream of the *trax* ORF
Traxcheck-R	5′-TCT AAC ATA TAG AAA GCA GCG-3′	Downstream of the *trax* ORF
Ade6Clone-F	5′-TCC CCC CGG GGG GAA TAC GCA CAT TGA AAC ATG GAC G-3′	Downstream of *bub1*

Ade6Clone-R	5′-TCC CCC CGG GGG GAC ATT GAA TTC TCC AAT ATA TTT AGA ATT AGC-3′	Upstream of *vtc4*
Ade6ext-F	5′-CAC GAG AGA ACT CAG CAG CG-3′	~1 kb upstream of *ade6*
Ade6A-F	5′-TGG TAG TAC GCA GTT TAG ACG-3′	Within *ade6*
Ade6B-R	5′-CAA CCC TCT TGA AGC TGC-3′	Within *ade6*
Ade6C-F	5′-TGG AGG TGG TCA ATT GGG C-3′	Within *ade6*
Ade6D-R	5′-GCA ACA GAT TTC GTA ACG GC-3′	Within *ade6*
Ade6E-R	5′-CTT CAA AAG TGC GCA CCG C-3′	Within *ade6*
Ade6F-F	5′-CAT CTC ATT AAG CTG AGC TGC-3′	Within *ade6*
Ade6G-F	5′-TCT TGA AAC AAT GCT AAT CGC-3′	Within *ade6*
Rad32-ext-F	5′-ACT TTC CGT CTA CCG ATC CC-3′	~500 bp upstream of *rad32* start codon
Rad32-int-R	5′-TCA TTC TCT GGA ACA CGA CC-3′	~500 bp downstream of *rad32* start codon
Rhp14-ext-F	5′-CTT GGT CAT TAC AAA ACC CG-3′	~300 bp upstream of *rhp14* start codon
Rhp14-int-R	5′-ATC TTG CAA TTC CGG TTC CG-3′	~300 bp downstream of *rhp14* start codon
Rhp51-ext-F	5′-GCC ATA CAA ATA GTT GGC GC-3′	~500 bp upstream of *rhp51* start codon
Rhp51-int-R	5′-GAA TAT GGT ACT CAG TGG CG-3′	~600 bp downstream of *rhp51* start codon
Rad13-ext-F	5′-GTC TCG TTC ACC AAT GGT GG-3′	~500 bp upstream of *rad13* start codon
Rad13-int-R	5′-TTG GCG TTT CAA TGA CGG CG-3′	~500 bp downstream of *rad13* start codon
Nbs1-ext-F	5′-TTA ACC GGG ACA TTA CGC GC-3′	~500 bp upstream of *nbs1* start codon
Nbs1-int-R	5′-ACT TCA AAC TCG CAT GGG CC-3′	~500 bp downstream of *nbs1* start codon
Rad50-ext-F	5′-CGT GAA GAC TAT CCT GAG CG-3′	~600 bp upstream of *rad50* start codon
Rad50-int-R	5′-AGC AGG CTC ACT TAA TGG CC-3′	~700 bp downstream of *rad50* start codon
Ura4-mid-R	5′-GTA TAA TAC CCT CGC CTG GC-3′	Located centrally in *ura4*
His3-int-F	5′-GGG TAA TAA TTG ATA TGA GGG C-3′	Located centrally in *his3*
His3-int-R	5′-GGC CTG AAA TCG CCA GCT CCA AC-3′	Located centrally in *his3*
tRNA-his3-BstX1-F	5′-CTG CAG AAC CAA GAG TTT GGC AGC CTT ATC GCT GTG CG-3′	Within *his3*
tRNA-his3-BstX1-R	5′-TTC TGC AGC CAA ACT CTT GGG GAC ACC TCT ATT AAG ATT AGC-3′	Within *his3*
tRNAGlu1-F	5′-CTG CAG AAC CAA GAG TTT GGT TAG TTT TAT TGT ATG ATC	Upstream of tRNAGlu, *Bst*XI in orientation 1.

	GCC C-3′	
tRNAGlu 1-R	5′-TTC TGC AGC GAA ACT CTT GGC CGC TTC TTC CCA TAA GG-3′	Downstream of tRNAGlu, *Bst*XI in orientation 1.
tRNAGlu 2-F	5′-TTC TGC AGC CAA ACT CTT GGT TAG TTT TAT TGT ATG ATC GCC C-3′	Upstream of tRNAGlu, *Bst*XI in orientation 2.
tRNAGlu 2-R	5′-CTG CAG AAC CAA GAG TTT GGC CGC TTC TTC CCA TAA GG-3′	Downstream of tRNAGlu, *Bst*XI in orientation 2.
5B-box1-F	5′-CTG CAG AAC CAA GAG TTT GGA CCC CCA CTA TAC ATT AAA AAC-3′	Upstream of 5B-box sequence, *Bst*XI in orientation 1.
5B-box1-R	5′-TTC TGC AGC CAA ACT CTT GGT CCC TAA TTT GTA TAT AAC G-3′	Downstream of 5B-box sequence, *Bst*XI in orientation 1.
5B-box2-F	5′-TTC TGC AGC CAA ACT CTT GGA CCC CCA CTA TAC ATT AAA AAC C-5′	Upstream of 5B-box sequence, *Bst*XI in orientation 2.
5B-box2-R	5′-CTG CAG AAC CAA GAG TTT GGT CCC TAA TTT GTA TAT AAC G-3′	Downstream of 5B-box sequence, *Bst*XI in orientation 2.
Ade6-clone-PstI	5'-TAT GCT GCA GCA TTG AAT TCT CCA ATA TAT TTA GAA TTA GC- 3'	Primer for pSRS5 *ade6* frameshift
Ade6-frameshift-F	5'-CAG ATG CCT CGA GGT GTC CCT GTC CCA CTG TT-3'	Primer for pSRS5 *ade6* frameshift
Ade6-clone-SacI	5'-ATA CGA GCT CAT ACG CAC ATT GAA ACA TGG ACG-3'	Primer for pSRS5 *ade6* frameshift
Ade6-Frameshift-R	5'-AAC AGT GGG ACA GGG ACA CCT CGA GGC ATC TG- 3'	Primer for pSRS5 *ade6* frameshift

Chapter 3 Characterisation of the *S. pombe* Translin null mutant and its interacting partner TRAX

3.1 Introduction

Tranlsin binding sites have been identified in several cancer-related translocation breakpoints (Aoki *et al.*,1995; Atlas *et al.*, 1998; Kanoe *et al.*, 1999; Hosaka *et al.*, 2000; Abeysinghe *et al.*, 2003; Wei *et al.*, 2003). Translin has also been found to bind GT-rich single-stranded regions of the genome and has been implicated in many cellular pathways including DNA repair (Kasai *et al.*, 1997), recombination (Aoki *et al.*, 1995) and telomere stability (Jacob *et al.*, 2004).

It was independently identified in mice as the testes brain RNA binding protein (TB-RBP) (Wu *et al.*, 1997). Translin is found in every cell type but is enriched in the brain and testes. Translin has a binding partner known as Translin-associated factor X (TRAX). Both proteins are highly conserved and nearly every organism that possesses a homologue of Translin also has one of TRAX. Translin binds both RNA and DNA (Kasai *et al.*, 1997; Wu *et al.*, 1998; Aoki *et al.*, 1999; Sengupta and Rao, 2002; Gupta *et al.*, 2005), and exists as an octameric toroid (Kasai *et al.*, 1997; Van Loock *et al.*, 2001; Sugiura *et al.*, 2004). TRAX has no intrinsic DNA binding activity. Together they are thought to bind to mRNAs and regulate translation, mRNA sorting and transport both in the testes and neurones (Kwon and Hecht, 1993; Han *et al.*, 1995; Morales *et al.*, 2002; Yang *et al.*, 2003, Chennathukuzhi *et al.*, 2003).

All Translin and TRAX deficient organisms studied have been found to be viable. Both Translin deficient *Drosophila* and mice show several minor neurological and behavioural abnormalities (Chennathukuzhi *et al.*, 2003; Stein *et al.*, 2006; Suseendranathan *et al.*, 1997). In Translin-deficient mice there was a considerable down-regulation of many brain mRNAs and fertility defects (Chennathukuzhi *et al.*, 2003). These mice also showed reduced monoaminergic neurotransmitter levels and exhibited exaggerated fear responses and defensive behaviour when exposed to threats (Stein *et al.*, 2006). Many germ cells in the testes do not proceed beyond the first meiotic metaphase stage in Translin-deficient mice, this

strongly suggests a defect in meiotic chromosome segregation and cytokinesis (Chennathukuzhi *et al.*, 2003). Also early passage Translin-null mouse embryonic fibroblast cells (MEFs) show slower rates of proliferation with a block in the G2 stage of the cell cycle (Yang *et al.*, 2004; Chennathukuzhi *et al.*, 2003), proliferation rates are however restored on the re-introduction of TB-RBP (Yang *et al.*, 2004). It has also been demonstrated in human embryonic kidney (HEK) 293 cells that Translin levels closely parallel the proliferative state and that the over expression of Translin accelerates cell proliferation (Ishida *et al.*, 2002), this has also been observed in MEFs (Yang *et al.*, 2004).

Translin is thought to stabilise TRAX in both *Drosophila* and Murine cells (Clauβen *et al.*, 2006; Yang *et al.*, 2004), where, in Translin-deficient cells, no TRAX protein can be detected despite TRAX mRNA levels being normal. Upon re-introduction of Translin into MEFs devoid of Translin, TRAX protein levels are also restored (Yang *et al.*, 2004). The converse however is not true, Translin protein levels are normal in TRAX knock out mice.

The human *Translin* gene encodes a protein of 228 amino acids and has a molecular size of 26 kDa (Aoki *et al.*, 1995). The native molecular weight however was estimated to be 220 kDa by gel filtration chromatography. This suggested that Translin exists as an octamer. Electron microscopic (Aoki *et al.*, 1997a) and crystallographic studies (Aoki *et al.*, 1997a; Pascal *et al.*, 2002) support this, indicating that Translin possesses an octameric ring shaped structure. Octamers of *S. pombe* Translin have also been isolated (Laufman *et al.*, 2005).

In this chapter we looked at the highly conserved Translin protein structure using several bioinformatic tools. We have also created and characterised Translin deletion mutants and have found some conserved functions in the stabilisation of TRAX. Using both deleted strains and over expression clones we have investigated a role for Translin and TRAX in cellular proliferation, and tested sensitivity of Translin-deficient cells to microtubule destabilizing drugs, and found no growth deficiencies in any of the tests. We have also tested the response of Translin-deficient cells to some forms of stress, a previously uninvestigated area, and found no evidence of a role in stress response for Translin.

3.2 The gene and protein structures of *S. pombe* Translin

3.2.1 Gene structure

S. pombe has three chromosomes. The *S. pombe* homolog of *H. sapiens Translin* was identified by the fission yeast genome sequencing project (Wood *et al.*, 2002) and is located on the right arm of chromosome I (SPAC30.03c). Human, mouse and fission yeast (predicted) *tsn1* all contain six exons and five introns (Aoki *et al.*, 1997a), and the intron/exon boundaries in human and mouse are well conserved (Aoki *et al.*, 1997a). *S. pombe* unspliced *tsn1* gene is 886 bp in size, and the spliced form is predicted to be 663 bp. It has 81 BLAST hits to 62 unique species (using NCBI BLAST), however it has no identified ortholog in *Saccharomyces cerevisiae*.

3.2.2 Protein structure

The *S. pombe tsn1* gene is predicted to code for a protein of 220 amino acids with a predicted mass of 25.4 kDa. The amino acid sequences of human, mouse and *Drosophila* were aligned with the *S. pombe* sequence (Fig. 3.1). Human and mouse Translin show 98% identity, *Drosophila* Translin has 52% identity to human, and *S. pombe* has the lowest identity with human Translin at 36% identity and 54% similarity.

Several functional sites have been identified in the human and murine proteins and these have been partially conserved in *S. pombe* (refer to Fig. 3.2). There are two basic regions towards the N-terminus which are putative RNA and RNA/DNA binding domains which are KRCLKAREH and RFHEHWRVLQR respectively (Aoki *et al.*, 1995; 1999; Pascal *et al.*, 2002; Sugiura *et al.*, 2004). These residues are not very well conserved in *S. pombe*.

Human and mouse Translin protein also contain a putative GTP-binding site near the C-terminus which is highly conserved in human, mouse, rat, hamster, chicken, *Xenopus*, *Drosophila* and fission yeast (Chennathukuzhi *et al.*, 2001). The human sequence is vnsVTAGDysr and the fission yeast sequence is vnsVISGNyhi. GTP

binding in murine Translin suppresses the RNA binding but not DNA binding of Translin (Chennathukuzhi *et al.*, 2001).

Murine Translin and TRAX proteins have functional nuclear export signals (NES) and nuclear localization signals (NLS) respectively, and their relative levels is thought to determine their distribution in spermatocytes (Cho *et al.*, 2004). The human sequence for the NES site is largely conserved in *S. pombe*, but TRAX is believed to lack the NLS (Laufman *et al.*, 2005).

Several post-translational modification sites were searched for in both Human and fission yeast Translin amino acid sequences using several bioinformatic prediction servers. Several phosphorylation sites were predicted throughout both sequences. The only functional regions to have sites in both sequences were the NES and GTP-binding sites. This may be significant with phosphorylation at these points possibly having an effect on function. Prenylation sites were predicted in human Translin for farnesyl and geranylgeranyl isoprenoids which was not found in the fission yeast form (using PrePS-Prenylation Prediction Suite). Potential PEST sequences were not identified (using EMBnet AUSTRIA PESTfind). Myristoylation, sumolylation and ubiquitination sites were not identified in either sequence, (using ExPASy Myristoylator, SUMOsp 2.0 and UN Ubiquitin Nugget Servers respectively).

The *S. pombe* Translin monomer was modelled using the 3D-JIGSAW comparative modeling server and using the human Translin protein crystallographic data as a template (Fig. 3.3). The two structures are very similar, with highly helical secondary structures with seven α helices which consist of 70% of the protein (Pascal *et al.*, 2002; Sugiura *et al.*, 2004). This model for *S. pombe* Translin was also obtained by Laufman *et al.*, (2005).

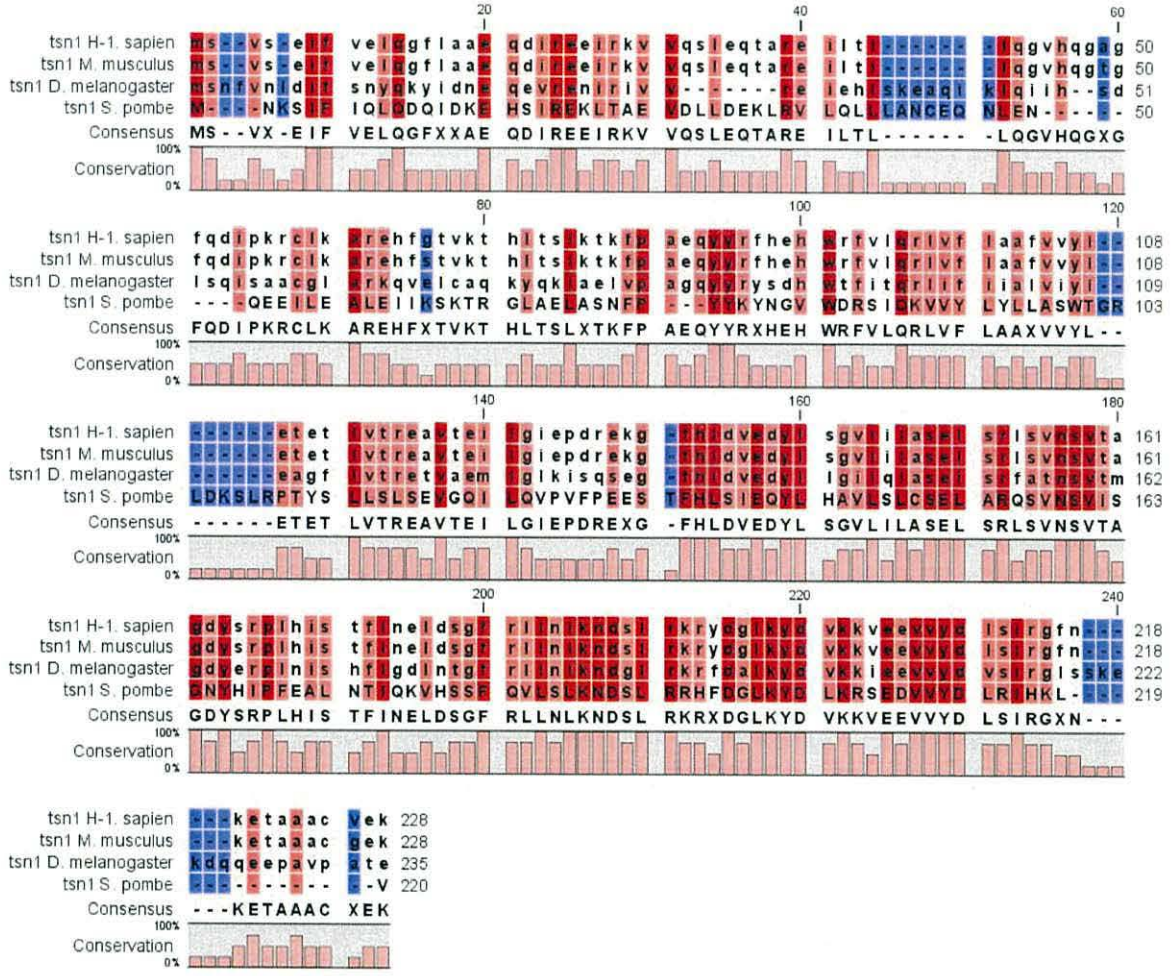


Figure 3.1 Translin amino acid sequence alignment. Translin amino acid sequences of *Homo sapiens* (NP_004613), *Mus musculus* (NP_035780), *Drosophila melanogaster* (AAM50730) and *Schizosaccharomyces pombe* (SPAC30.03c) were aligned using CLC bio alignment tool. Highly conserved amino acids are shown as dark red and unique amino acids are shown in blue. The conservation is also depicted graphically.

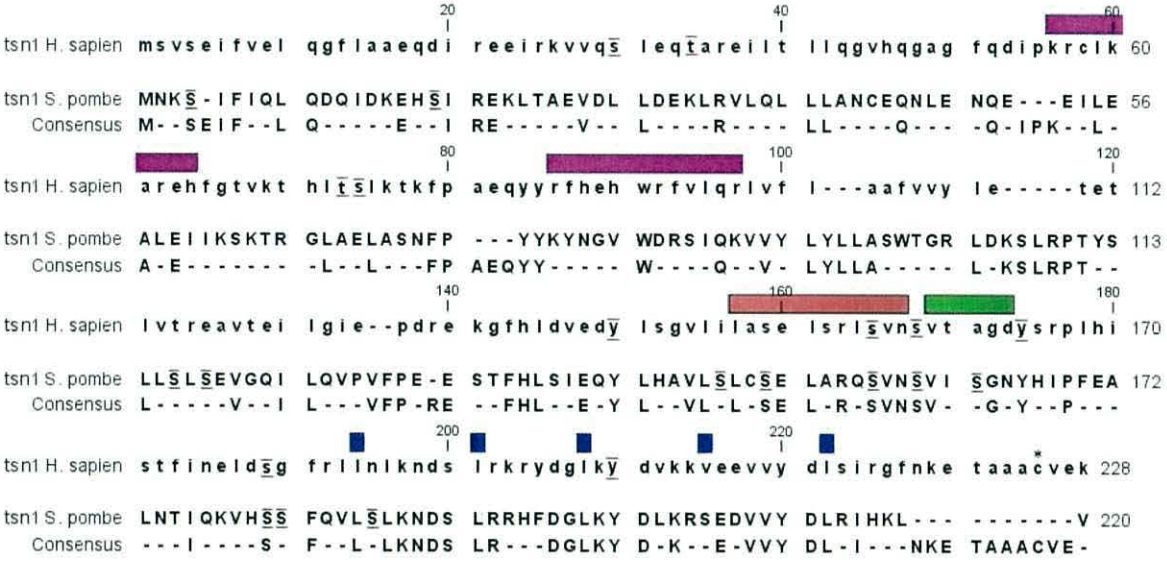


Figure 3.2 Alignment of human and *S. pombe* sequences showing known functional sites and predicted phosphorylation sites. The *S. pombe* (SPAC30.03c) Translin protein sequence is 51% homologous to *Homo sapiens* (NP_004613). The sequences were aligned using the CLC combined workbench 3 protein alignment tool. Know functional regions in the human sequence are highlighted; the basic regions believed to be involved in DNA and RNA binding (pink), nuclear export signal (NES) (red), GTP-binding site (green), amino acids involved in the leucine zipper (blue) and the asterix shows the cysteine residue at aa 225 which forms a disulfide bond between monomers in the mammalian protein. Phosphorylation sites were found using three different servers (NetPhos 2.0 Server, NetPhosYeast 1.0 Server and PROSITE motif search.) the consensus of the different searches are underlined. Several phosphorylation sites were found in the same region as the NES and GTP-binding site (between positions 155-175).

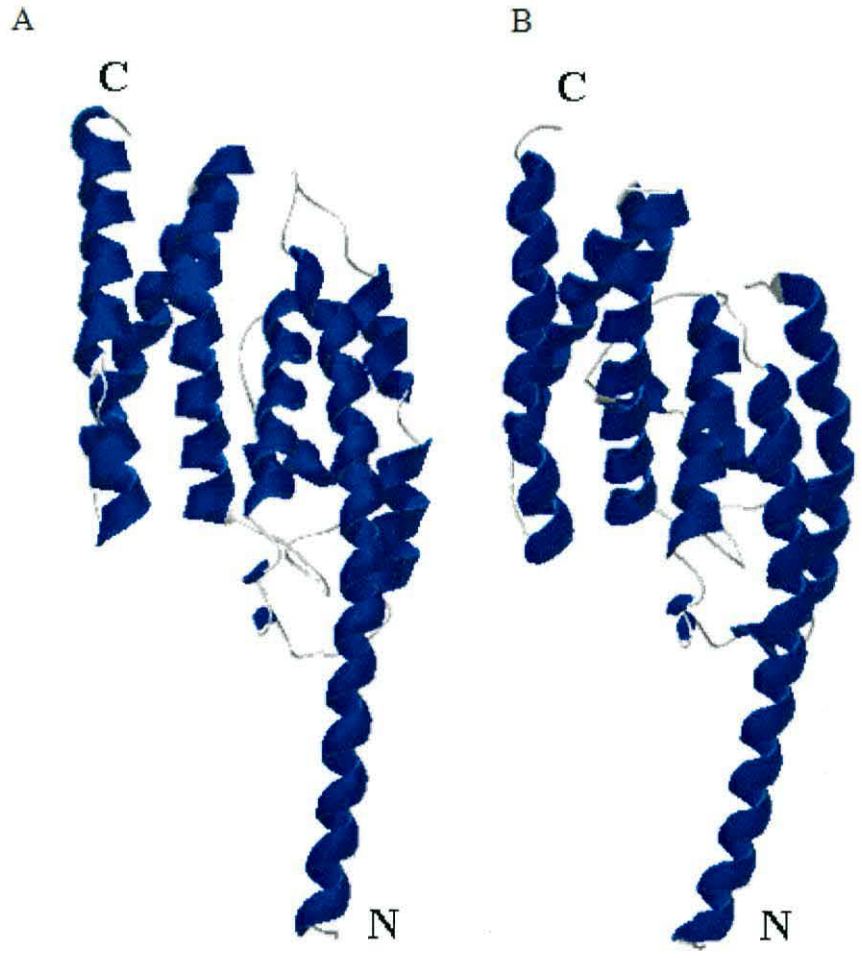


Figure 3.3 Comparative model of the *S. pombe* Translin monomer. The 3D structure of *Homo sapiens* Translin based on crystallographic data is shown in **A** (modelled on pdb 1j1j), and was used as a template to predict the tertiary structure of *S. pombe* Translin by the 3D-JIGSAW comparative modelling server **B** (Guex and Peitsch, 1997), and visualized using Swiss-pdbViewer 3.7.

3.3 Construction and characterisation of *S. pombe* Translin-null mutants.

3.3.1 Construction

Translin-null mutants were constructed using a PCR based gene targeting method (Bähler *et al.*, 1998). Primers were designed that had 70 bp of homology to the sequence immediately upstream and downstream of the *tsn1* open reading frame (ORF) (SPAC30.03c), and 20 bp of homology to the plasmid pFA6a-kanMX6 which carries the geneticin resistance marker gene *kanMX6*. The PCR product gave a cassette of roughly 1.62 kb which contained the *kanMX6* marker gene (1.48 kb) flanked by 70 bp of *tsn1* homologous sequence. This was chemically transformed into a *S. pombe* haploid strain (BP90), and a diploid strain (BP296), carrying the following background markers h^- *ade6-M26 ura4-D18 leu1-32* (BP90) and h^+/h^- *ade6-M216/ade6-M210 ura4-D18/ura4-D18 leu1-32/leu1-32* (BP296). Geneticin-resistant transformants were selected and checked by PCR.

3.3.2 Verification of the Translin null strains by PCRs, Southern and Western blot analysis

Five haploid and one diploid translin null strain were confirmed by PCR (refer to Fig. 3.4). PCR products of the expected sizes for *tsn1* gene replacement were obtained in three separate reactions for all five haploid strains. Nested PCRs were also carried out to ensure that no portion of the gene was still present (Fig. 3.5). Fourteen primers were designed in the exon regions of the *tsn1* ORF. No product for any of the primer pairs was obtained for the five haploid transformants.
Southern blots were carried out on all five knockouts and a wild-type strain (see Fig. 3.6). Genomic DNA was extracted and digested with *Bsp*HI. In the wild-type a band of 3.2 kb was detected with a *tsn1* probe, and in the knockout strains a band of 3.8 kb was detected with a probe carrying homology to the *kanMX6* marker gene. This further indicated that these strains are true knockouts.
Both anti-C-terminal and anti-N-terminal Translin antibodies were raised in rabbit (See Chapter 2). However, only the anti-C-terminal antibody worked successfully on Western blots. Tsn1-specific bands were found to migrate with a mobility of

approximately 25 kDa on a 12 % SDS-page gel (Fig. 3.7), and Translin protein was not detected in the knockout strains.

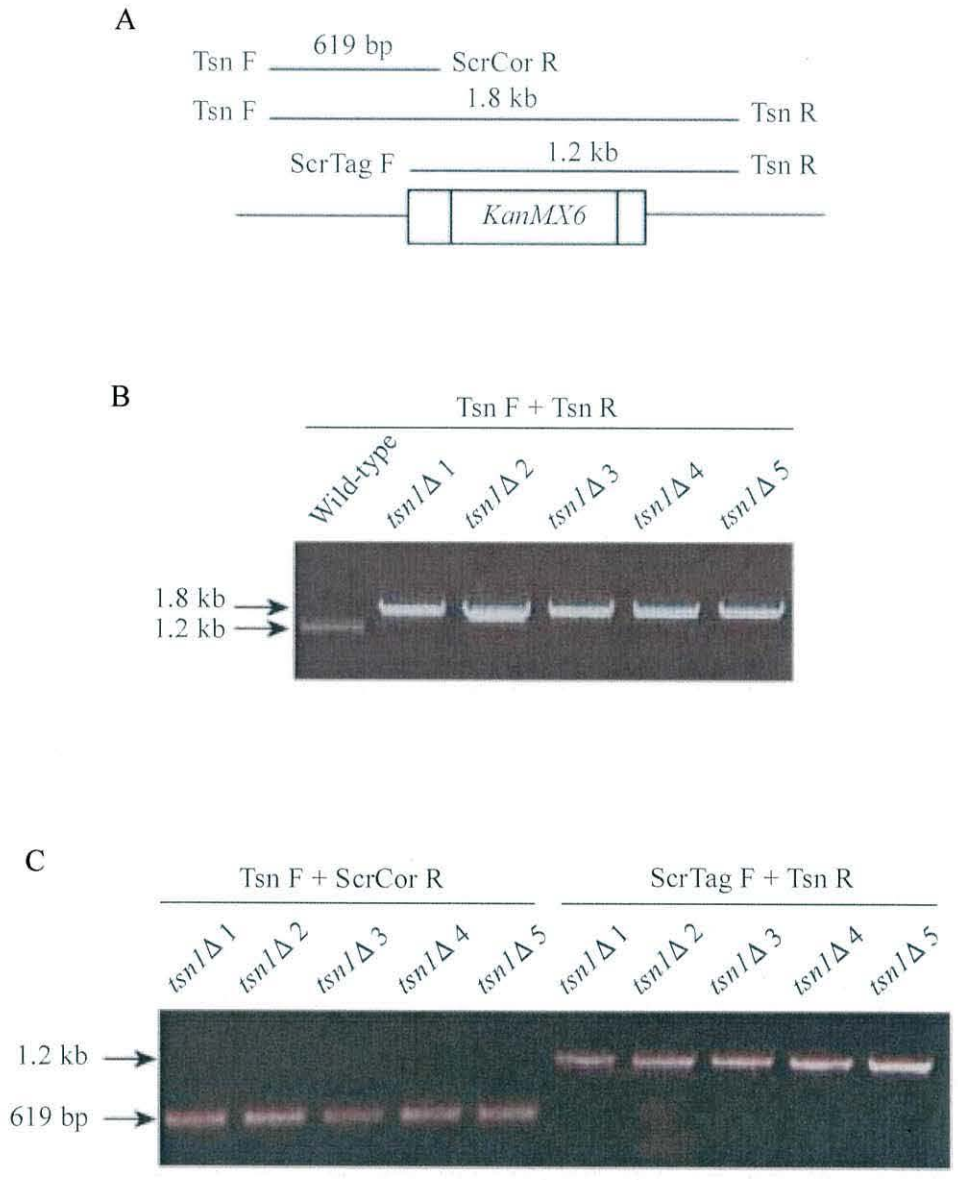
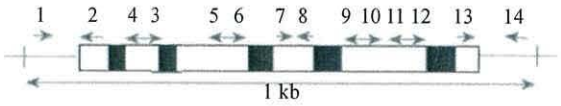


Figure 3.4 PCR checks on Translin knockout strains. A. Diagram showing the positions of the screening primers used and the predicted PCR product sizes. **B**. PRC products from a *wt* strain and the *tsn1*Δ haploid strains with Translin external primers TsnF and TsnR. The product in the *wt* is1.2 kb and is 1.8 kb in the knockout as the kanamycin cassette is larger than the *tsn1* gene. **C**. PCR products with the *tsn1*Δ haploid strains using primers TsnF and ScreencorrectR (ScrCor R), and TsnR and ScreentagF (ScrTag F), giving products of 619 bp and 1.2 kb respectively.

A



B

Primer set	Primers	Product size (bp)
A	1 + 2	131
B	1 + 4	197
C	3 + 5	358
D	6 + 8	137
E	7 + 8	75
F	7 + 9	144
G	10 + 11	133
H	12 + 14	160
I	13 + 14	73
J	7 + 11	209
K	10 + 14	309

C

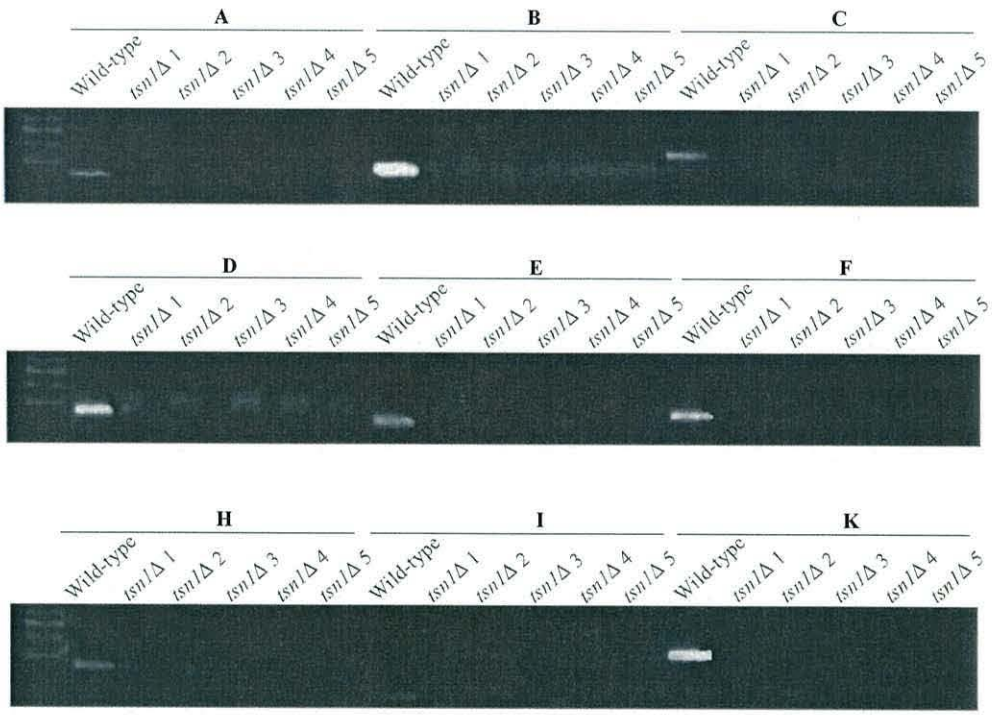


Figure 3.5 Nested PCRs for the *tsn*Δ strains. A. Diagram showing the locations of the primers used. Introns are shown filled in. **B.** Shows primers sets and the expected product size for each primer pair. **C.** Shows PCR products for reactions A-K. Primer 11 failed to give a product in all of the reactions attempted, and so reactions G and J are missing from the figure.

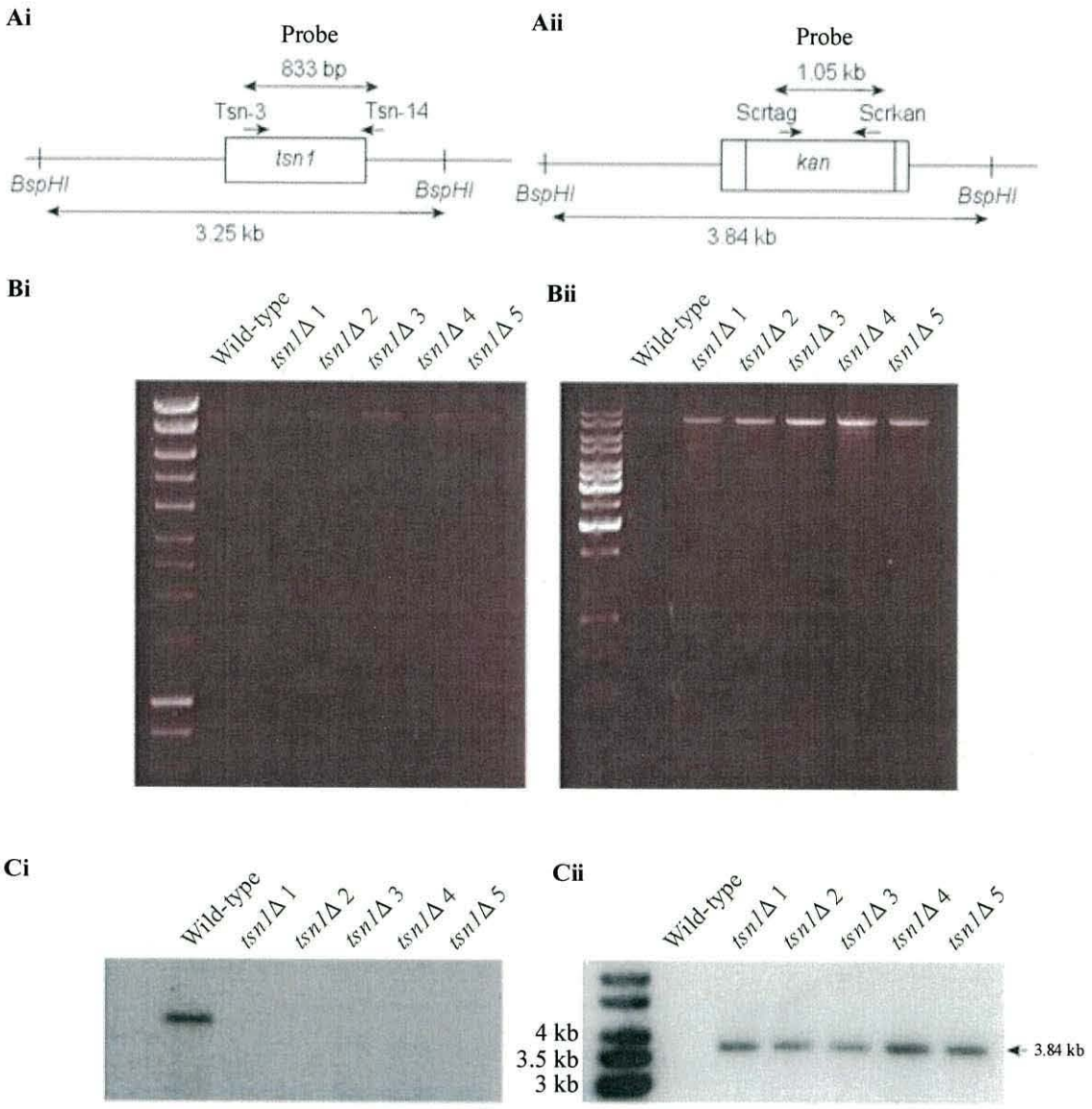


Figure 3.6 Southern blots of candidates *tsn1*Δ strains. Ai and **ii** show the *Bsp*HI digestion sites and probe locations for *tsn1* and *tsn1::kanmx6* respectively. Giving a fragment of 3.25 kb with a tsn probe of 833 bp produced from primers Tsn-3-F and Tsn-14-R (**Ai)**, and giving a fragment of 3.84 kb with a 1 kb probe homologous to the kanamycin sequence produced from primers Screentag-F (Scrtag) and Scrkan1458-R (**Aii**). Faint but fairly equal loading of digested genomic DNA for *wt* (BP90), and *tsn1*Δ strains 1-5 can be seen in **Bi,** and equal loading of the *tsn1*Δ strains 1-5 and fainter loading of the wild-type (BP90) for the southern probed for *kanMX6* can be seen in **Bii**. **Ci** shows a clear single band for *tsn1* in the *wt* control but nothing in the deleted strains, and a band of expected size was detected with the *kanMX6* probe in all five *tsn1*Δ in **Cii**.

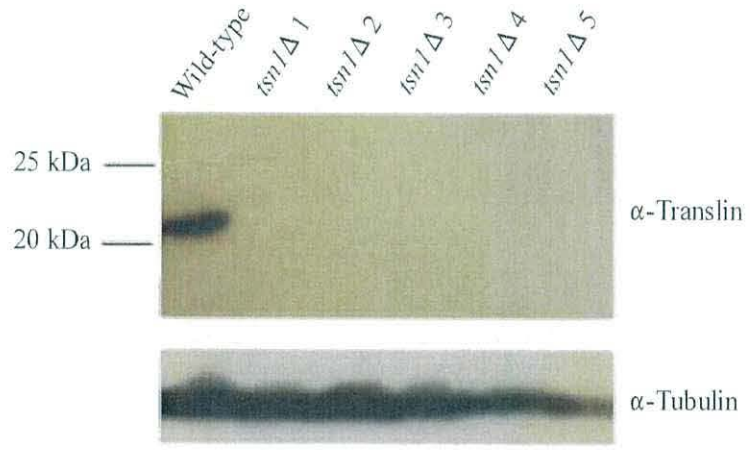


Figure 3.7 Western blots of two haploid *tsn1*Δ strains. Protein blots of the *tsn1*Δ strains 1 - 5 and a control strain were probed with anti-C-terminal Translin antibody. Bands corresponding to the predicted size for Translin were detected only in the wild-type (BP90) control. Anti-α-tubulin blot is shown as a loading control.

3.4 Regulation of TRAX transcription by Translin

Strains deleted for TRAX were also created and characterised using the same methodology described for the *tsn1*Δ mutants, and like the *tsn1*Δ mutants, the *trax*Δ strains are also viable (S. Ramayah personal communication). Whole cell protein extracts (WCEs) of mitotically growing *tsn1*Δ and *trax*Δ cells were made and Translin and TRAX protein levels were analysed by western blot. In *trax*Δ cells no TRAX protein could be detected with TRAX-specific antibodies, as expected; however, Translin protein levels were equal to wild-type levels (see Fig. 3.8A). In *tsn1*Δ cells no TRAX protein could be detected in a limited exposure, but with a prolonged exposure low levels of TRAX protein was detected. When the *trax* mRNA levels were analysed by northern blot in these strains, it was found that *tsn1*Δ cells contained levels similar to wild-type. These observations suggest that TRAX expression is post transcriptionally regulated by Translin.

Prolonged exposure of western blots of whole cell extracts from the wild-type exposed at least three modified forms of TRAX, migrating at higher molecular weights (Fig. 3.8B). This suggests that TRAX protein is modified in mitotically growing cells. The molecular weight shift between these species was roughly 5 kDa. *S. pombe* ubiquitin monomers are 8.4 kDa but it is still possible that at least one of these species is an ubiquitinated form of TRAX, possibly targeting it for degradation by the proteasome. Previous studies have demonstrated that in TB-RBP-deficient MEFs treated with the proteasome inhibitor MG-132 TRAX protein levels were restored (Yang *et al.*, 2004). Moreover, ubiquitinated forms of TRAX were also found in MEFs (Yang *et al.*, 2004), suggesting that TRAX is regulated by ubiquitin-mediated proteolysis. We addressed this possibility in two different ways. Firstly, we tried to inhibit protein degradation by treating wild-type cells with MG-132. Cycloheximide was used in conjunction with MG-132 to inhibit further protein synthesis. In western blots of WCEs of cultures treated with the inhibitors showed no increase in TRAX protein (Fig. 3.9). This approach is potentially flawed, as it is not known if MG-132 is effective in *S. pombe* cells.

Both Cdc13 and Rum1 are regulated by ubiquitination-mediated proteolysis. We attempted to determine whether MG-132 was effective in *S. pombe* by probing Western blots of WCEs of MG-132 treated cells with anti-Cdc13 and anti-Rum1 antibodies. Unfortunately, these antibodies did not work in our hands. A second experiment was subsequently designed using a *mts3* temperature sensitive mutant. *mts3* encodes a subunit of the proteasome complex, and when the *mts3-1* mutant is shifted to the non-permissive temperature of 37°C the activity of the proteasome is greatly reduced (Gordon *et al.*, 1996). *mts3-1 tsn1*Δ double mutants were made and TRAX protein was detectable in this strain at both the permissive and non-permissive temperatures, but there was no increase in the levels and no restoration to wild-type levels (Fig. 3.10). This suggests another Translin-dependent and proteasome-independent pathway is controlling TRAX protein levels.

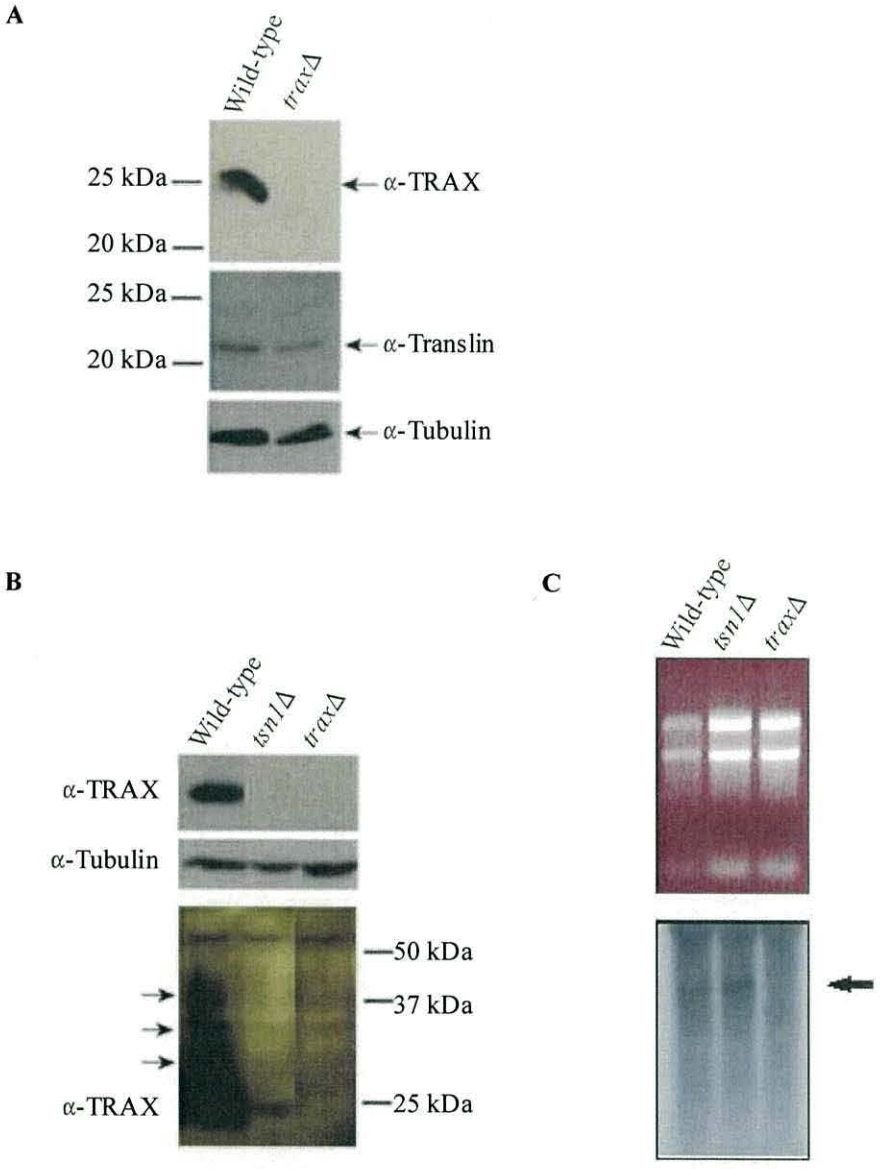


Figure 3.8 Analysis of TRAX expression in Tsn-deficient cells. Western blots were carried out on WCEs from *wt*, *tsn1*Δ and *trax*Δ cells. (**A**) In *trax*Δ cells no TRAX protein was detected, but translin protein is still present at wild-type levels. (**B**) In *tsn1*Δ cells TRAX protein is undetectable following limited exposure (upper panel), but can be detected following prolonged exposure (lower panel). Higher molecular weight modified forms of TRAX (black arrows) are also apparent in the prolonged exposure. In both figures anti-α-tubulin blots are shown as loading controls. (**C**) A Northern blot probed with a TRAX-specific probe, showed that TRAX mRNA is detected in the wt and *tsn1*Δ cells at roughly equal levels (lower panel). As expected, no TRAX mRNA is detected in the *trax*Δ. Total RNA loaded is shown in an ethidium stained gel, the upper panel.

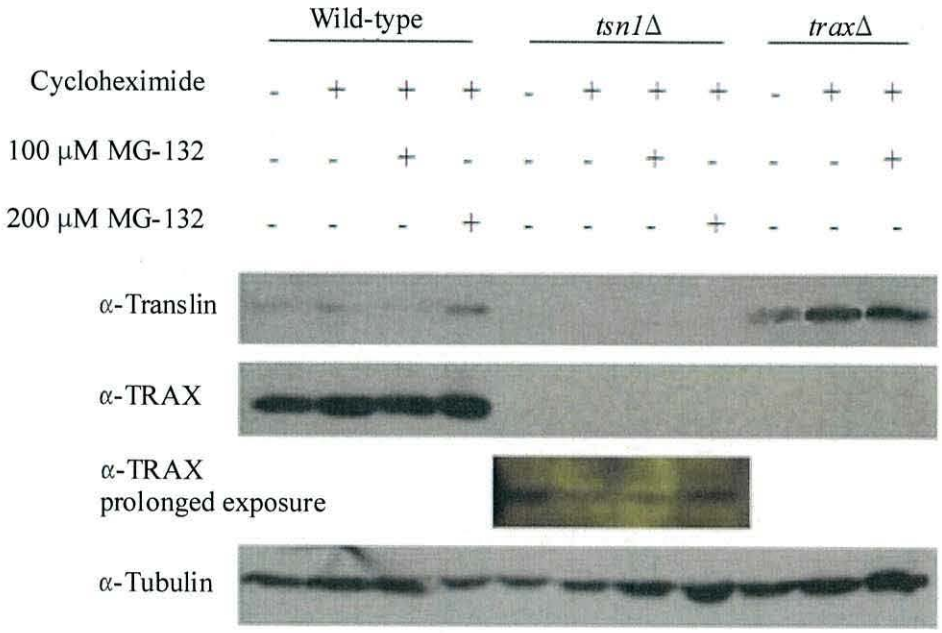


Figure 3.9 Cultures treated with MG-132. Exponentially growing cultures were treated with 100 μg/ml of the eukaryotic protein synthesis inhibitor Cycloheximide and 100 μM or 200 μM of the proteasome inhibitor MG-132. After 2 hrs of treatment at 33°C Whole cell extracts (WCEs) were prepared and Translin and TRAX protein levels analysed. No significant change was seen in Translin protein levels in wild-type and *traxΔ* cells. There was also no detectable increase in TRAX protein in wild-type and *tsn1Δ* cells in both a short and a prolonged exposure. Anti-α-tubulin blot is shown as a loading control.

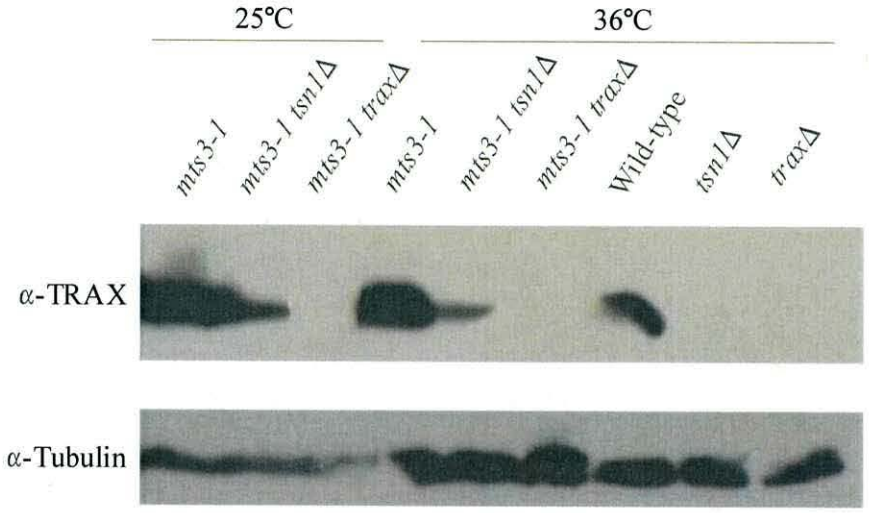


Figure 3.10 Western blot of *mts3* mutant strains. TRAX protein is slightly restored on loss of proteasome function translin-deficient cells. Cultures were grown at 25°C then split and left at 25°C and 36°C for 4 hrs and then WCEs were prepared. There is elevated TRAX protein levels in all *mts3-1 trax*$^{+}$ strains. In the *mts3-1 tsn1::kanmx6* double mutant some TRAX protein is restored, but there is no change in TRAX protein levels at the permissive or non-permissive temperature.

3.5 Investigation into the role of Translin and TRAX on cell growth

3.5.1 Growth proficiency of Translin null mutants

Translin deficient mice show reduced cell proliferation (Chen *et al.*, 2003) and over expression of Translin in murine cells has been shown to increase the rate of cell proliferation (Ishida *et al.*, 2002). A Translin genomic clone was made (pDP1; see Chapter 2), and transformed into the *tsn1*Δ strains. The presence of *tsn1*$^+$ and Translin protein was verified by PCR and western blot respectively (Fig. 3.11). The mitotic growth rate was followed over 10.5 hrs for wild-type cells (BP90), *tsn1::kanmx6* strains 1 and 5, *tsn1::kanmx6* (pFY20) empty vector and *tsn1::kanmx6* (pDP1) translin genomic clone. Samples were taken every 1.5 hrs for a total cell count, viable cell count and OD_{595nm} measurements (Fig. 3.12 shows the OD measurements, total cell count and viable cell count gave similar results). The rate of growth was found to be the same for all the strains tested. All strains entered stationary phase with similar kinetics. This suggests that the lack of Translin does not affect growth rate or the cell division rate.

3.5.2 Translin clones and overexpression of Translin and TRAX

tsn1 and *trax* clones were made that were under the control of the repressible *nmt*-promoter (no message in thiamine), so that *tsn1*$^+$ and *trax*$^+$ could be over expressed and any effect on growth rate determined. Only the ORF of *S. pombe* *tsn1* was amplified by PCR using primers with the *Bam*HI restriction site added to the 5′ and 3′ ends. This was digested and inserted into the *Bam*HI site located in the polylinker of pREP3X and pREP81X which contain the full strength and low strength *nmt*-promoters respectively. Inserts in the correct orientation were selected based on plasmid digests and on PCR results (see Fig. 3.13). pAJ1 was selected as the *nmt::tsn1*$^+$ full strength promoter and pAJ2 as the low strength. Both plasmids were fully sequenced over the insert using overlapping PCR products.

The growth rates were followed over 10 hrs for wild-type strains containing empty vector or induced pAJ1, pSRSB3 (full strength *nmt::trax*$^+$ clone created by S. Ramayah) or both for the co-over expression of *tsn1*$^+$ and *trax*$^+$. No

difference in growth rate was observed in any of the strains (see Fig. 3.14-3.16). Samples were taken every 2 hrs and WCEs prepared. Western blots showed an increase in Translin levels at 6 and 10 hrs of growth in the *tsn1*$^+$ over expression (see Fig. 3.14). Translin levels were also up in the *trax*$^+$ over expression experiment at 6 hrs but down again at 10 hrs of growth (Fig. 3.15). If Translin is needed to stabilise TRAX then perhaps levels of Translin increase in response to *trax*$^+$ mRNA over expression. TRAX levels were also elevated when *tsn*$^+$ was over expressed at 6 hrs of growth and then reduced again at 10 hrs. Perhaps this increase is directly due to the increase in Translin levels and that more TRAX is produced possibly because its regulation is Translin-dependent. TRAX levels were also elevated when pSRSB3 was induced, but again these levels dropped at 10 hrs of growth compared to 6 hrs. So in both experiments Translin and TRAX levels were greater mid-log phase than when the culture is nearing stationary phase. In the co-over expression experiment only Translin protein was found to be over expressed (Fig. 3.16). This could be due to conditions or perhaps the two plasmids inhibit each other or some other reason which is not apparent.
In further investigation (by S. Ramayah) of the *nmt::trax*$^+$ clones (pSRSB3 and pSRSB2, full and medium strength respectively), it was found that there was a considerable increase in *trax* mRNA produced in both but that this did not translate into increased TRAX protein after 16 hrs nor 20 hrs post Thiamine removal (see Fig. 3.17). This is consistent with the results from the over expression experiments, where increased TRAX protein is seen at six hrs but not at 10 hrs growth.

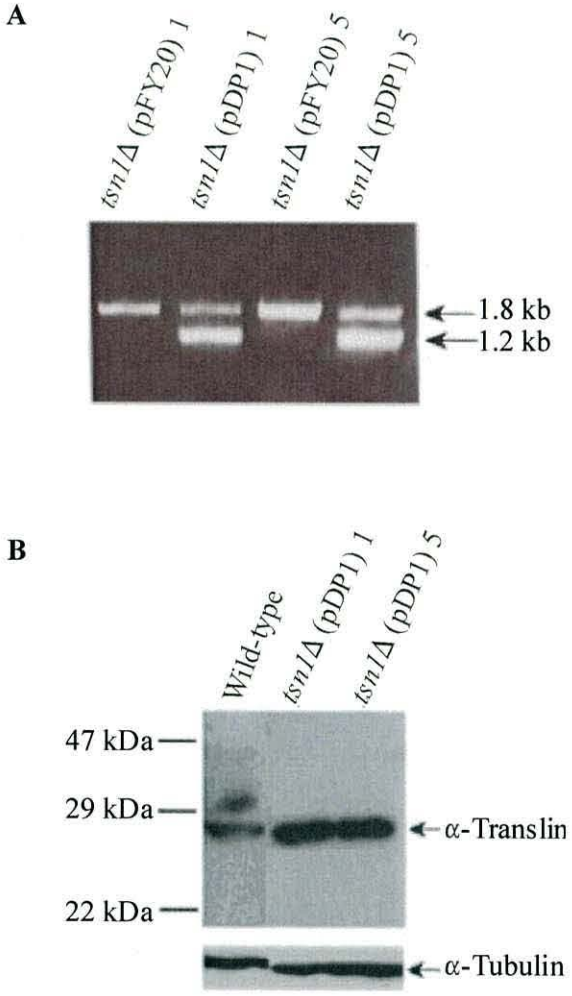


Figure 3.11 Verification of the *tsn1* gene and Translin protein in *tsn1*Δ strains transformed with the genomic *tsn1* clone pDP1. DNA was extracted from a pFY20 (empty vector) and pDP1 transformant from each of the five *tsn1*Δ strains and a PCR with TsnF and TsnR carried out (only the strains 1 and 5 are shown). A single band of 1.8 kb can be seen in the pFY20 transformants corresponding to the larger kanamycin cassette, and a double band of 1.2 kb and 1.8 kb in the pDP1 strains corresponding to *wt tsn1* and the kanamycin insert respectively (**A**). A western blot was also carried out (**B**) on *tsn1::kanmx6* (pDP1) strains 1 and 5, and in both cases a clear band of Translin protein can be seen in an α-C-teminal Translin blot. Anti-α-tubulin is also shown as a loading control.

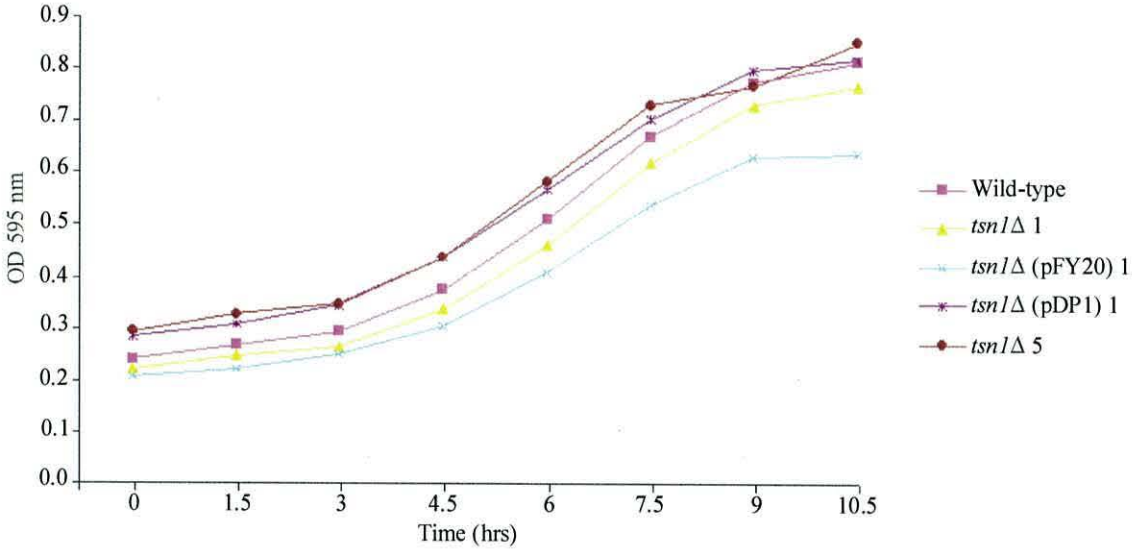


Figure 3.12 Time course of *tsn1*Δ strains 1 and 5 and *tsn1*Δ strains carrying the *tsn1* genomic clone (pDP1) and empty vector (pFY20). Growth was followed over 10.5 hrs at 30 °C, during which time two doublings occurred.

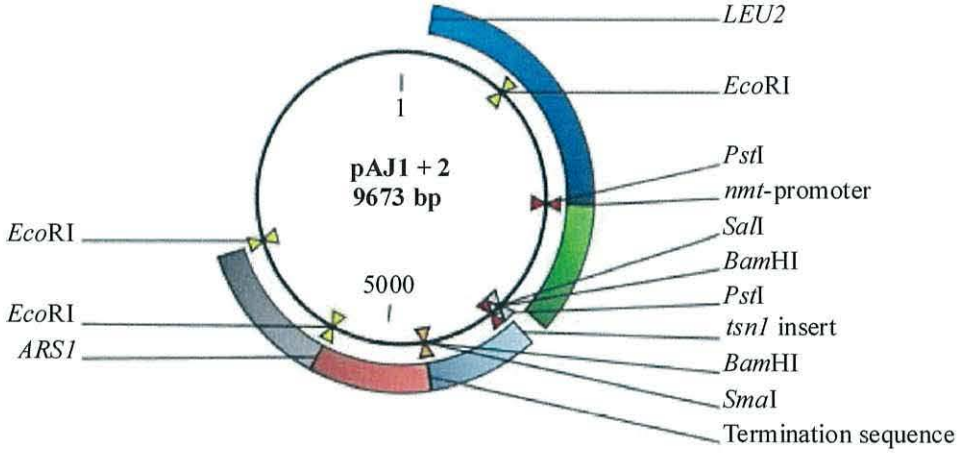


Figure 3.13 Translin clones with repressible *nmt*-promoters. The *tsn1* ORF was inserted into the *Bam*HI site in the multicloning site of the *nmt*-promoter (no message in thiamine) plasmids pREP3X (full strength promoter) and pREP81X (low strength promoter), creating plasmids pAJ1 (full strength) and pAJ2 (low strength) respectively (A). The plasmids also contain the *LEU2* gene (dark blue), a termination sequence (red) and the autonomous replication sequence (*ARS*) (grey).

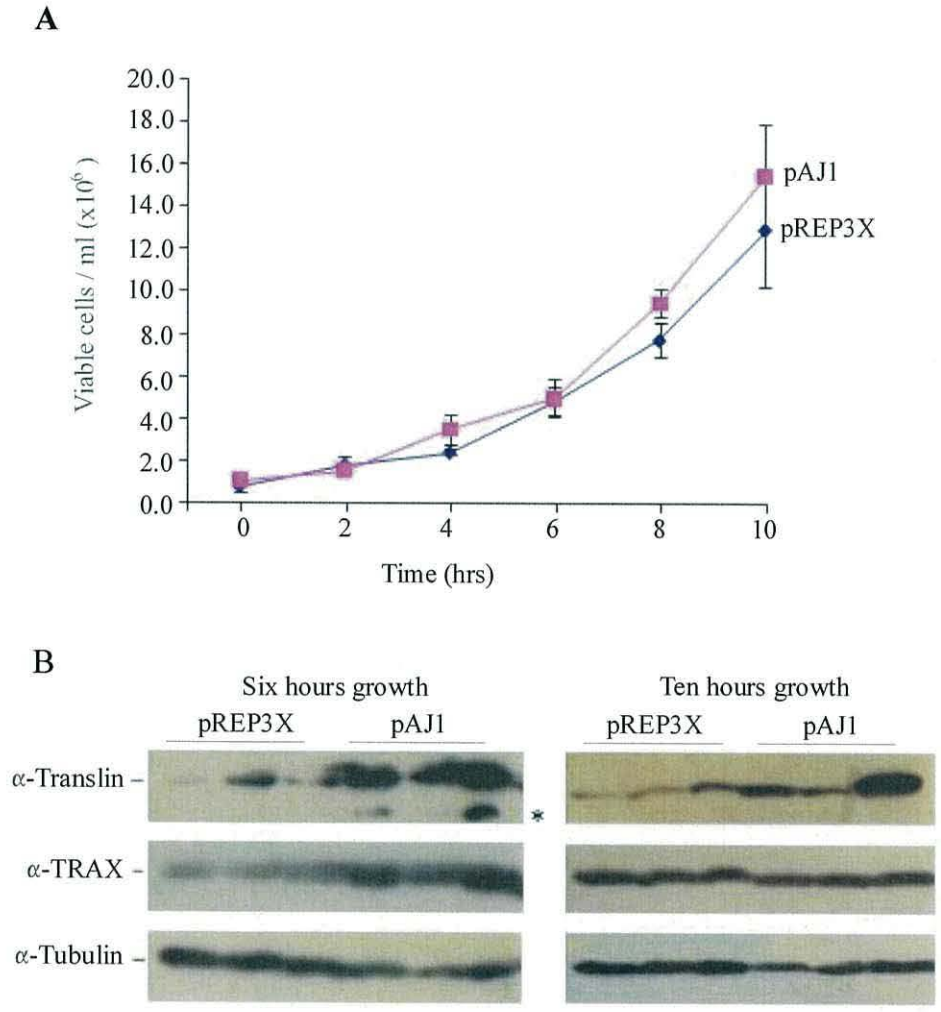


Figure 3.14 Over expression of Translin. Translin was over expressed using the pAJ1 plasmid under control of the full strength *nmt*-promoter. pREP3X empty vector was used as a negative control. Exponential growth at 33°C was followed over 10 hours, and the OD_{595} and viable cell count measured for three cultures of each strain (A), the standard deviation between the three measurements are shown as error bars. No difference in growth rates or cell morphology was seen. WCEs were prepared from each time point. (B) Increased levels of Tsn were detected in western blots at both six and ten hours of growth, (asterix indicates a Tsn-related band which we believe to be a Tsn degradation product detectable in some WCEs). Interestingly TRAX protein levels seem to be increased at six hours as well but not at ten hours, perhaps this is directly related to Tsn over expression. An anti α-tubulin blot is shown as a loading control.

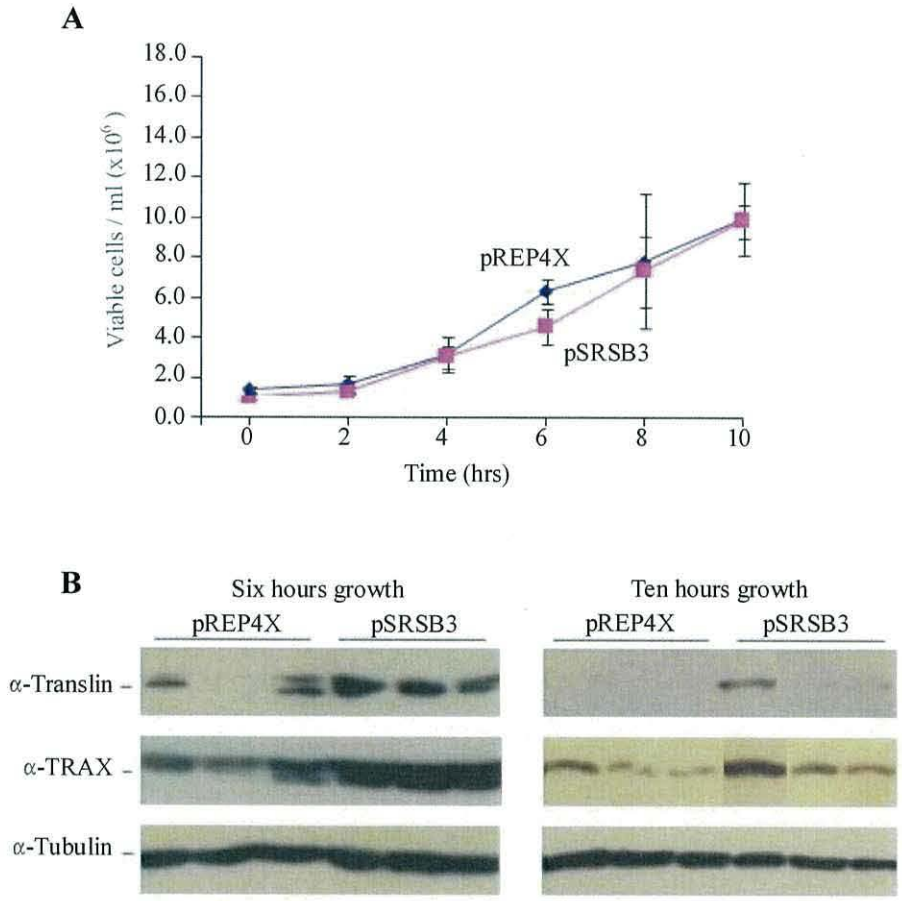


Figure 3.15 Over expression of TRAX. TRAX was over expressed using the pSRSB3 plasmid under control of the full strength *nmt*-promoter. pREP4X empty vector was used as a negative control. Exponential growth at 33°C was followed over 10 hours, and the OD_{595} and viable cell count measured for four cultures of each strain (A), the standard deviation between the three measurements are shown as error bars. No difference in growth rates or cell morphology was seen. WCEs were prepared from each time point. (B) An increase in TRAX levels was only seen at six hours of growth with reduced levels at ten hours. Interestingly, Translin levels appeared to show the same pattern. Increased levels of Tsn are perhaps needed to stabilise increased levels of TRAX. An anti α-tubulin blot is shown as a loading control.

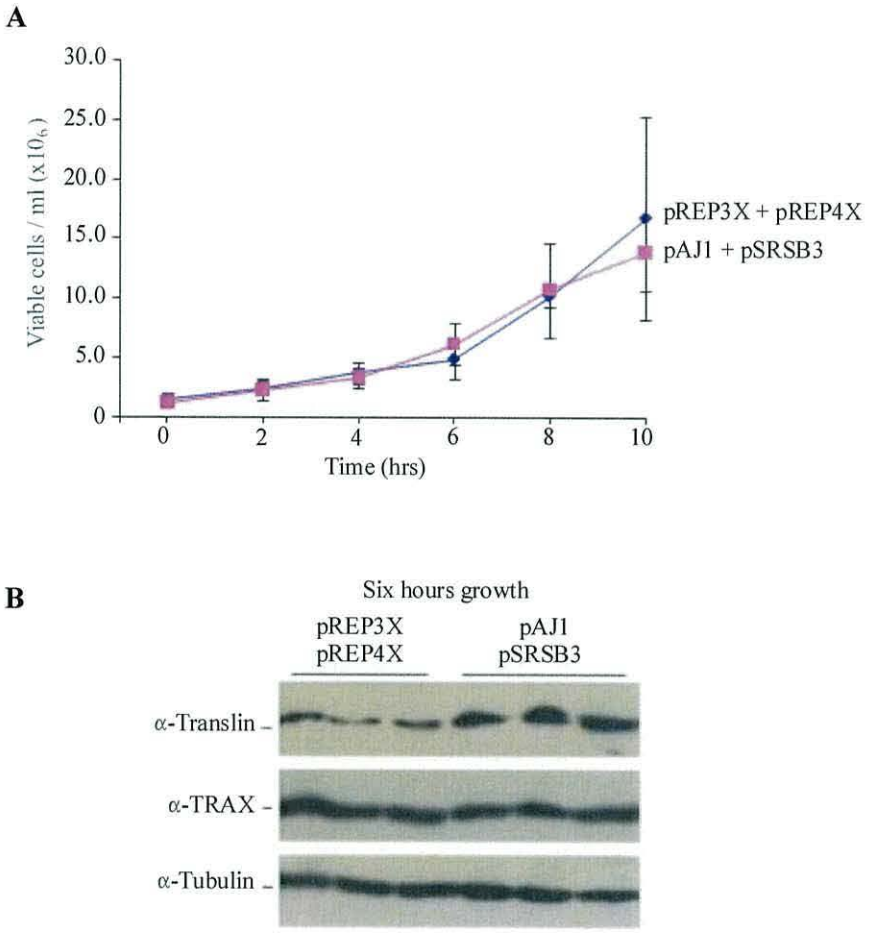


Figure 3.16 Co-over expression of Translin and TRAX. Translin and TRAX were over expressed using the pAJ1 and pSRSB3 plasmids respectively. pREP3X and pREP4X empty vector were used as a negative control. Exponential growth at 33°C was followed over 10 hours, and the OD_{595} and viable cell count measured for four cultures of each strain (A), the standard deviation between the three measurements are shown as error bars. No difference in growth rates or cell morphology was seen. WCEs were prepared from each time point. (B) At six hours increased Tsn levels were detected but no increase in TRAX levels were detected. An anti α-tubulin blot is shown as a loading control.

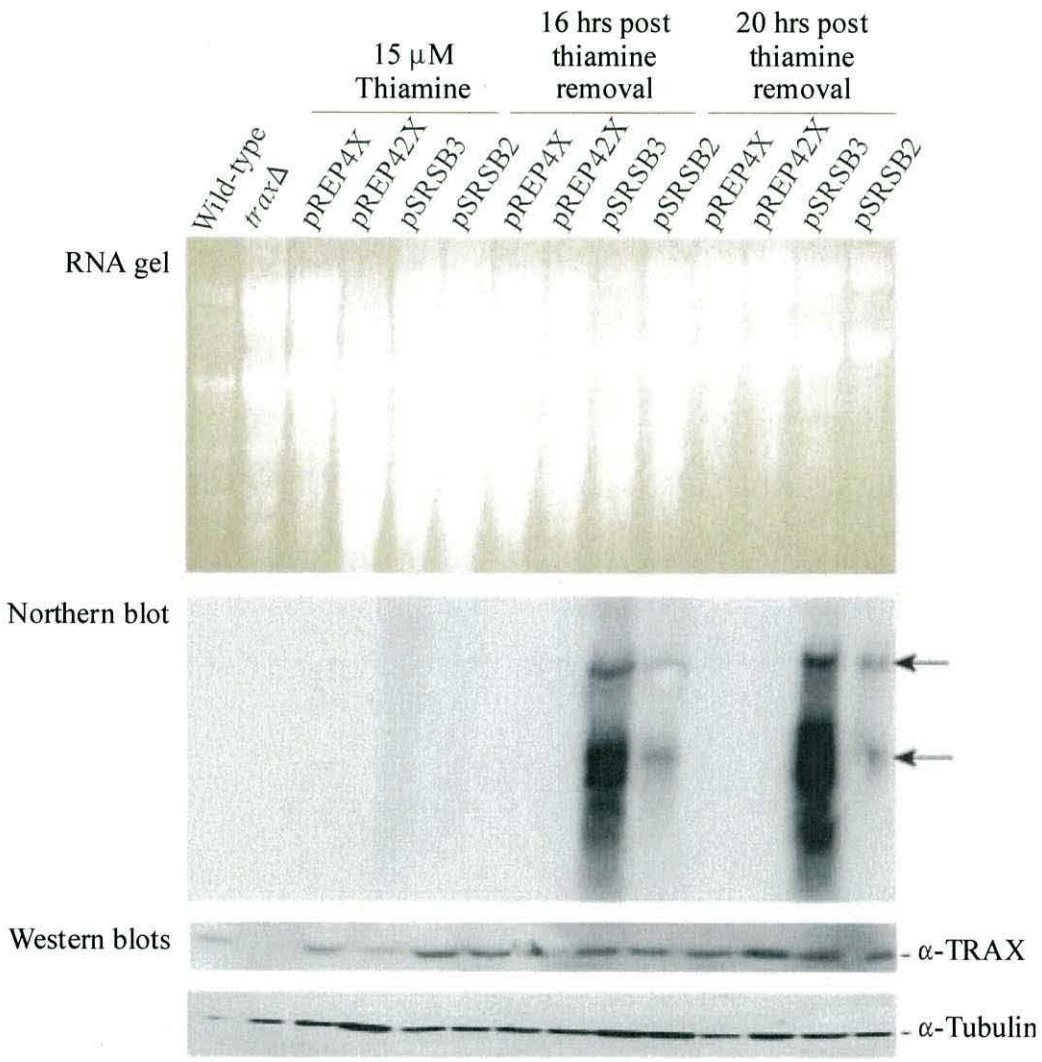


Figure 3.17 Analysis of TRAX expression of the TRAX clones pSRSB3 and pSRSB2. Cultures containing pREP4X, pREP42X (full and medium strength *nmt*-promoter empty vector respectively,) pSRSB3 and pSRSB2 (full and medium strength *nmt::trax* clones respectively,) were grown in selective media containing 15 μM thiamine until stationary phase. Samples were taken for Northern and Western blots at 16 and 20 hours post thiamine removal. Two bands of 1 kb and 1.5 kb were detected with a TRAX-specific probe (black arrows) in both TRAX clones after thiamine removal. Increased *trax* RNA levels resulted in only a very slight increase in TRAX protein levels in these strains. RNA gel shows loading of RNA samples, and an anti α-tubulin blot is shown as a loading control of protein samples (work carried out by S. Ramayah.)

3.6 Temperature sensitivity

All five *tsn1*Δ mutants were tested for temperature sensitivity at 20°C, 30°C and 37°C. Growth was the same as wild-type for all five strains (Fig. 3.18).

3.7 Microtubule destabilisers

It has been shown that the rate of cell proliferation in mouse cells correlates with the levels of Translin (Ishida *et al.*, 2002). The same study showed that when the cells entered mitosis, Translin was seen on the astral microtubules which radiate from the centromeres at prometaphase/metaphase transition. This may imply that Translin accelerates microtubule organization and chromosome segregation during mitosis (Ishida *et al.*, 2002). To test whether Translin functions in microtubule stabilisation, all five *tsn1*Δ mutants were tested for sensitivity to the microtubule inhibitor thiabendazole (TBZ) and the more sepecific microtubule inhibitor methyl benzimidazol-2-yl carbamate (MBC). A *bub1*Δ mutant strain was used as a positive control. Fission yeast Bub1 is a kinetochore scaffold protein and is required for the recruitment of other proteins to the kinetochore (Kadura *et al.*, 2005; Vanoosthuvse *et al.*, 2004). None of the *tsn1*Δ strains showed increased sensitivity to either agent compared to the wild-type control (Fig. 3.19)

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3.8 Salt and osmotic stress

To test whether Translin may have a role in a wider cellular stress response, the *tsn1*Δ mutants were tested for sensitivity to agents that cause salt and osmotic stress. A *spc1*Δ mutant was used as a positive control. In *S. pombe*, Spc1 is activated by Wis1 in response to various forms of stress, including osmostress (Millar *et al.*, 1995; Shiozaki and Russell, 1996), Spc1 then phosphorylates the nuclear transcription factor Atf1, which in turn regulates genes required for cellular resistance to various types of stress (Shiozaki and Russell, 1996; Wilkinson *et al.*, 1996). No increase in sensitivity to potassium chloride or sorbitol was detected in any of the *tsn1*Δ mutants compared to the wild-type control (Fig. 3.20).

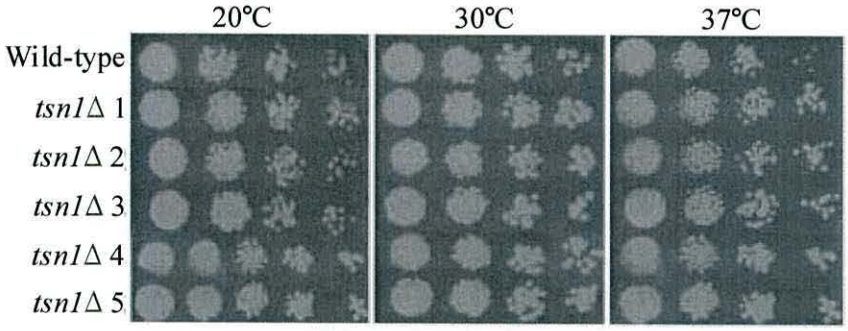


Figure 3.18 Temperature sensitivity test of Translin null mutants. Plates were incubated at 20°C, 30°C and 37°C for approximately 3 days. Serial dilutions were of log phase cultures (10^{-2} to 10^{-5}, from left to right).

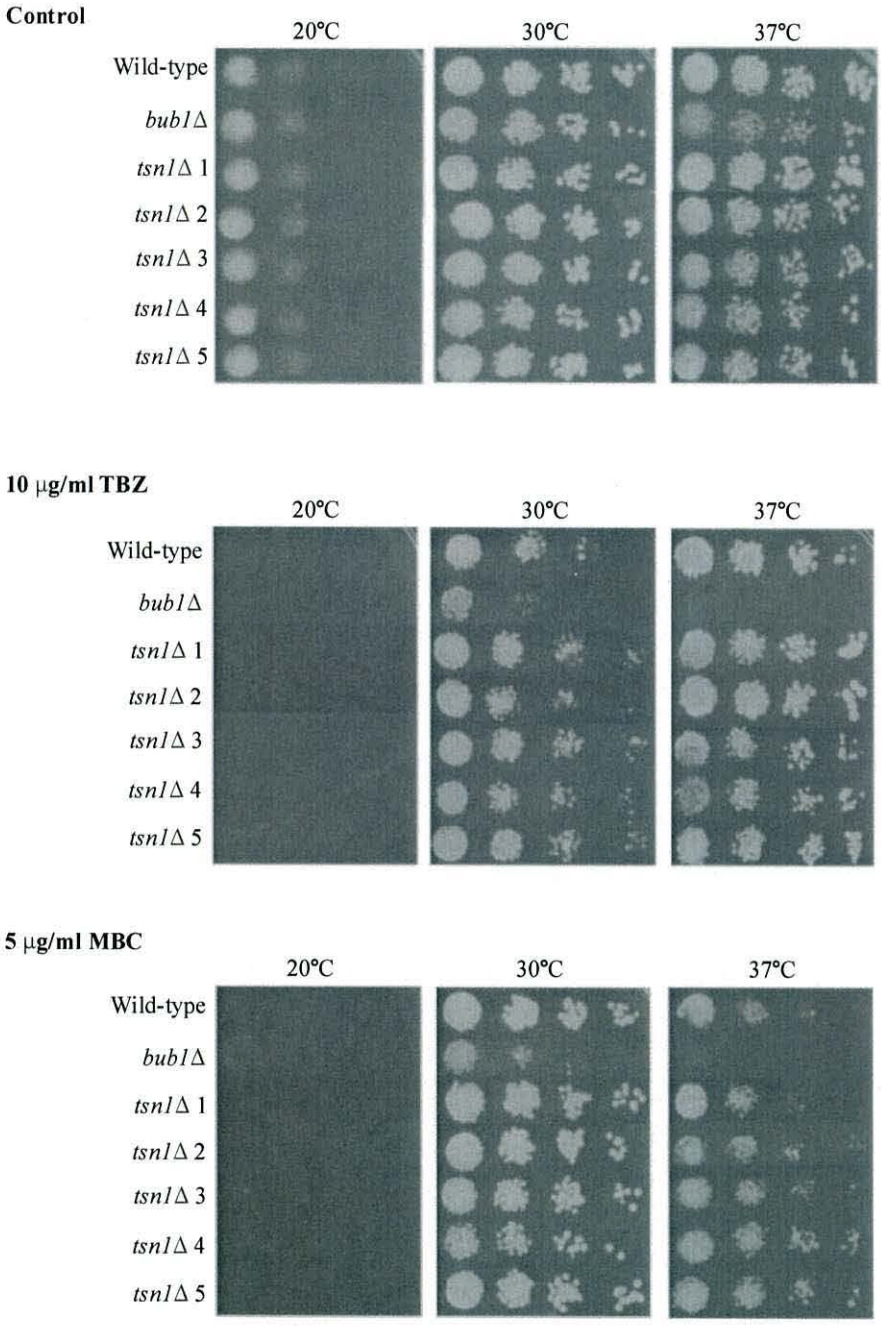


Figure 3.19 TBZ and MBC spot tests of *tsn1*Δ single mutants. All five *tsn1*Δ strains were tested for sensitivity to the microtubule inhibitors thiabendazole (TBZ) and methyl benzimidazol-2-yl carbamate (MBC). Plates were incubated at 20°C, 30°C and 37°C for approximately 3 days. Serial dilutions were of log phase cultures (10^{-2} to 10^{-5}, from left to right).

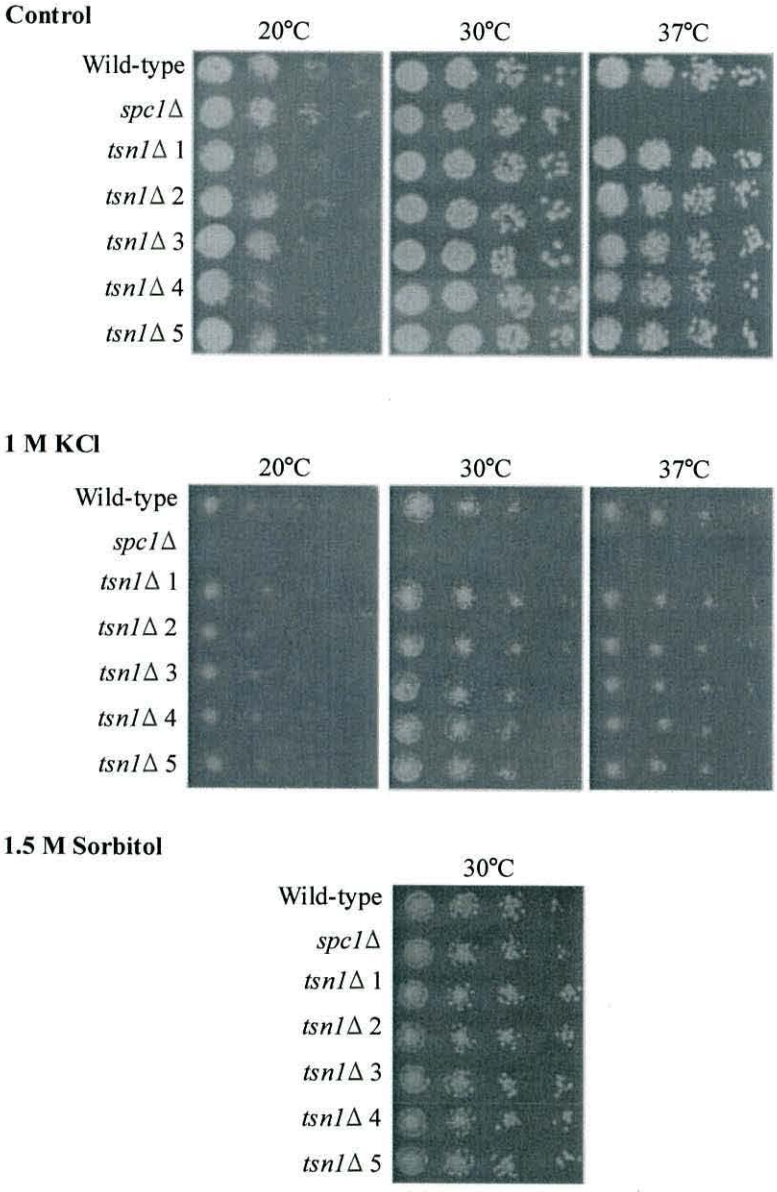


Figure 3.20 Potassium chloride and sorbitol spot tests of *tsn1*Δ single mutants. Plates were incubated at 20°C, 30°C and 37°C for approximately 3 days. Serial dilutions were of log phase cultures (10^{-2} to 10^{-5}, from left to right).

3.9 Discussion

S. pombe Translin is 36% identical and 54% similar to the human form. Even though there is no x-ray crystallographic data for *S. pombe* Translin, it is predicted to have a highly helical tertiary structure, similar to that of human Translin. Furthermore, it has been found to exist as an octamer and that only the octameric structure has DNA and RNA binding activity (Laufman *et al.*, 2005), as opposed to mouse Translin, where Translin dimers have been found to bind DNA (Wu *et al.*, 1998). The putative RNA and RNA/DNA binding domains are not very well conserved in *S. pombe*, however, recombinant *S. pombe* Translin has been shown to bind $d(GT)_{15}$ and $d(GTT)_{10}$ oligodeoxy nucleotide repeats with high affinity and binds telomeric repeats $d(GGTTACA)_4$ with lower affinity. Interestingly, *S. pombe* Translin had a higher affinity for oligoribonucleotide $(GU)_{15}$ than for the corresponding oligodeoxynucleotide $d(GT)_{15}$ (Laufman *et al.*, 2005), possibly suggesting that *S. pombe* Translin functions in RNA metabolism rather than DNA. These binding differences may be related to the differences in structure and ultimately differences in function. It is not known if DNA and RNA binding in *S. pombe* is due to the same basic regions clearly identified in human Translin (Aoki *et al.*, 1995, 1999). Interestingly *Drosophila* Translin shares 52% identity with human Translin but shows no DNA or RNA binding ability (Sengupta *et al.*, 2006), however the basic regions thought to be involved in nucleic acid binding are less poorly conserved than in *S. pombe*. However the ssDNA and RNA probes used were different to those used in studies on the *S. pombe* protein (Sengupta *et al.*, 2006).

The GTP binding site and the NES sequences are fairly well conserved in *S. pombe*. GTP binding is thought to suppress RNA binding ability but not DNA binding (Chennathukuzhi *et al.*, 2001). In *S. pombe* this may be an important factor if it naturally has higher affinity for RNA. There were also multiple predicted phosphorylation sites in both human and *S. pombe* sequences, with several being in the NES and GTP binding sites, these may be real and have a significant effect on the function of these sites. It is thought that *S. pombe* TRAX

lacks a NLS (Laufman *et al.*, 2005), which may suggest that the Translin/TRAX complex functions almost exclusively in the cytoplasm. However, as yet no localisation studies have been completed.

In the C-terminus there is also a leucine zipper region, which is believed to be required for protein dimerization in mammalian Translin and is stabilised by a disulphide bond involving the cysteine residue 225. Dimerization or oligermerisation is essential for DNA or RNA binding, as the monomer will not bind to nucleic acids *in vitro* (Wu *et al.*, 1998; Aoki *et al.*, 1999). The leucine residues, which make up the zipper are largely conserved in the fission yeast protein but the cysteine is not. Recombinant *S. pombe* Translin forms octamers which bind both DNA and RNA. In Yeast two-hybrid assays Translin was found to specifically bind to TRAX and other Translin subunits (Laufman *et al.*, 2005), this all suggests that even though cysteine 225 is missing, Translin subunits still form stable multimeric structures. Cysteine 225 is also replaced in *Drosophila* Translin and dimers are not stabilised by disulphide interactions (Suseendranathan *et al.*, 2007). In fact there are doubts about whether a disulphide bridge could form in the reducing environment of the cell (Pascal *et al.*, 2002). Excimer fluorescence studies indicate that disulphide bonds are not required for octamer formation and that Translin monomers interact in a tail-to-tail orientation (Han *et al.*, 2002).

In the quaternary structure of the human protein, two tetramers come together to form an octamer, with the basic residues thought to be involved in RNA and DNA binding facing inwards into a cavity where it is thought RNA and DNA fits into (see Fig. 3.21). The multimerization of different Translin molecules is flexible, which may aid binding of different nucleotide structures (Sugiura *et al.*, 2004).

We found that *S. pombe* Translin-null mutants are viable, show normal cell morphology and growth rate. The facts that this protein is well conserved and that all Translin-deficient organisms studied to date are viable suggests that Translin is possibly part of a redundant pathway.

In Translin-deficient cells we detected greatly reduced levels of TRAX protein. The conclusion being that TRAX expression is Translin-dependent as in murine

and *Drosophila* cells (Yang *et al.*, 1997; Clauβen *et al.*, 2006). Translin-TRAX interactions are thought to prevent ubiquitin-mediated TRAX proteolysis and the inhibition of proteolysis results in a full restoration of TRAX levels in Translin-null MEFs (Yang *et al.*, 1997). We found that in our Translin-null strains, TRAX mRNA levels are the same as in wild-type, and that both proteasome inhibition with MG-132 and the use of a proteasome mutant did not restore TRAX levels to wild-type levels. It is unknown whether MG-132 is effective at proteasome inhibition in *S. pombe*, therefore we also used a *mts3-1* mutant which has reduced proteasome activity at a permissive temperature. The fact that the level of TRAX protein is only slightly restored in the *mts3-1 tsn1*Δ strains compared to the *mts3-1* single mutant, suggests that the *mts3-1* conditional mutant is possibly functionally leaky and residual proteasome activity is possibly sufficient to mediate some TRAX degradation in the absence of Translin, this is possibly indicative of TRAX being highly labile. Based on the evidence we believe that TRAX levels are regulated in a proteasome-independent, Translin-dependent manner, and that Translin possibly regulates the translation of $trax^{+}$ mRNA. Since organisms that possess a Translin homolog also have a TRAX homolog, it suggests that these two proteins are functionally related and possibly function in the same pathway. *S. pombe* Translin and TRAX have been shown to specifically interact *in vitro* as observed in mouse and human (Laufman *et al.*, 2005; Aoki *et al.*, 1997b; Chennathukuzhi *et al.*, 2001; Finkenstadt *et al.*, 2002). Since this interaction is conserved in unicellular eukaryotes, it is likely that any conserved function is not tissue-specific.

In a cell cycle study in HEK cells, Translin was found to localise to the centrosome, bipolar mitotic spindles and midzone, and possibly increases the rate of cellular proliferation by stabilising microtubules (Ishida *et al.*, 2002). Translin-null mice were found to develop into mature adults but were 10-30% smaller at birth compared to wild-type littermates (Chennathukuzhi *et al.*, 2003), and MEFs also displayed decreased proliferation (Yang *et al.*, 2004). In human HeLa cells TRAX was also found to be essential for normal proliferation (Yang *et al.*, 2004). In *S. pombe* Translin and TRAX deficient cells, cellular proliferation was normal,

and we found no increase in sensitivity to the microtubule inhibitors TBZ and MBC. We also over expressed both Translin and TRAX and found no change in growth. Interestingly, both Translin and TRAX levels were elevated at 6 hrs of growth in both single over expression experiments. Perhaps Translin increases in response to there being increased TRAX mRNA in the single TRAX over expression experiment and TRAX levels perhaps increase when translin is over expressed because there is more Translin present to stabilise TRAX mRNA. In MEFs from Translin-null heterozygous mice, TRAX is reduced to 50% despite *trax* mRNA levels being normal (Chennathukuzhi *et al.*, 2003), showing that the level of TRAX protein is dependent on the amount of Translin present, and that perhaps a given amount of Translin can stabilise only a given amount of TRAX. There was a co-ordinated expression of both Translin and TRAX in synchronized HeLa cells (Yang *et al.*, 2004). Microarray studies with HeLa cells show *tsn1* mRNA levels to fluctuate through the cell cycle while *trax* mRNA stays constant (Whitfield *et al.*, 2002). It is interesting that in both single over expression experiments Translin and TRAX levels are greatest at mid-log phase where many cells are dividing, rather than near stationary phase. This may imply that over expressed Translin and TRAX are degraded later on in the culture development and that Translin/TRAX do function at some point in the cell cycle if not necessarily in a way that accelerates growth. Interestingly wild-type mice treated with ionising radiation showed a down-regulation of Translin protein and mice deficient for the checkpoint protein Atm(-/-), showed no change in Translin levels after treatment (Ishida *et al.*, 2002). In another experiment investigating the differentiation of PC12 cell by nerve growth factor and megakaryotic differentiating K562 cells, Translin levels were relatively high in the proliferating cells and rapidly down-regulated during cell cycle arrest and differentiation (Ishida *et al.*, 2002), which is similar to our over-expression data, however, we did not look at endogenous Translin levels through the cell cycle. However Ishida *et al.*, (2001) found the same cell cycle-dependent pattern of Translin expression in both endogenous and over-expressed protein. Another study in *S. pombe* reported a slight stimulation in the rate of proliferation of *tsn1*$^{-}$ *trax*$^{-}$ double

mutants and in *tsn1*$^{-}$ single mutant cells, but this has not been confirmed during this investigation (Laufman *et al.*, 2005).

The co-over expression of Translin and TRAX was not successful and in general even the single over expression of TRAX was unreliable. It may be that in only cloning the ORF of *trax*$^{+}$, we may have lost important untranslated regions (UTRs) associated with TRAX, which may be essential for TRAX translation. Also because the clones are in strains with a wild-type *tsn*$^{+}$ *trax*$^{+}$ background the protein produced will not be exclusively from the plasmid, which may also complicate the interpretation.

A whole range of down stream stress responses can be induced upon activation of the transcription factor Atf1 by Spc1. We have found no evidence for a primary function in the cellular stress response for Translin in heat, salt, or osmotic stress. However, it is possible that it may have a redundant role which remains unidentified.

3. 10 Main Conclusions:

- *S. pombe* Translin is highly conserved and has a highly helical tertiary structure similar to human Translin
- Our *tsn1::kanmx6* strains are true Translin knock-outs
- Translin-deficient cells are viable, show normal cell morphology and growth rates
- TRAX expression is Translin-dependent and proteasome-independent
- Over expression of Translin and TRAX, does not result in accelerated proliferation
- Translin-deficient cells exhibit no temperature sensitivity, are not sensitive to osmotic stress or microtubule destabilisers

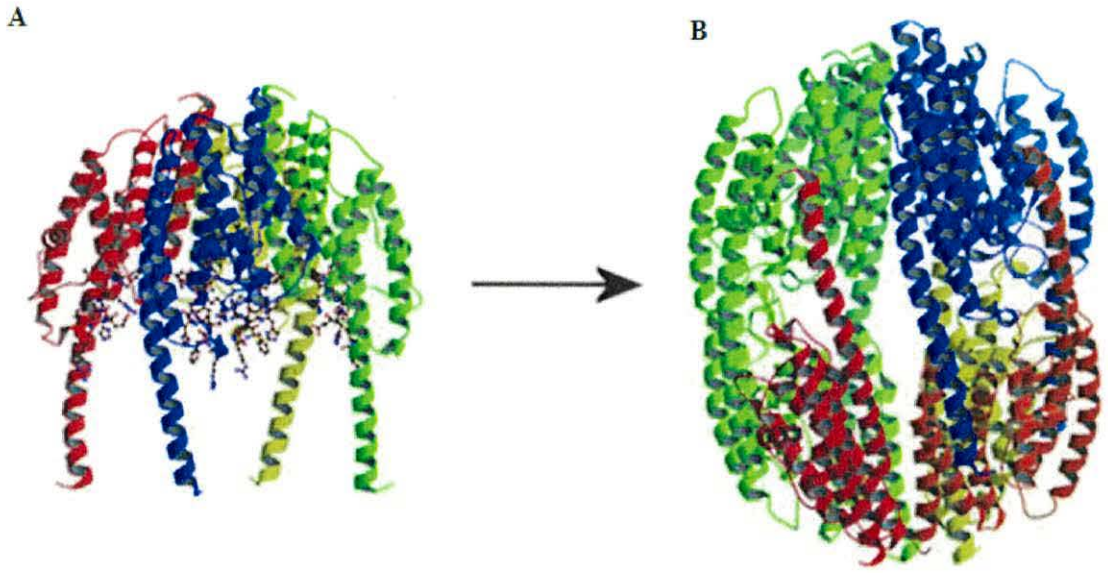


Figure 3.21 Tetramer and octamer of human Translin. Four Translin monomers interact to form a tretramer (A), two tretramers then interact to form an octameric toroidal structure (B). Side chains shown in (A) are those thought to be involved in DNA and RNA binding. (Adapted from Sugiura *et al.*, 2004).

Chapter 4 Analysis of GT repeats stability, homologous recombination and non-homologous end joining in Translin and Trax deficient *S. pombe*

4.1 Introduction

Repetitive microsatellite DNA is found mainly at the telomeres and at the centromeres, and many factors are required for their stability. Instability of microsatellites is thought to be a result of slippage of the replication machinery causing errors which are then left unrepaired. When slippage and unpaired bases occur within the newly synthesized strand, then repeat units are added. Slippage in the template strand results in deletions. When microsatellites are located within genes, instability of these regions are a common cause of several human genetic disorders, for example Huntingdon's Disease (McMurray, 1995). Microsatellites are mainly found in non-coding regions and changes do not result in loss of function. Translin is known to bind to G-rich consensus sequences in single-stranded DNA found at translocation breakpoint junctions and recombination hotspots (Aoki *et al.*, 1995). Since *S. pombe* Translin has been found to have a high affinity for binding GT repeat sequences (Laufman *et al.*, 2005), we have looked at GT repeat stability in both the *tsn1*Δ and *trax*Δ mutants, using a strain containing a GT repeat sequence in the *ade6* gene (Mansour *et al.*, 2001). We investigated whether the loss of Translin and Trax has an effect on the stability of a dinucleotide repeat sequence and on intragenic recombination at the repeat. Crossovers and gene conversion occur during meiosis when Holliday junctions are resolved. This gives two main benefits, i) it promotes genetic diversity, ii) it forms chiasmata, a physical connection between homologues essential for the correct bipolar attachment to the meiosis I spindle (reviewed by Roeder, 1997). Involvement of Translin in intragenic meiotic recombination has not been tested in any organism. Three Translin binding sites were found at a subtelomeric male meiotic recombination hotspot (*Fok*I-*Fnu*4HI), when only one in every 1200 kb would be expected to have a translin binding site if the sequence were randomly generated (Badge *et al.*, 2000). Two of these sites were tested for recombinant Translin binding and one showed strong sequence specific binding, and the

recombination rate for the 3 kb interval was enhanced by more than 300-fold. Generally, subtelomeric regions of dicentric autosomes show a consistent male-specific enhancement in recombination rate and are rich in highly variable minisatellites (Badge, 2000). Crossover levels were found to be normal in Translin-deficient *Drosophila* (Clauβen *et al.*, 2006), but *Drosophila* translin was also found to have no DNA binding activity (Suseendranathan *et al.*, 2007). Human Translin has been implicated in homologous recombination (Aoki *et al.*, 1995). In this chapter we tested mitotic recombination rates at the *ade6* locus and meiotic recombination rates (both intragenic and intergenic) in *tsn1*Δ and *trax*Δ backgrounds.

DNA double-strand breaks (DSBs) can be repaired by either non-homologous end joining (NHEJ) or homologous recombination repair (HRR) pathways (van Gent *et al.*, 2001). If DSBs are left unrepaired then parts of the chromosome that are left unconnected to the centromere will fail to segregate to the daughter cell, resulting in chromosome deletions. Incorrect repair of DSBs can result in chromosome translocations and other aberrations (Pierce *et al.*, 2001). HR is the predominant repair pathway in fission yeast S-phase/G2 cells (Manolis *et al.*, 2001), as it provides error-free repair by using the sister chromatid as a template for DNA replication. If DSBs occur at points in the cell cycle when there is no template for HR, then the break can be repaired by NHEJ (Ferreira and Cooper, 2004), where the two ends of a break are joined via intra-chromatid rejoining regardless of sequence homology, resulting in errors. Even in diploid cells, NHEJ is favoured during G1 as this will prevent loss of heterozygosity (Moynahan and Jasin, 1997). The proteins involved in NHEJ in mammals include the DNA-PK complex which comprises the Ku70-Ku80 heterodimer and a catalytic subunit (DNA-PKcs), Artemis nuclease and the DNA ligase IV-Xrcc4 complex. These proteins are also involved in V(D)J recombination, a site-specific recombination event which generates antibody and T-cell receptor diversity (Smith and Jackson, 1999). C1D is an activator of DNA-PK (Yavuzer *et al.*, 1998) and has also been found to interact with Trax when DNA damage occurs. Interestingly, it has been found that in *S. pombe* HR proteins Rad50 and Rad32 are not required for NHEJ

as in other organisms suggesting other proteins fulfil their function (Manolis *et al.*, 2001). Translin has been suggested to be involved in NHEJ due to its association with translocation breakpoint junctions.

In this chapter we have looked at GT repeat stability and different types of recombination in both the *tsn1*Δ and *trax*Δ mutants. We have also investigated a role in NHEJ. The aim being to identify any possible role of *S. pombe* Translin and Trax in GT repeat stability, recombination and NHEJ.

4.2 GT repeat stability analysis

S. pombe Translin has been shown to bind $d[GT]_n$ and $d[GTT]_n$ DNA repeats (Laufman *et al.*, 2005). We hypothesised that Translin may function to stabilise repetitive sequences during replication or recombination. To test this we looked at the stability of GT repeats artificially introduced into the *ade6* open reading frame and at intragenic recombination between GT repeats in *ade6* and other *ade6* test alleles.

4.2.1 GT repeat stability in *tsn1*Δ and *trax*Δ mutants

We tested the stability of $(GT)_8$ repeats in a *tsn1*Δ background, using a strain carrying a $(GT)_8$ microsatellite repeat inserted into the *ade6* gene (created by Mansour *et al.*, 2001; Fig. 4.1). During replication of repeat sequences, slippage can occur resulting in the deletion or addition of repeat units. The *ade6* gene is inactivated by the insertion of seven GT repeats at the position of an already existing GT. An in-frame open reading frame can be achieved by the loss of one repeat ($[GT]_7$; 2 nucleotides lost) or four repeats ($[GT]_4$; 8 nucleotides lost) or the gain of two repeats ($[GT]_{10}$; 4 nucleotides gained), resulting in adenine prototrophy. Following the method of Schaer *et al.*, (1997), a fluctuation analysis was carried out where individual colonies were grown to stationary phase and the number of adenine prototrophs determined. The median value of seven colonies represents one repeat (Lea and Coulson 1949; see Chapter 2). The *ade6-(GT)*$_8$ strains were sequenced before use to ensure the GT_8 status was maintained. The results show a small rise in $[GT]_8$ instability in both *tsn1*Δ and *trax*Δ cells (Fig. 4.1C), but the difference is not statistically significant. However, the broad range of values obtained in the *tsn1*Δ mutant might indicate a subtle underlying effect.

4.2.2 Meiotic intragenic recombination assays in an *ade6-(GT)*$_8$ *tsn1*Δ background

An intragenic recombination assay was carried out between the *ade6-((GT)*$_8$*-1397)* allele and *ade6-52* and *ade6-469* alleles positioned 433 bp and 945 bp away

from the $(GT)_8$ inserts respectively (see Fig. 4.2), in both a wild-type and a *tsn1*Δ backgrounds. As perhaps expected in both backgrounds the recombination frequency is higher in the *ade6-*$(GT)_8$ X *ade6-469* cross than in the *ade6-*$(GT)_8$ X *ade6-52* (Fig. 4.3), as the alleles are further apart. In both experiments the recombination frequency is down in the *tsn1*Δ strains but the difference is not statistically significant.

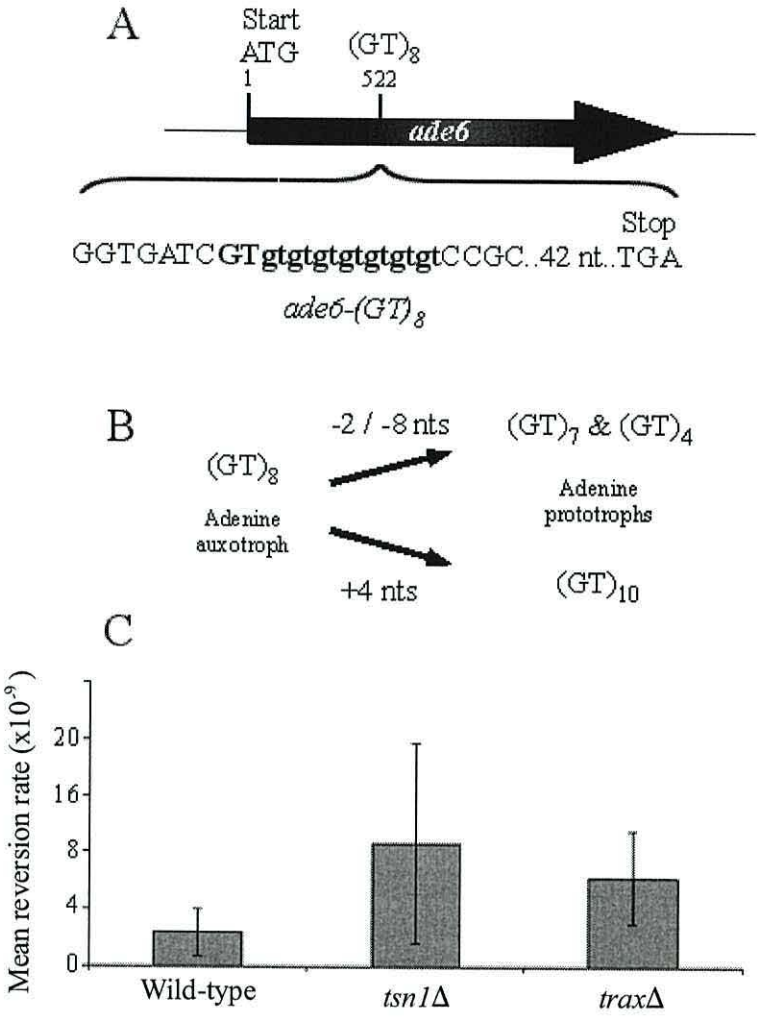


Figure 4.1 Stability of a GT microsatellite in *tsn1*Δ and *trax*Δ strains. A. Seven GT repeats were integrated into a*de6* at position 552 adjacent to an endogenous GT, giving a sequence of $[GT]_8$ repeats (Mansour *et al.*, 2001.) B. Frame shifts can result in the loss or gain of [GT] repeats. The loss of one [GT] or four $[GT]_4$ repeats or gain of two $[GT]_2$ results in adenine prototrophy. C. A small but insignificant increase in the rate of prototroph production (or GT microsatellite instability) is seen in *tsn1*Δ and *trax*Δ strains. Student's *t*-test gives *P*-values > 0.05 in pair wise comparison of mutants with wild-type. Mean reversion rates are calculated from at least three independent median values obtained from independent experiments (error bars show the standard deviation, n ≥ 3 in all cases). (The *trax*Δ fluctuation assay was carried out in collaboration with S. Ramayah.)

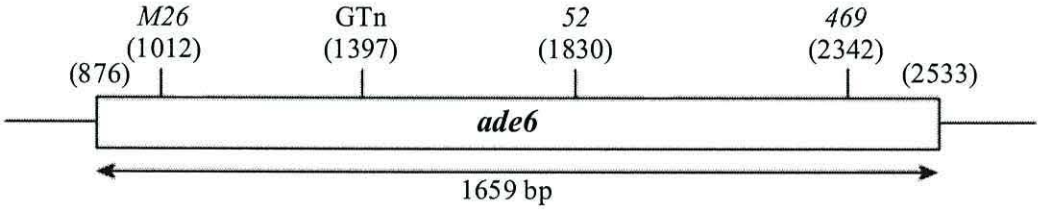


Figure 4.2 Map of *ade6* alleles. Showing the positions of the different test alleles used (in brackets). The ATG start codon being at position 876, and the stop codon at 2533.

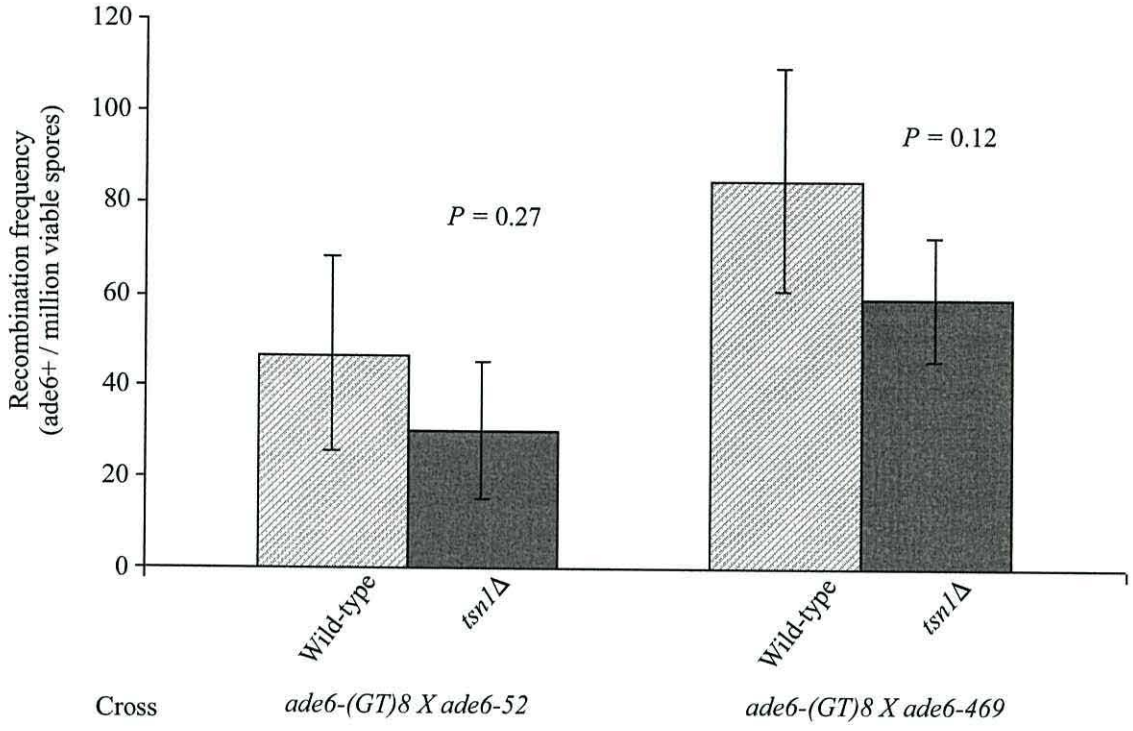


Figure 4.3 Meiotic intragenic recombination assays with *tsn1Δ ade6-(GT)$_8$* background strains. *tsn1Δ ade6-(GT)$_8$* strains were crossed with *tsn1Δ* strains carrying the *ade6-52* or the a*de6-469* alleles. In both crosses the recombination frequency is down in the *tsn1Δ* strains, but the reduction is not statistically significant. Student's *t*-test gives *P*-values > 0.05 in pair wise comparison of mutants with wild-type. Error bars show the standard deviation (n = 4 in all cases).

4.3 Homologous recombination assays

4.3.1 Plasmid-by-chromosome mitotic recombination assay

It has been suggested that Translin is involved in recombination (Aoki *et al.*, 1995). Mitotic recombination can be measured using a plasmid-by-chromosome assay, where a strain carrying a chromosomally encoded *ade6-M26* allele is transformed with the plasmid pade6-469 (Szankasi *et al.*, 1988), which carries the *ade6-469* allele. The two mutations are 1330 bp apart so recombination between the two will result in *ade*$^+$ prototrophy which can be measured. Fluctuation analyses were carried out on wild-type, *trax*Δ and *tsn1*Δ strains carrying pade6-469 to determine if Translin or TRAX function in this pathway (Lea and Coulson, 1949). No difference in the frequency of adenine prototroph production was observed in either mutant background (Fig. 4.4), suggesting Translin and Trax do not affect inter molecular mitotic recombination at the *ade6* locus.

4.3.2 Intragenic recombination assay

Translin binding sites have been identified at a subtelomeric male meiotic recombination hotspot in humans (Badge *et al.*, 2000). To test whether *S. pombe* Translin may have a function in intragenic meiotic recombination, a two factor cross was carried out using the *ade6-52* test allele and the meiotic recombination hotspot *ade6-M26* allele, which are 818 bp apart (Fig. 4.2). The results show no difference in the recombination frequencies in a wild-type and a *tsn1*Δ background (Fig. 4.5), so there is no loss of meiotic intragenic recombination or *ade6-M26* hotspot activity.

4.3.3 Intergenic recombination assays

Intergenic recombination or crossing over was measured at two intervals; at *pro1*$^+$-*ura1*$^+$ on chromosome I and at *ade6*$^+$-*arg1*$^+$ on chromosome III (Fig. 4.6A). These intervals were chosen because they are adjacent to different prominent meiotic double-strand break sites; *mbs1* in the case of *pro1*$^+$-*ura1*$^+$, and *ade6-M26* in the case of *ade6*$^+$-*arg1*$^+$, giving regional meiotic recombination (DeVeaux and

Smith, 1994). The genetic distances were calculated using the Haldane equation (see Chapter 2). Recombination frequencies were statistically indistinguishable between wild-type and the *tsn1*Δ mutant.

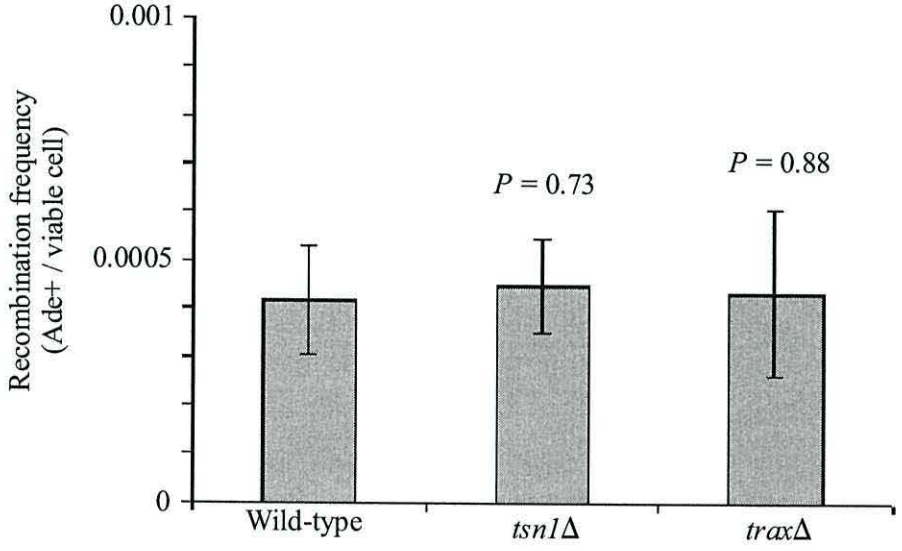


Figure 4.4 Mitotic plasmid-by-chromosome recombination assays. Mitotic recombination was measured between an *ade6-469* allele located on a plasmid (pade6-469) and *ade6-M26* present in the genome, by measuring adenine prototroph frequency after log phase growth. In both a *tsn1*Δ and *trax*Δ backgrounds there was no difference in recombination frequencies compared to wild-type. (Error bars show standard deviation, $n \geq 3$ in all cases). Student's *t*-test gives *P*-values > 0.05 in pair wise comparison of *tsn1*Δ and *trax*Δ mutants with wild-type.

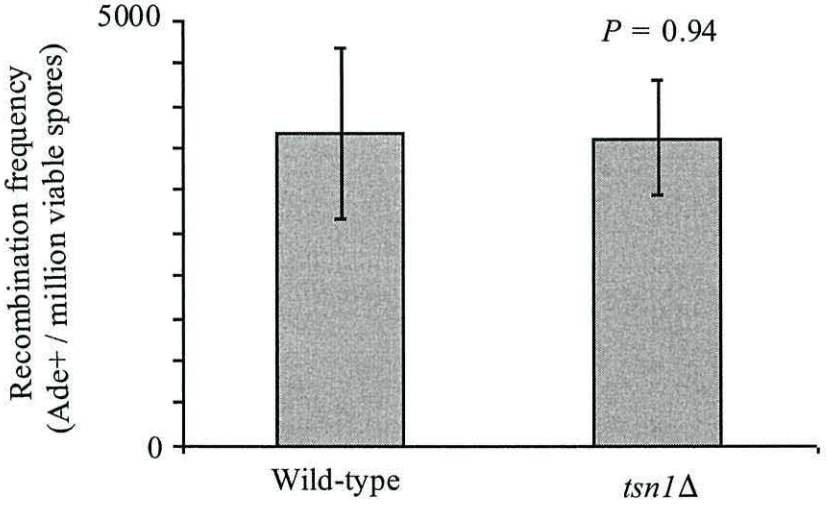


Figure 4.5 Intragenic recombination assay at *ade6*. Meiotic intragenic recombination between *ade6-M26* and *ade6-52* is not altered in a *tsn1*Δ background. (Error bars show standard deviation, n ≥ 3 in all cases). Student's *t*-test gives *P*-values > 0.05 in pair wise comparison of *tsn1*Δ mutants with wild-type.

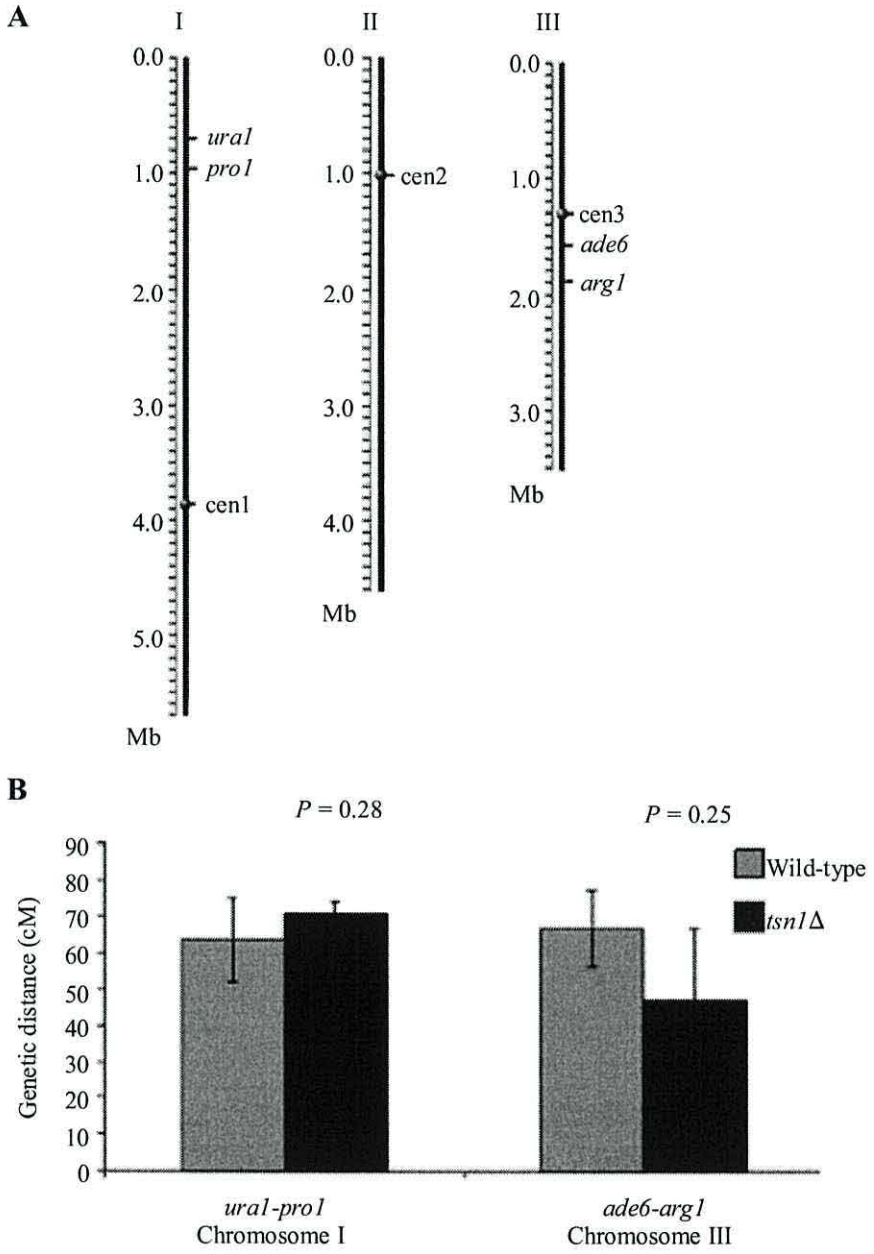


Figure 4.6 Meiotic intergenic recombination in a *tsn1*Δ mutant background. **A**. Shows a limited *S. pombe* genetic map (NCBI nucleotide database). Intergenic meiotic recombination was measured on chromosome I at *ura1-pro1*, and on chromosome III at *ade6-arg1*. There was no significant change in the genetic distance in a *tsn1*Δ background compared to wild-type (**B**). Genetic distances were calculated using Haldane's mapping function (see Chapter 2), and error bars show the standard deviation ($n \geq 3$ in all cases). Student's *t*-test gives *P*-values > 0.05 in pair wise comparison of *tsn1*Δ mutants with wild-type.

4.4 Analysis of non-homologous end joining in the *tsn1*Δ mutant

NHEJ can be measured using a plasmid recircularisation assay. *S. pombe* mutants defective in NHEJ, such as *lig4*Δ strains, show no increased sensitivity to DNA damaging agents when compared to wild-type strains (Manolis *et al.*, 2001). A *lig4*Δ strain can be used as a control. Wild-type, *lig4*Δ and *tsn1*Δ cells were transformed with *Pst*I, *Sma*I and *Xma*I linearised plasmid (pFY20; Li *et al.*, 1997), giving 3′ overhangs, blunt ended or 5′ overhangs respectively. All enzymes cut within a small region next to the *ARS* of pFY20. The plasmid also contains the selectable marker *ura4*$^+$. Since the plasmid cut sites are not homologous to any particular region of the *S. pombe* genome, recircularisation is either by perfect religation or by a non-homologous recombination reaction, resulting in deletion of some plasmid DNA, both are referred to as NHEJ. Cells with recircularised plasmids will give *ura4*$^+$ transformants. Uncut plasmid was also transformed in the strains to normalise for differences in transformation efficiencies. NHEJ efficiency is given by the *L/C* ratio, where the transformation frequency of cells transformed with linearised plasmid (*L*) is divided by the transformation frequency of cells transformed with circular uncut plasmid (*C*).

The results show that *lig4*Δ has greatly reduced NHEJ efficiency as previously reported by Manolis *et al.*, (2001), indicating that the assay has worked and that linear plasmid is relatively free of uncut contaminants (refer to Table 4.1). The *tsn1*Δ cells were found to recircularise all forms of the cut plasmid with equal efficiency and also showed the same efficiency in NHEJ as the wild-type.

Table 4.1 NHEJ assay results. Non-homologous DNA end joining of linear DNA with 3′, 5′ and blunt termini is not impaired in translin-deficient cells.

	***Pst*I (3′ over hang)**		***Sma*I (blunt ended)**		***Xma*I (5′ over hang)**	
	Mean L / C[a, b]	**% of wild-type**	**Mean L / C[a, b]**	**% of wild-type**	**Mean L / C[a, b]**	**% of wild-type**
Wild-type	0.21 (0.20)	-	0.37 (0.37)	-	3.0×10^{-3} (1.0×10^{-3})	-
lig4Δ[d]	$< 3.1 \times 10^{-4}$[c] (3.0×10^{-4})	< 0.15	4.7×10^{-4} (4.7×10^{-4})	1.3	$< 2.2 \times 10^{-4}$[c] (3.0×10^{-4})	< 7.3
tsnΔ	0.2 (0.15)	95.2	0.49 (0.27)	132.4	6.0×10^{-3} (6.0×10^{-3})	200.0

[a]The L/C ratio is the ratio of the number of transformants per viable cell obtained using linearised pFY20 plasmid DNA / the number of transformants per viable cell obtained for covalently closed circle pFY20 plasmid DNA.

[b]n = 3 in all cases. Standard deviation is given in parentheses. Pair wise comparison of wild-type and *tsn1Δ* values using Student's *t*-test gave *P*-values of > 0.05 in all cases.

[c]No transformants were obtained; the value of 3 was used as the number of transformants obtained for the calculation of L / C ratio.

[d]This strain carries the *ade6-704* allele, whereas the wild-type and the *tsnΔ* strain carry the *ade6-M26* allele.

4.5 Discussion

In a compact genome like *S. pombe*, microsatellites within genes would be a high risk factor for loss of function, Mansour *et al.*, (2001) identified no repetitive regions within *S. pombe* ORFs, this is perhaps an evolutionary change to eliminate the risk. This however is not true in higher eukaryotes and even though *S. pombe* Translin does not have a major function in GT stability, Translin in higher eukaryotes may. We detected a statistically insignificant increase in GT repeat stability and a decrease in intragenic recombination frequency. There was a large amount of heterogeneity within the *tsn1*Δ population, suggesting a possible variable requirement for Translin.

We found no defect in mitotic recombination and meiotic recombination (in crossovers and gene conversions) in the *tsn1*Δ and *trax*Δ strains tested. This conforms to the findings in *Drosophila* where no defect in meiotic intergenic recombination was observed in Translin deficient flies (Clauβen *et al.*, 2006), but then *Drosophila* Translin was found to have no DNA binding activity (Sengupta *et al.*, 2006). Also no defects in the NHEJ of 3′, 5′ and blunt termini was found, suggesting that Translin does not have a primary role in this DNA repair pathway. TRAX mutants were not tested for proficiency in NHEJ, but as already discussed in Chapter 3, TRAX protein levels are greatly depleted in a Translin-null strain, which might imply that the same result would be obtained in a *trax*Δ mutant. Since it is known that the MRN complex is not involved in NHEJ in *S. pombe* as it is in other organisms, other factors must be involved (Manolis *et al.*, 2001). Budding yeast lack Translin and TRAX homologs but budding yeast MRX complex (MRN homolog), is involved in NHEJ, there is the possibility therefore that Translin and TRAX in *S. pombe* are involved in a redundant NHEJ pathway. *S. pombe* homologues for all the NHEJ proteins have not yet been fully identified. This has been investigated further in the next chapter.

The association of TRAX with C1D which is an activator of DNA-PK also strongly suggests that TRAX (and Translin) may function in DNA repair. In response to DNA damage caused by γ-irradiation, C1D is induced and targets DNA-dependent protein kinase to specific nuclear regions (Yasui *et al.*, 1991;

Yavuzer *et al.*, 1998). DNA-PK along with other proteins involved in NHEJ also function in V(D)J recombination. Translin is proposed to be required for early lymphocyte development, and transported into the nucleus for processes such as immunoglobulin or TcR rearrangements (Aoki *et al.*, 1995). Early T and B cell precursors in the thymus lack the cell surface expression of the coreceptor molecules CD4 and CD8, they are $CD4^-$, $CD8^-$ double negative (DN). Production of $CD4^+$ and $CD8^+$ double positives (DP), requires a recombination event at the TcRβ locus in order to differentiate into DP thymocytes. Additional rearrangements in DP at the TcRα locus give rise to TcRα- TcRβ heterodimers which differentiate into $CD4^+$ $CD8^-$ or $CD4^-$ $CD8^+$ single positives (SP) T cells (Kruisbeek, 1993; von Boehmer and Fehling, 1997). If Translin played a crucial role in T-cell development then in null mice T-cell development would be arrested at the DN stage as seen in V(D)J recombinase-activating gene (Rag) deficient mice (Mombaerts *et al.*, 1992; Shinkai *et al.*, 1992). However, in both wt and TB-RBP-null mice T-cell development was normal with an equivalent percentage of DN, DP and SP cells. Similar rearrangements occur in B-cell development and this again was not found to require Translin (Chennathukuzhi *et al.*, 2003). These findings further support our results that Translin does not play a primary role in recombination.

4.6 Main conclusions

- Translin deficient cells show a statistically insignificant increase in GT repeat instability
- Translin and Trax appear to have no major role in mitotic and meiotic recombination
- NHEJ is normal in the *tsn1*Δ strain

Chapter 5 Investigation into a possible role of Translin and TRAX in DNA repair

5.1 Intoduction

Translin was first identified as a cancer-associated translocation breakpoint binding protein (Aoki *et al.*, 1995), and has also been found to bind human male meiotic hot spots (Badge *et al.*, 2000), as well as other chromosomal rearrangement breakpoints (Visser *et al.*, 2005; Gajecker *et al.*, 2006a; 2006b; Wu *et al.*, 1997). Other evidence also suggests that Translin and TRAX may function in the DNA damage response. Firstly, Translin has been found to interact with murine GADD34 (Hasegawa and Isobe 1999; 2000). GADD34 is a member of the growth arrest and DNA damage inducible proteins, and functions to prevent cells undergoing apoptosis in response to DNA damage (Chou and Roizman, 1994; Selvakumaran *et al.*, 1994; Zhan *et al.*, 1994). On exposure to MMS, GADD34 expession was elevated but Translin levels remained the same. It is thought that GADD34 may possibly be involved in the nuclear transport of Translin from the cytoplasm in response to DNA damage (Hasegawa and Isobe, 1999). Secondly, treatment of mammalian cells with the chemotherapeutic drugs mitomycin C, etoposide, irradiation and peroxide all resulted in elevated levels of Translin in the nucleus, which decreased during long exposure (Kasai *et al.*, 1997). What function Translin may have in the nucleus in response to damaging agents is as yet unclear. Translin lacks a nuclear localization signal so is perhaps transported to the nucleus as a result of interacting with other proteins such as GADD34 or TRAX, which both have nuclear targeting signals (Aoki *et al.*, 1997a, 1997b; Hasegawa and Isobe, 1999). Thirdly, it has been found that Translin null mice have severely delayed hematopoietic colony formation after exposure to ionizing irradiation, these results suggest that Translin contributes to hematopoietic regeneration following ionizing irradiation (Fukuda *et al.*, 2008). Finally, the Translin interacting protein TRAX, has also been found to interact with C1D protein upon γ-irradiation (Erdemir *et al.*, 2002). In response to DNA damage caused by γ-irradiation, C1D is induced and targets DNA-dependent protein

kinase (DNA-PK) to specific nuclear regions (Yasui *et al.*, 1991; Yavuzer *et al.*, 1998). DNA-PK is involved in the repair of DNA DSBs and V(D)J recombination which is essential for the development of immune cells (Smith and Jackson, 1999). Association of TRAX with C1D or Translin is mutually exclusive because a C1D/TRAX interaction only requires the TRAX leucine zipper motif, whereas TRAX/Translin interaction requires the full-length of TRAX.

Contrary to the evidence implicating Translin and TRAX in the DNA damage response, are studies using *Drosophila* embryos and MEFs defective for Translin and TRAX showing no sensitivity to DNA damaging agents (Yang *et al.*, 2004; Clauβen *et al.*, 2006). In previous work only limited investigation was carried out into a possible role of Translin and TRAX in DNA repair using only a few DNA damaging agents. In this chapter we have used several different DNA damaging agents to address the question of whether or not Translin and TRAX are involved in the DNA damage response. Moreover, we investigated a possible redundant function for Translin and TRAX in DNA damage recovery.

5.2 DNA damage sensitivity tests of Translin null mutants

Using spot test sensitivity assays, *S. pombe* can be used to detect whether a mutant exhibits any sensitivity to DNA damaging agents. Since it is unknown if Translin functions in a specific repair pathway if any, a range of different DNA damaging agents were used to test for sensitivity at a range of temperatures, as each one is known to cause different types of lesions which are targeted by different DNA repair pathways (refer to Fig. 5.1 and Table 5.1). All five *tsn1*Δ strains were tested for sensitivity to UV, mitomycin C, cisplatin, methyl methane sulfonate (MMS), hydroxyurea, camptothecin and phleomycin, at 20°C, 30°C and 37°C.

UV irradiation induces the formation of pyrimidine dimers. In *S. pombe*, UV dimers are targeted by the nucleotide excision repair (NER) pathway and UVER (see Chapter 1; Bowman *et al.*, 1994; de Laat *et al.*, 1999). We found that none of our five *tsn1*Δ strains were sensitive to UV radiation at 20°C, 30°C and 37°C.

Interstrand cross-links caused by agents such as mitomycin C and cisplatin are repaired by either NER or homologous recombination (HR) repair (Jachymczyk *et al.*, 1981; McHugh *et al.*, 2000). Studies in *S. cerevisiae* show that NER proteins are important in the initial incision at interstrand cross-links (Jachymczyk *et al.*, 1981; McHugh *et al.*, 1999), and that HR completes the repair initiated by a DSB (Dronkert and Kanaar 2001; Jachymczyk *et al.*, 1981; Kaur *et al.*, 1998). Interstrand cross-linking agents mainly elicit a checkpoint response from the G2 DNA damage checkpoint and cell cycle arrest (Grossmann *et al.*, 1999; Meniel *et al.*, 1997). Again no sensitivity to mitomycin C or cisplatin was detected in the Translin deficient strains at the temperatures tested (see Fig. 5.2).

MMS is an alkylating agent that modifies both guanine (to 7-methylguanine) and adenine (to 3-methyladenine) to cause base mispairing and replication blocks respectively (Beranek, 1990). Damage caused by alkylating agents is predominantly repaired by BER and DNA alkyltransferases (Lindahl and Wood, 1999). It is thought that the spontaneous hydrolysis of alkylated bases to AP sites during BER could lead to DSB formation (Brookes and Lawley, 1963). AP sites are heat-labile and can be transformed into SSBs (Lindahl and Andersson, 1972;

Rydberg, 2000), subsequent alkylation on the opposite strand close to the first alkylation would result in a DSB. A recent study suggested that MMS does not directly cause DSBs (Lundin *et al.*, 2005), however, HR mutants are highly sensitive to MMS suggesting that HR is involved in the repair of MMS-induced DNA damage. We detected no sensitivity to MMS in any of the five *tsn1*Δ strains (see Fig. 5.3).

Hydroxyurea (HU) is thought to inhibit ribonucleotide reductase causing the depletion of nucleotides and subsequent S-phase arrest (Adams *et al.*, 1971). This results in replication fork stalling and DSB formation, which are then targeted by HR or NHEJ for repair. Again, we found that none of the *tsn1*Δ strains were sensitive to HU (see Fig. 5.4).

Camptothecin (CPT) is an inhibitor of topoisomerase I enzyme (Top1) and leads to SSBs by trapping Top1-DNA intermediates and inhibiting the enzymes religation activity. Such protein-DNA complexes are converted into DSBs during DNA replication (Pommier *et al.*, 2003). In *S. pombe* and vertebrates CPT exposure results in the activation of the DNA damage checkpoint pathway in which Chk1 kinase acts as a downstream effector (Liu *et al.*, 2000; Takai *et al.*, 2000; Walworth and Bernards, 1996; Wan *et al.*, 1999; Wang *et al.*, 2002; Zachos *et al.*, 2003). At all three temperatures tested, no sensitivity to CPT was detected in any of the five *tsn1*Δ strains (see Fig. 5.5).

Phleomycin is a structurally related form of the anticancer drug bleomycin. Bleomycin is known to cause AP sites, SSBs and DSBs in DNA (Umezawa *et al.*, 1974). Phleomycin is known to cause DNA lesions but the exact nature of these lesions is unclear. Again no sensitivity was detected in any of the *tsn1*Δ strains (see Fig. 5.6).

Two *trax*Δ strains were tested for DNA damage sensitivity using the same agents and temperature range. These strains also showed no sensitivity to any of the agents (S. Ramayah, personal communication). These results suggest that both Translin and TRAX do not have a primary role in DNA repair pathways in *S. pombe*.

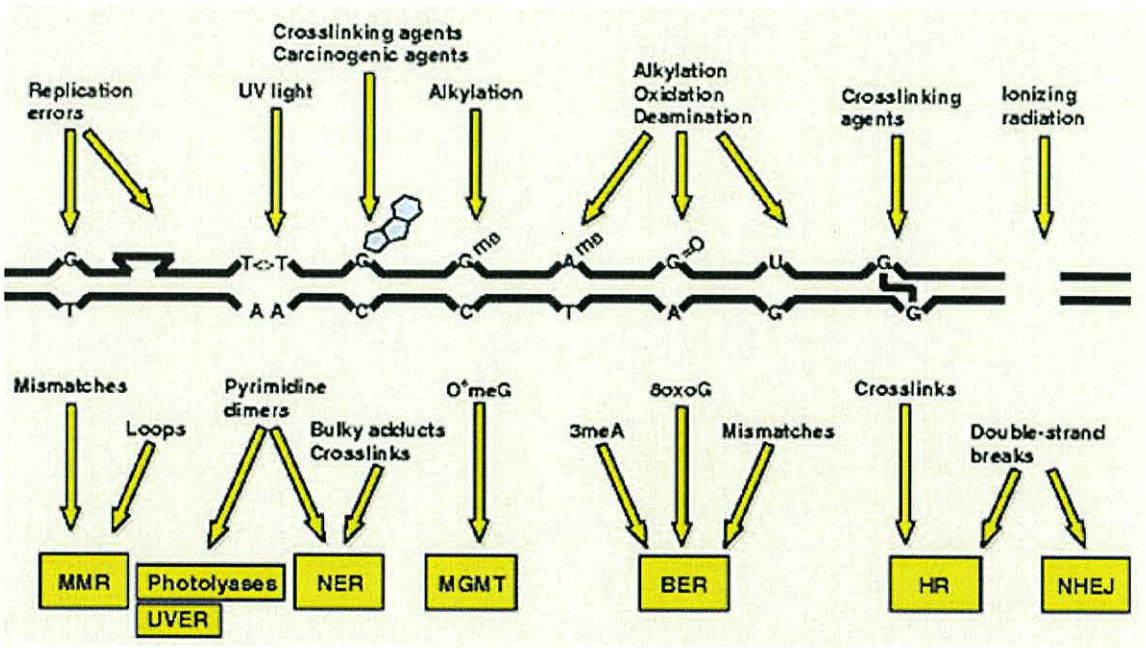


Figure 5.1 Summary of the DNA damage lesions targeted by the major DNA repair pathways. MMR, mismatch repair; NER, nucleotide excision repair; BER, base excision repair; HR, homologous recombination; NHEJ, non-homologous end joining. (See text for further explanation; taken from Fleck and Nielsen, 2004).

Table 5.1 Summary of the lesions caused by different DNA damaging agents and the DNA repair pathways that target them. MMS, methyl methanesulphonate; NER, nucleotide excision repair; HR, homologous recombination; BER, base excision repair; NHEJ, non-homologous end joining.

Damaging agent	Effect	Main repair pathway	References
UV radiation	Pyrimidine dimerisation	Photolyases UVER or NER	Bowman *et al.* 1994; de Laat *et al.*, 1999
Mitomycin C	DNA crosslinker	NER or HR	Jachymczyk *et al.*, 1981; McHugh *et al.*, 2000
Cisplatin	DNA crosslinker	NER or HR	
MMS	Alkylating agent	MGMT, BER or NER	Fung *et al.*, 2002; Morishita *et al.*, 2002
Hydroxyurea	NTP depletion	HR or NHEJ	Adams *et al.*, 1971
Camptothecin	Topo I inhibitor	HR or NHEJ	Wan *et al.*, 1999
Phleomycin	Causes DSBs	HR or NHEJ	Umezawa *et al.*, 1974

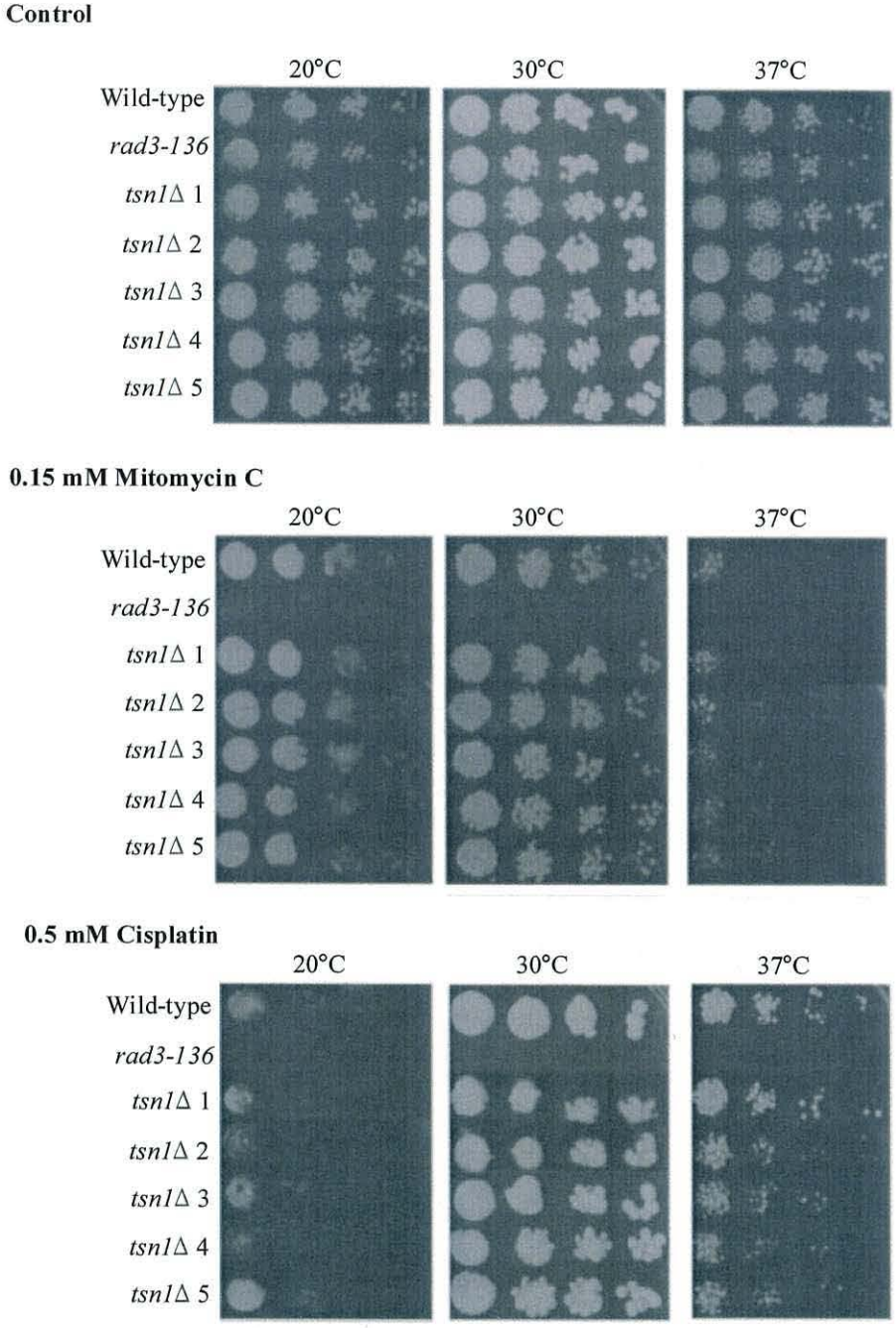


Figure 5.2 Mitomycin C and cisplatin spot tests of *tsn1*Δ single mutants. No increase in sensitivity compared to wild-type was detected in any of the five *tsn1*Δ at 20°C, 30°C and 37°C. *rad3-136* cells were used as a positive control. Serial dilutions were of log phase cultures (10^{-2} to 10^{-5}, from left to right).

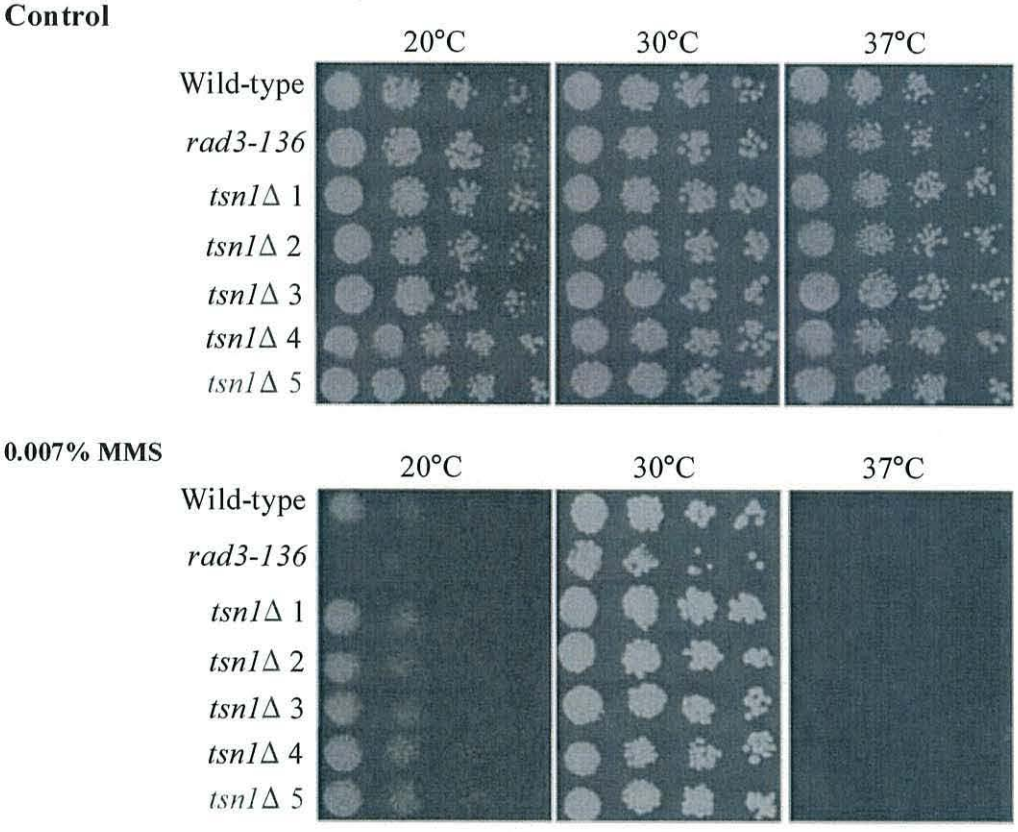


Figure 5.3 MMS spot tests of *tsn1*Δ single mutants. None of the five *tsn1*Δ strains tested increased sensitivity to MMS compared to wild-type at 20°C, 30°C and 37°C. *rad3-136* cells were used as a positive control. Serial dilutions were of log phase cultures (10^{-2} to 10^{-5}, from left to right).

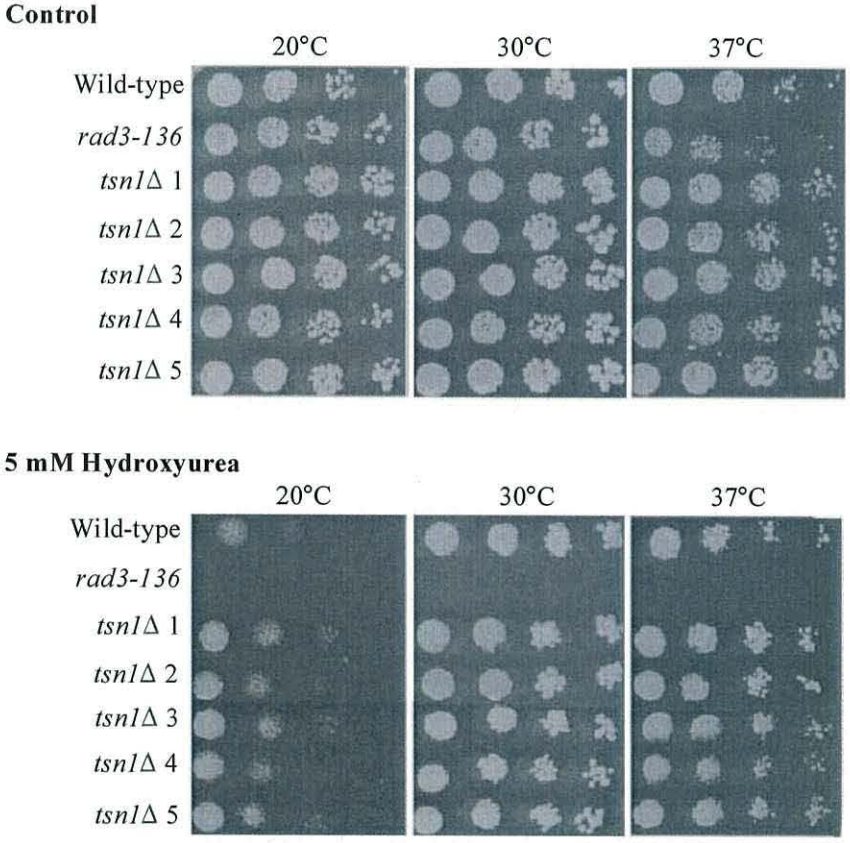


Figure 5.4 Hydroxyurea spot tests of *tsn1*Δ single mutants. None of the five *tsn1*Δ strains tested showed increased sensitivity to HU when compared to wild-type at 20°C, 30°C and 37°C. Again *rad3-136* cells were used as a positive control. Serial dilutions were of log phase cultures (10^{-2} to 10^{-5}, from left to right).

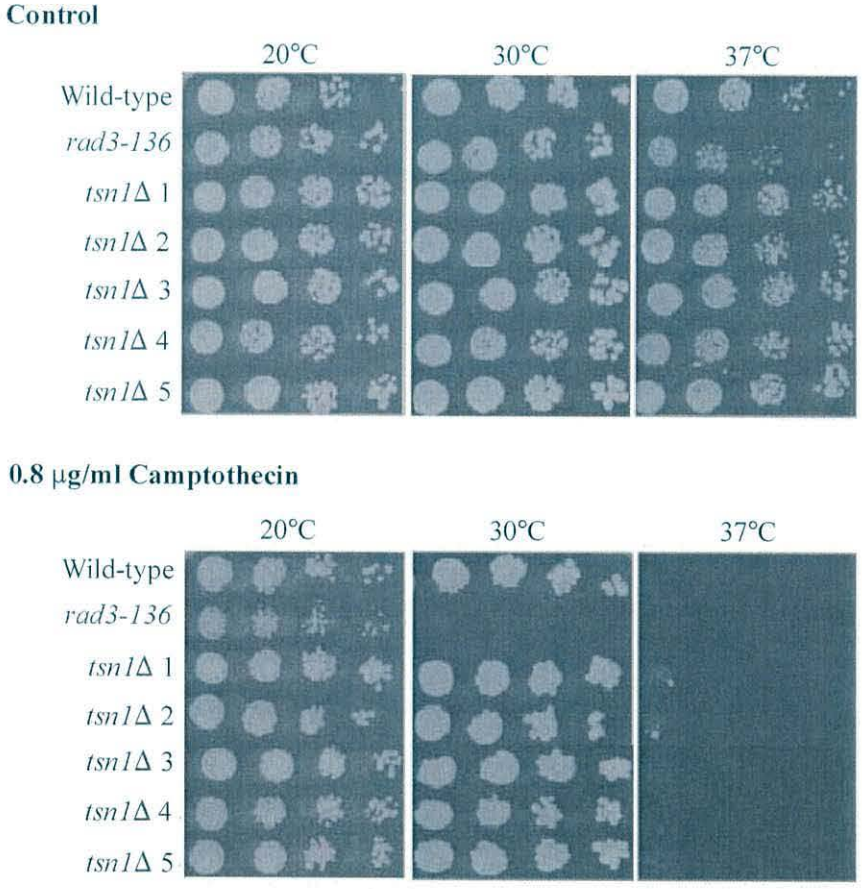


Figure 5.5 Camptothecin spot tests of *tsn1*Δ single mutants. The five *tsn1*Δ strains tested showed no increase in sensitivity to CPT compared to wild-type at 20°C, 30°C and 37°C. *rad3-136* cells were used as a positive control. Serial dilutions were of log phase cultures (10^{-2} to 10^{-5}, from left to right).

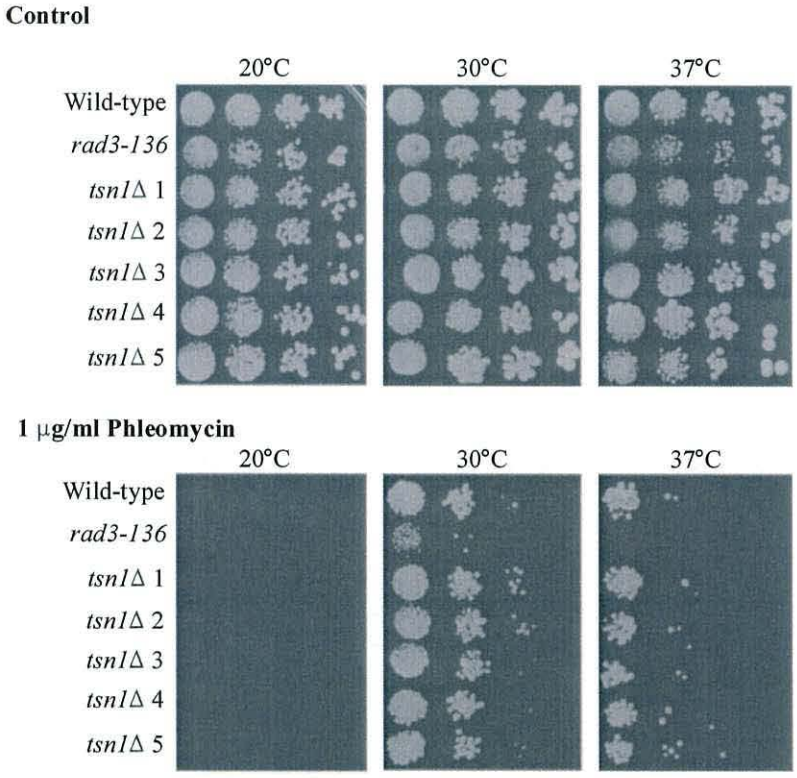


Figure 5.6 Phleomycin spot tests of *tsn1*Δ single mutants. No increase in sensitivity to phleomycin was detected compared to wild-type cultures in any of the five *tsn1*Δ strains tested at 20°C, 30°C and 37°C. *rad3-136* cells were used as a positive control. Serial dilutions were of log phase cultures (10^{-2} to 10^{-5}, from left to right).

5.3 DNA damage sensitivity tests of a *tsn1*Δ *trax*Δ double mutant

A *tsn1*Δ *trax*Δ double mutant was made by crossing a *tsn1*Δ and *trax*Δ single mutants, and PCR screening kanamycin resistant colonies. This strain was tested for sensitivity to UV, mitomycin C, cisplatin, methyl methane sulfonate (MMS), hydroxyurea, camptothecin and phleomycin in the same way as the single mutants (see Fig. 5.7). Even though *tsn1*Δ and *trax*Δ single mutants do not appear to have a primary function in the DNA repair pathways targeted in these tests, it is still possible that they might function in a redundant pathway. If this is true for both Translin and TRAX then the lack of any sensitivity in the double mutant would suggest that they are epistatic.

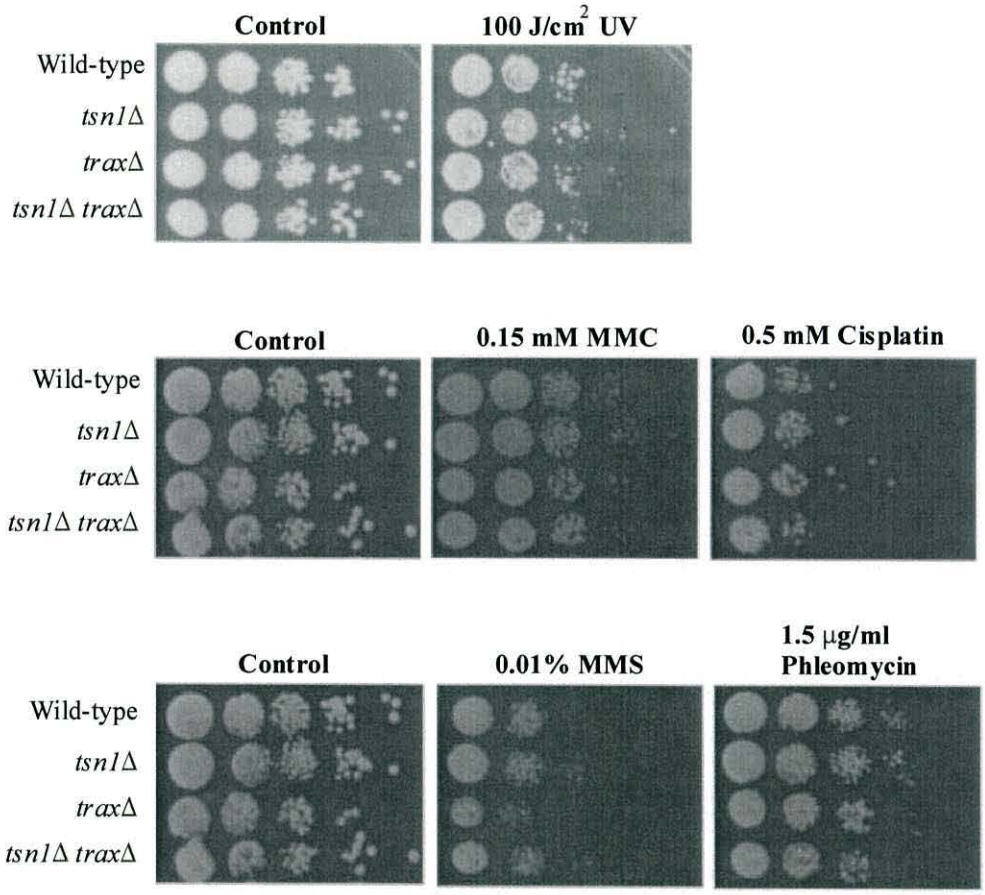


Figure 5.7 DNA damage sensitivity tests of a *tsn1*Δ and *trax*Δ double mutant. No increase in sensitivity to UV, MMC, cisplatin, MMS, phleomycin or camptothecin (result not shown) compared to wild-type was detected for a *tsn1*Δ *trax*Δ double mutant tested at 30°C. Serial dilutions were of log phase cultures (10^{-2} to 10^{-5}, from left to right).

5.4 Investigation into a redundant function of Translin and Trax in the DNA damage response

To test whether Translin and/or TRAX function in a redundant repair pathway, we constructed a number of double mutants. Formerly characterized mutants from the major repair pathways in *S. pombe* (refer to table 5.2) were selected and crossed with either a *tsn1*Δ or a *trax*Δ strain. DNA damage response mutants selected included *msh2*Δ involved in the MMR pathway, *lig4*Δ which functions in NHEJ, *rhp7*Δ, *rad13*Δ and *rhp14*Δ involved in NER, and *rhp51*Δ, *rad50*Δ, *nbs1*Δ and *rad32*Δ from the HR pathway (see Chapter 1 for more information on these pathways).

Table 5.2 Summary of *S. pombe* DNA repair protein homologs.

S. pombe	***S. cerevisiae***	**Human**	**Main Pathway**	**Function**
Msh2	Msh2	MSH2	MMR	Forms a heterodimer and binds base-base mismatches
Lig4	Lig4	Ligase IV	NHEJ	Ligase joins ends in NHEJ reaction
Rhp7	Rad7	-	NER	Maybe involved in damage recognition
Rad13	Rad2	XPG	NER	Mediates 3' incision of damaged DNA
Rhp14	Rad14	XPA	NER	Verifies correct assembly of the NER complex
Rhp51	Rad51	RAD51	HR	Forms a filament pairing homologous DNA
Rad50	Rad50	RAD50	HR	Binds DNA molecules together
Nbs1	Xrs2	NBS1	HR	Interacts with ATM checkpoint protein
Rad32	Mre11	MRE11	HR	Exonuclease and is central to MRN function

5.4.1 Analysis of *msh2*Δ *tsn1*Δ and *msh2*Δ *trax*Δ double mutants

The *S. pombe msh2*$^{+}$ gene is involved in mitotic and meiotic mismatch repair, mating-type switching, and meiotic chromosome organization (Rudolph *et al.*, 1998). The Msh2 protein forms a heterodimer with other MutS homologs and binds to base-base mismatches in DNA (see Table 5.2 and Chapter 1). Msh2 is involved in the major pathway of mismatch repair, and along with Swi4 (the *S. pombe* homolog of Msh3), is also involved in mating-type switching. The *msh2*Δ *tsn1*Δ and *msh2*Δ *trax*Δ double mutants were tested for sensitivity to UV, MMC, cisplatin, MMS and phleomycin. MMR removes base mismatches and small insertion/deletion loops introduced during replication. Phleomycin is thought to possibly inhibit DNA polymerase and interestingly the *msh2*Δ *trax*Δ double mutant exhibited a subtle increase in sensitivity to this damaging agent (see Fig. 5.8 and Table 5.4). The *msh2*Δ *tsn1*Δ double mutant displayed a slight rescue phenotype when exposed to UV radiation (data not shown). Sensitivity to all other agents tested, were no different to the single mutants.

5.4.2 Analysis of *lig4*Δ *tsn1*Δ and *lig4*Δ *trax*Δ double mutants

Lig4 provides the ligase activity in NHEJ (see Chapter 1). NHEJ repairs DSBs mainly in the G1 phase of the cell cycle when homologous chromosomes are not available for repair of DSBs by HR. Therefore, all the *lig4*Δ strains used were transferred to minimal media lacking nitrogen, which depletes nucleotide production and arrests cells in G1. The *lig4*Δ *tsn1*Δ and *lig4*Δ *trax*Δ double mutants were tested for sensitivity to UV, MMC, cisplatin, MMS and phleomycin. With the MMC and phleomycin doses used, all the strains were inviable. In the others no sensitivities were detected (see Table 5.4). We also carried out a NHEJ assay and found no defect in the pathway in the *tsn1*Δ single mutant compared to wild-type cells, *lig4*Δ cells however were greatly defective at NHEJ (refer to Chapter 4).

5.4.3 Construction and testing of *rhp7*Δ *tsn1*Δ and *rhp7*Δ *trax*Δ double mutants

S. cerevisiae rad7 and *rad16* gene products have been found to function as a complex in NER (Verhage *et al.*, 1994; Wang *et al.*, 1997; Guzder *et al.*, 1997). They are thought to be essential for the GGR sub-pathway of NER as *rad7* and *rad16* mutants are deficient in the removal of cyclobutane prymidine dimers (CPDs) from the mating-type locus, non-transcribed strands and the promoter regions (Terleth *et al.*, 1990; Verhage *et al.*, 1994; Tijsterman *et al.*, 1996), and are proposed to have a role in damage recognition and may also be required for post-incision events (Reed *et al.*, 1998). The *S. pombe* proteins Rhp7 and Rhp16 are thought to be the structural and functional homologs of *S. cerevisiae* Rad7 and Rad16, however only a *rhp16* clone will complement a *S. cerevisiae rad16* mutant (Bang *et al.*, 1996), a *rhp7* clone did not complement a *rad7 S. cerevisiae* mutant (Lombaerts *et al.*, 1999). Rhp7 and Rhp16 are exclusively involved in NER and are essential for GGR. *rhp7* and *rhp16* genes have only 405 nucleotides between them and are transcribed in opposite directions, suggesting a co-regulation, and both are induced upon UV irradiation (Lombaerts *et al.*, 1999).

*rhp7*Δ *tsn1*Δ and *rhp7*Δ *trax*Δ double mutants were made by crossing a *rhp7::kanmx6* strain with the *tsn1::kanmx6* and *trax::kanmx6* strains. Geneticin-resistant colonies were selected for and tested by PCR for the position of the kanamycin alleles using primers located within the kanamycin-resistance gene and either upstream or downstream of the target gene (see Chapter 2). The *rhp7*Δ *tsn1*Δ and *rhp7*Δ *trax*Δ double mutants were tested for sensitivity to UV, MMC, Cisplatin, MMS and phleomycin (see Fig. 5.9 for UV and phleomycin results and Table 5.4 for a summary). Interestingly, the *rhp7*Δ *tsn1*Δ and *rhp7*Δ *trax*Δ double mutants showed slightly more sensitivity to UV compared to the single mutants. Damage caused by MMC and cisplatin can be targeted by NER, however here no increase in sensitivity was seen in the double mutants. These results may suggest that Translin and TRAX are involved in a repair pathway other than the canonical NER pathway.

5.4.4 Analysis of *rad13Δ tsn1Δ* and *rad13Δ traxΔ* double mutants

S. pombe Rad13 is the homolog of the human XPG protein and together with Swi10, carries out the 3' and 5' dual incision of damaged DNA during NER (Rödel *et al.*, 1992; Carr *et al.*, 1994; Carr *et al.*, 1993). Rad13 and Swi10 are both essential in short-patch repair during meiotic recombination but not during mitotic growth. Rad13 seems to have a distinct function in repair compared to Swi10. Interestingly, a *rad13Δ swi10Δ* double mutant was found to be more sensitive to MMS treatment than either single mutant, indicating that they have slightly different roles in the processing of MMS-induced lesions (Kunz and Fleck 2001). It has been demonstrated that human XPG plays a role in long-patch BER (Bessho 1999; Klungland *et al.*, 1999; Dianov *et al.*, 2000) and so it is possible that Rad13 might also be an accessory factor in BER.

rad13Δ tsn1Δ and *rad13Δ traxΔ* double mutants were made by crossing a *rad13::kanmx* with *tsn1::kanmx6* and *trax::kanmx6* strains. Geneticin resistant progeny were selected and double mutants were identified using PCR. The *rad13Δ tsn1Δ* and *rad13Δ traxΔ* double mutants were tested only for MMS sensitivity and it was found that they were as sensitive as the *rad13Δ* single mutant (Fig. 5.10). Evidence implies that Rhp7 is more likely to function exclusively in NER, so further tests using *rad13Δ* strains were not continued.

5.4.5 Analysis of *rhp14Δ tsn1Δ* and *rhp14Δ traxΔ* double mutants

S. pombe rhp14 is the homolog of human *XPA* and budding yeast *RAD14*. It is involved in the NER pathway and it is proposed to function in damage recognition and/or verification of the correct assembly of the NER proteins at the point of damage (Araújo and Wood 1999; de Laat *et al.*, 1999). It is also thought that Rhp14 may have a NER-independent function (O. Fleck personal communication). *rhp14Δ tsn1Δ* and *rhp14Δ traxΔ* double mutants were made using a *rhp14::kanmx6* strain crossed with *tsn1::kanmx6* and *trax::kanmx6* strains, and the double mutants were isolated by screening geneticin resistant colonies using PCR. The double mutants were tested for sensitivity to UV irradiation, cisplatin and MMS (see Table 5.4 for summary). Damage caused by

UV and MMS are both targets of the NER pathway. *rhp14*Δ *tsn1*Δ and *rhp14*Δ *trax*Δ double mutants were tested and both showed increased sensitivity to UV and MMS treatment (the *rhp14*Δ *tsn1*Δ strain was less sensitive to MMS than the *rhp14*Δ *trax*Δ strain; see Fig. 5.11).

Multiple *rhp14*Δ *tsn1*Δ and *rhp14*Δ *trax*Δ double mutants were made again using the same parental strains and selecting for isogenic progeny (work completed by Lukas Stanczuk). Four *rhp14*Δ *tsn1*Δ double mutants and seven *rhp14*Δ *trax*Δ double mutants were tested for sensistivity to mitomycin C, camptothecin and phleomycin (UV and MMS sensitivity was not tested). Out of the seven *rhp14*Δ *trax*Δ double mutants isolated and tested only two showed considerable sensitivity to mitomycin C, camptothecin and phleomycin, none of the four *rhp14*Δ *tsn1*Δ double mutants showed any sensitivity compared to the wild-type strains (see Fig 5.12). When these *rhp14*Δ *trax*Δ double mutants were transformed with a $trax^{+}$ clone, the strains were rescued (work completed by Lukas Stanczuk).

5.4.6 Analysis of *rhp51*Δ *tsn1*Δ and *rhp51*Δ *trax*Δ double mutants

S. pombe Rhp51 protein is the homolog of *E.coli* RecA protein and human RAD51. Mutations in the *S. cerevisiae RAD51* gene result in a range of defects including sensitivity to ionizing radiation and alkylating agents, a deficiency in mating-type switching and deficiencies in mitotic and meiotic recombination (Shinohara *et al.*, 1992; Game, 1993). Rad51 forms a filament pairing homologous DNA, permitting genetic recombination to occur anywhere along the length of the homologous chromosome (Kowalczykowski and Eggleston 1994; also refer to Chapter 1). *rhp51*Δ *tsn1*Δ and *rhp51*Δ *trax*Δ double mutants were made and tested for sensitivity to UV irradiation, mitomycin C, cisplatin, MMS, hydroxyurea, camptothecin and phleomycin (see Table 5.5 for summary of results). Damage by the cross-linking agents mitomycin C and cisplatin can be targeted by HR repair, but unfortunately the doses used were too high and all the *rhp51*Δ stains were inviable. The double mutants showed no change in sensitivity to UV, MMS or HU compared to the single *rhp51*Δ mutant. However, on both

camptothecin and phleomycin the *rhp51Δ tsn1Δ* double mutant exhibited a slight rescue effect (see Fig. 5.13).

5.4.7 Analysis of *rad50Δ tsn1Δ* and *rad50Δ traxΔ* double mutants

Rad50 is part of the MRN complex (see Chapter 1), which has several critical functions including DNA damage recognition factors which act on DSBs, ssDNA ends arising from stalled RFs and/or misfolded or obstructed DNA. The complex consists of the large coiled-coil ATP-binding cassette (ABC) ATPase RAD50 protein, the nuclease MRE11 (Rad32 in fission yeast), and the checkpoint mediator NBS1. Null mutations in any of MRE11-RAD50-NBS1 leads to embryonic lethality in mice (Luo *et al.*, 1999; Xiao and Weaver 1997; Zhu *et al.*, 2001). In budding and fission yeasts severe damage sensitivity phenotypes are observed upon deletion of MRN subunits (Chahwan *et al.*, 2003; D'Amours and Jackson 2002; Paques and Haber 1999). Defects in MRN in humans lead to the disorders Nijmegan Breakage syndrome, Ataxia Telangiectasia and Ataxia Telangiectasia-like disorder (ATLD) (Carney *et al.*, 1998; Stewart *et al.*, 1999). *S. cerevisiae* Rad50 and Mre11 bind to form a well conserved dimer, giving the ATP-stimulated nuclease activity (Aravind *et al.*, 1999; Blinov *et al.*, 1989). ATP-binding to Rad50 modulates its interaction with DNA and the ATP-driven conformational changes in Rad50 are probably an important stereochemical event (Alani *et al.*, 1990; Connelly *et al.*, 2003; Hopfner *et al.*, 2000).

rad50Δ tsn1Δ and *rad50Δ traxΔ* double mutants were made and their sensitivity to UV, mitomycin C, cisplatin, MMS, hydroxyurea, camptothecin and phleomycin was tested (see Table 5.5 for a summary of all tests). The concentration of mitomycin C used was too high and all the *rad50Δ* strains were inviable. In all the other tests the *rad50Δ tsn1Δ* and *rad50Δ traxΔ* double mutants showed a partial rescue phenotype compared to the *rad50Δ* single mutant (see Fig. 5.14). However, in all instances the *rad50Δ* single strain had poorer growth on the control plates compared to all the other strains and showed greater sensitivity to camptothecin than the other two MRN mutants (see Fig. 5.19). In

both the *rad50Δ tsn1Δ* and *rad50Δ traxΔ* double mutants, the cell viability seemed to be restored to wild-type levels.

5.4.8 Analysis of *nbs1Δ tsn1Δ* and *nbs1Δ traxΔ* double mutants

Mutagenesis and biophysical data suggest that in humans, NBS1 binds to the $RAD32_2$:$RAD50_2$ head, and modulates the ATP-stimulated nuclease activity and is required for the hairpin-processing activity of the complex (Lee *et al.*, 2003; Paull and Gellert 1999) NBS1 may be involved in targeting the MRN complex to damaged sites, in attracting other repair proteins or in interacting with damage-induced chromatin markers at DSBs (Bradbury and Jackson 2003; Kobayashi *et al.*, 2002). NBS1 has been shown to interact with the checkpoint kinase ATM. NBS1 is phosphorylated by ATM (Tel1 in fission yeast), and this is required for later ATM-dependent phosphorylation of CHK2 (Buscemi *et al.*, 2001; Lee and Paull 2004), histone H2AX (Lee and Paull 2004), SMC1 (Horejsi *et al.*, 2004; Yazdi *et al.*, 2002), and FANCD2 (Nakanishi *et al.*, 2002). Phosphorylation of p53 requires the presence of the MRN complex but not of NBS1 phosphorylation (Lee and Paull, 2004).

nbs1Δ tsn1Δ and *nbs1Δ traxΔ* double mutants were made by crossing a *nbs1::kanmx* strain with *tsn1::kanmx6* and *trax::kanmx6* strains and the geneticin resistant progeny screened by PCR. The double mutants were tested for sensitivity to UV, mitomycin C, cisplatin, MMS, hydroxyurea, camptothecin and phleomycin (see Table 5.5 for a summary of the results). No increase in sensitivity was seen with UV or MMS treatment compared to the single mutant. On both camptothecin and phleomycin the double mutants showed increased sensitivity. On cisplatin, mitomycin C and hydroxyurea the double mutants displayed the same sensitivity as the single the *nbs1Δ* mutant (see Fig. 5.15).

5.4.9 Analysis of *rad32Δ tsn1Δ* and *rad32Δ traxΔ* double mutants

Rad32 is the central interacting protein of the MRN complex, binding Rad50, Nbs1, DNA and homodimerizes (Williams *et al.*, 2007). *In vitro*, Mre11 possesses Mn^{2+}-dependent dsDNA 3' - 5' exonuclease, ssDNA endonuclease, DNA

annealing and unwinding activities (Assenmacher and Hopfner 2004; Connelly *et al.*, 1998; D'Amours and Jackson 2002; Furuse *et al.*, 1998). These activities are regulated through interactions with Rad50 and Nbs1 (Carney *et al.*, 1998; D'Amours and Jackson 2002; Moncalian *et al.*, 2004; Paull and Gellert 1998). *rad32*Δ *tsn1*Δ and *rad32*Δ *trax*Δ double mutants were made from two different knock-out strains of *rad32*. The first set of *rad32*Δ *tsn1*Δ and *rad32*Δ *trax*Δ double mutants were made using a *rad32::ura4* strain. These mutants were tested for sensitivity to mitomycin C, cisplatin, MMS, hydroxyurea and phleomycin (see Table 5.5 for a summary of the results). Treatment with MMS and hydroxyurea showed no increase in sensitivity in any of the *rad32*Δ strain, however, the concentration of MMS used was perhaps too low. On both phleomycin and mitomycin C the *rad32*Δ *trax*Δ double mutant showed increased sensitivity compared to the *rad32*Δ *tsn1*Δ double and *rad32*Δ single (see Fig. 5.16). Interestingly, no increase in sensitivity to cisplatin (another DNA cross-linking agent) was detected, perhaps suggesting that mitomycin C and cisplatin result in different forms of damage.

A second set of *rad32*Δ *tsn1*Δ and *rad32*Δ *trax*Δ double mutants were constructed by crossing a *rad32::kanmx4* strain with *tsn1::kanmx6* and *trax::kanmx6* strains and PCR screening geneticin resistant mutants. These strains were tested for UV, mitomycin C, cisplatin, MMS, hydroxyurea, camptothecin and phleomycin sensitivity (see Table 5.5 for a summary of the results). Overall these strains appeared more sensitive to DNA damaging agents compared to the *rad32::ura4*. It is possible that in the *rad32::ura4* strain the *rad32* open reading frame (ORF) is not fully deleted, alternatively, in the *rad32::kanmx4* strain, the *rad32* ORF is not fully deleted and is a dominant negative. Interestingly, treatment with UV, MMS and hydroxyurea showed increased sensitivity in only the *rad32*Δ *trax*Δ double mutant compared to the *rad32*Δ *tsn1*Δ double and *rad32*Δ single (see Fig. 5.17 and Fig. 5.18). Treatment with cisplatin, mitomycin C, camptothecin and phleomycin showed increased sensitivity in both the *rad32*Δ *tsn1*Δ and *rad32*Δ *trax*Δ double mutants (see Fig. 5.17 and Fig. 5.18).

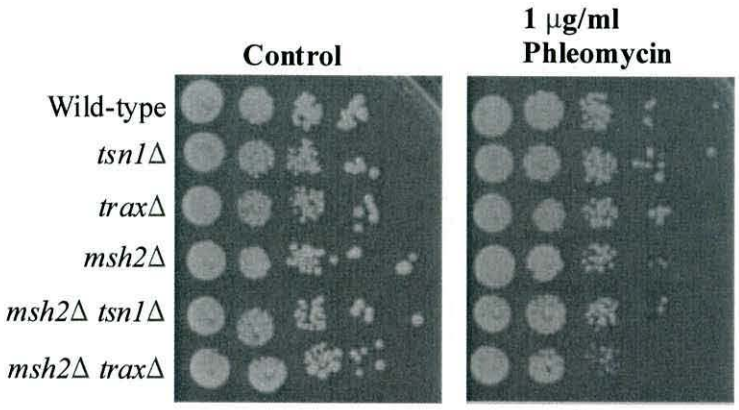


Figure 5.8 Phleomycin spot test of *msh2*Δ *tsn1*Δ and *msh2*Δ *trax*Δ double mutants. Plates were incubated at 30°C. Serial dilutions were of log phase cultures (10^{-1} to 10^{-5}, from left to right).

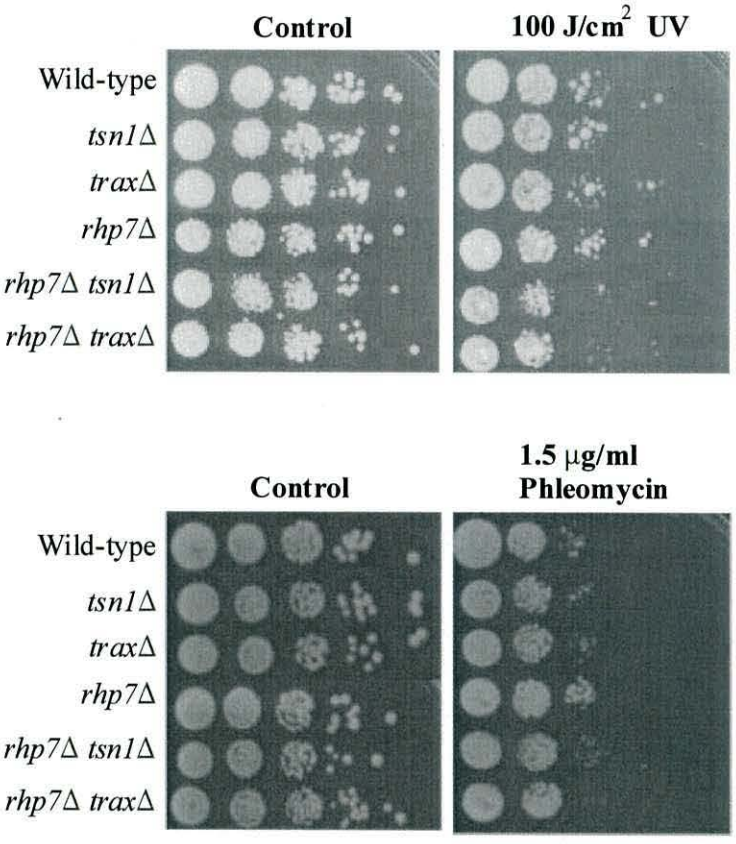


Figure 5.9 UV irradiation and phleomycin spot tests of *rhp7Δ tsn1Δ* and *rhp7Δ traxΔ* double mutants. Plates were incubated at 30°C. Serial dilutions were of log phase cultures (10^{-1} to 10^{-5}, from left to right).

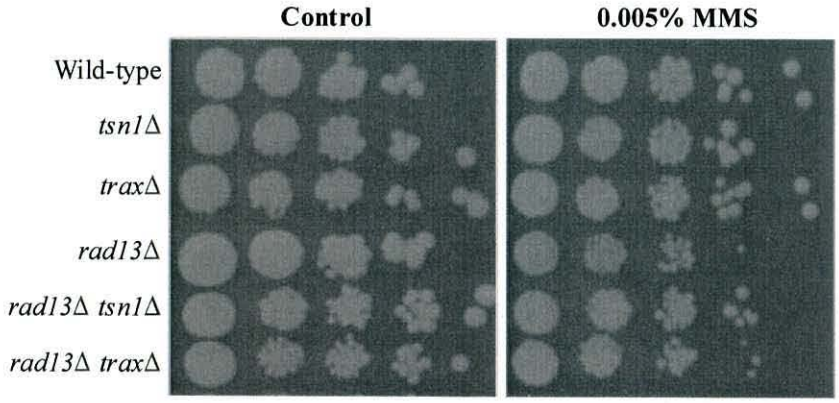


Figure 5.10 MMS spot test of *rad13*Δ *tsn1*Δ and *rad13*Δ *trax*Δ double mutants. Plates were incubated at 30°C. Serial dilutions were of log phase cultures (10^{-1} to 10^{-5}, from left to right).

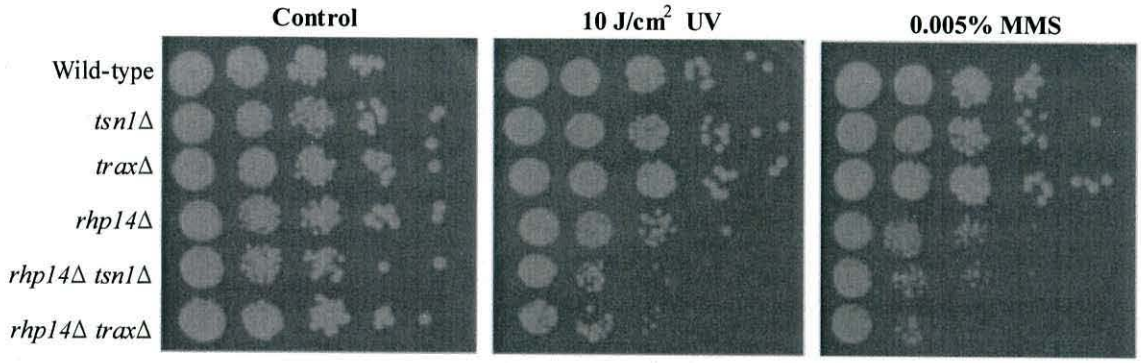


Figure 5.11 UV and MMS spot tests of *rhp14*Δ *tsn1*Δ and *rhp14*Δ *trax*Δ double mutants. Plates were incubated at 30°C. Serial dilutions were of log phase cultures (10^{-1} to 10^{-5}, from left to right).

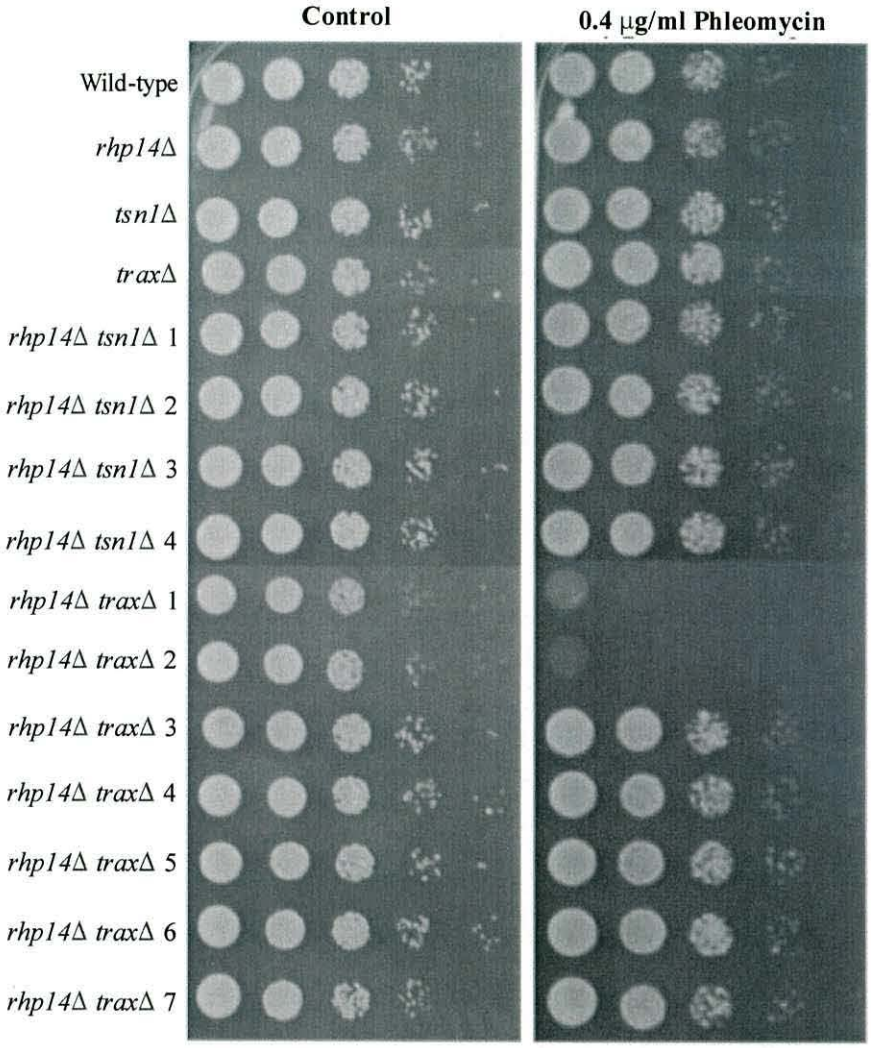


Figure 5.12 Phleomycin spot test of multiple *rhp14*Δ *tsn1*Δ and *rhp14*Δ *trax*Δ double mutants. Plates were incubated at 30°C. Serial dilutions were of log phase cultures (10^{-1} to 10^{-5}, from left to right. Performed by Lukas Stanczuk).

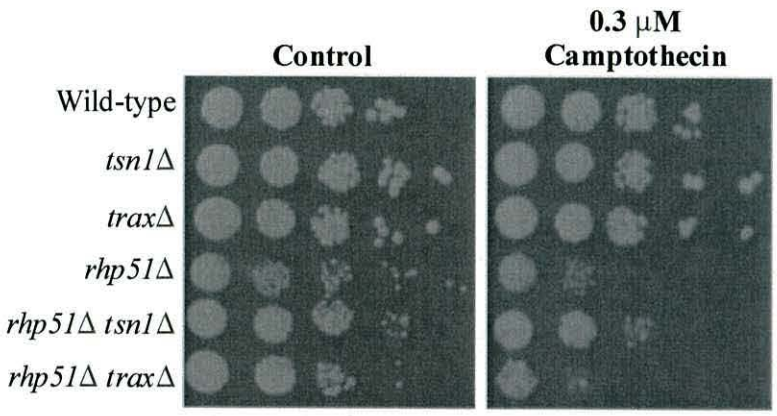


Figure 5.13 Camptothecin spot test of *rhp51Δ tsn1Δ* and *rhp51Δ traxΔ* double mutants. Control plate contains DMSO at the same concentration as the test plate. Plates were incubated at 25°C. Serial dilutions were of log phase cultures (10^{-1} to 10^{-5}, from left to right).

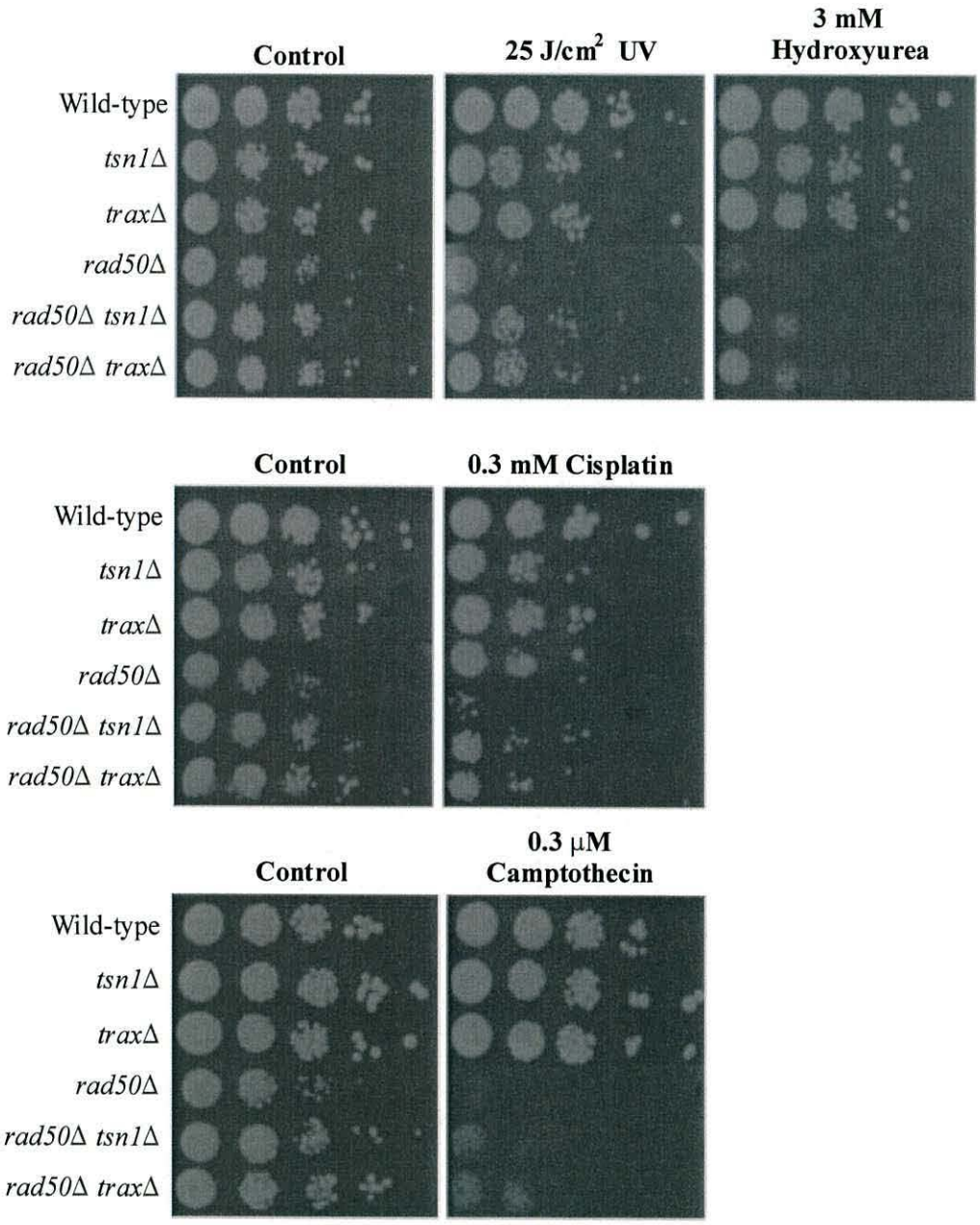


Figure 5.14 UV, hydroxyurea, cisplatin and camptothecin spot tests of *rad50Δ tsn1Δ* and *rad50Δ traxΔ* double mutants. Camptothecin and cisplatin control plates contain DMSO and DMF respectively, at the same concentration as the test plate. Plates were incubated at 25°C for the camptothecin test and at 30°C for the rest. Serial dilutions were of log phase cultures (10^{-1} to 10^{-5}, from left to right).

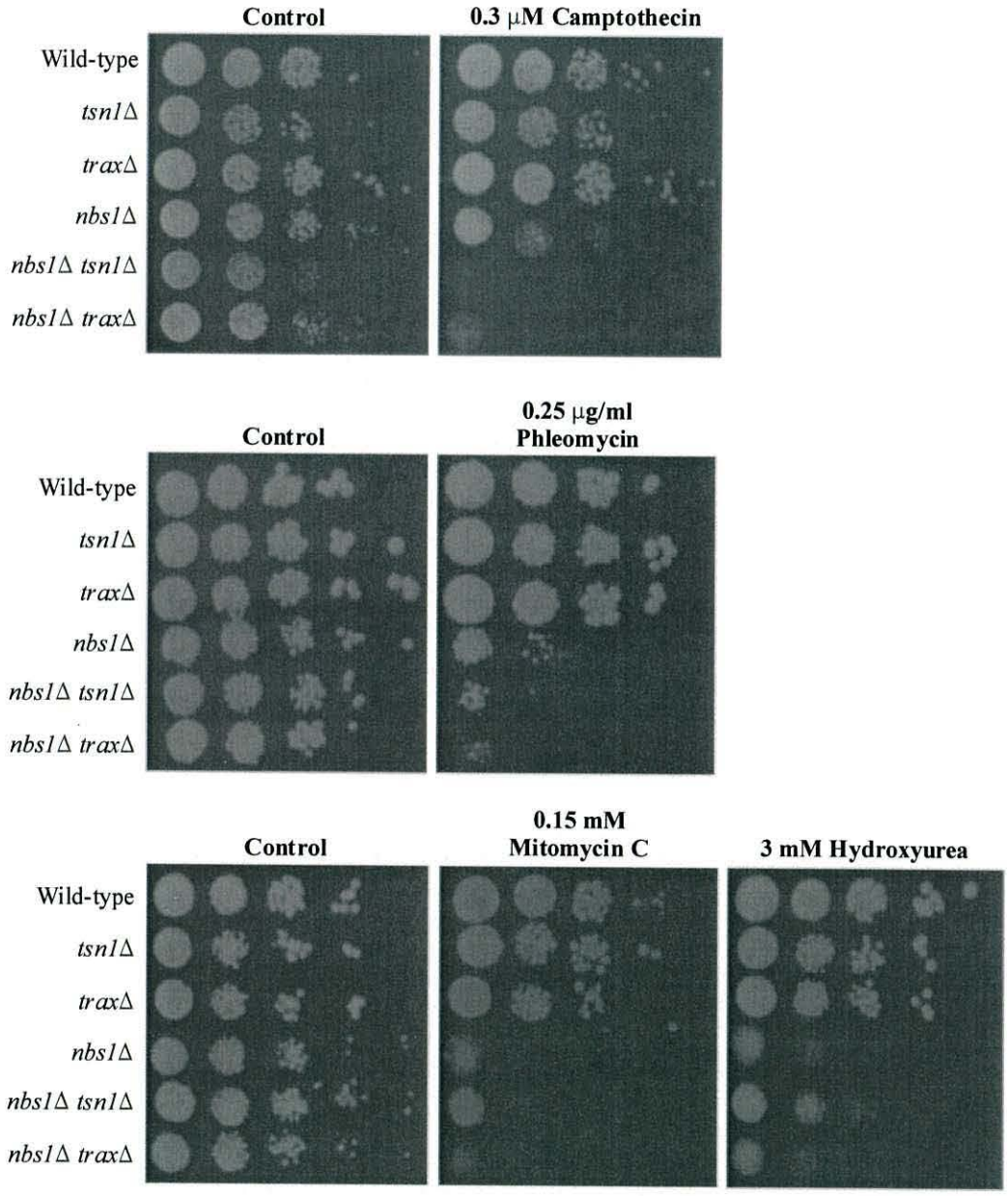


Figure 5.15 Camptothecin, phleomycin, mitomycin C and hydroxyurea spot tests of *nbs1*Δ *tsn1*Δ and *nbs1*Δ *trax*Δ double mutants. Camptothecin control plate contains DMSO at the same concentration as the test plate. Plates were incubated at 25°C for the camptothecin test and at 30°C for the rest. Serial dilutions were of log phase cultures (10^{-1} to 10^{-5}, from left to right).

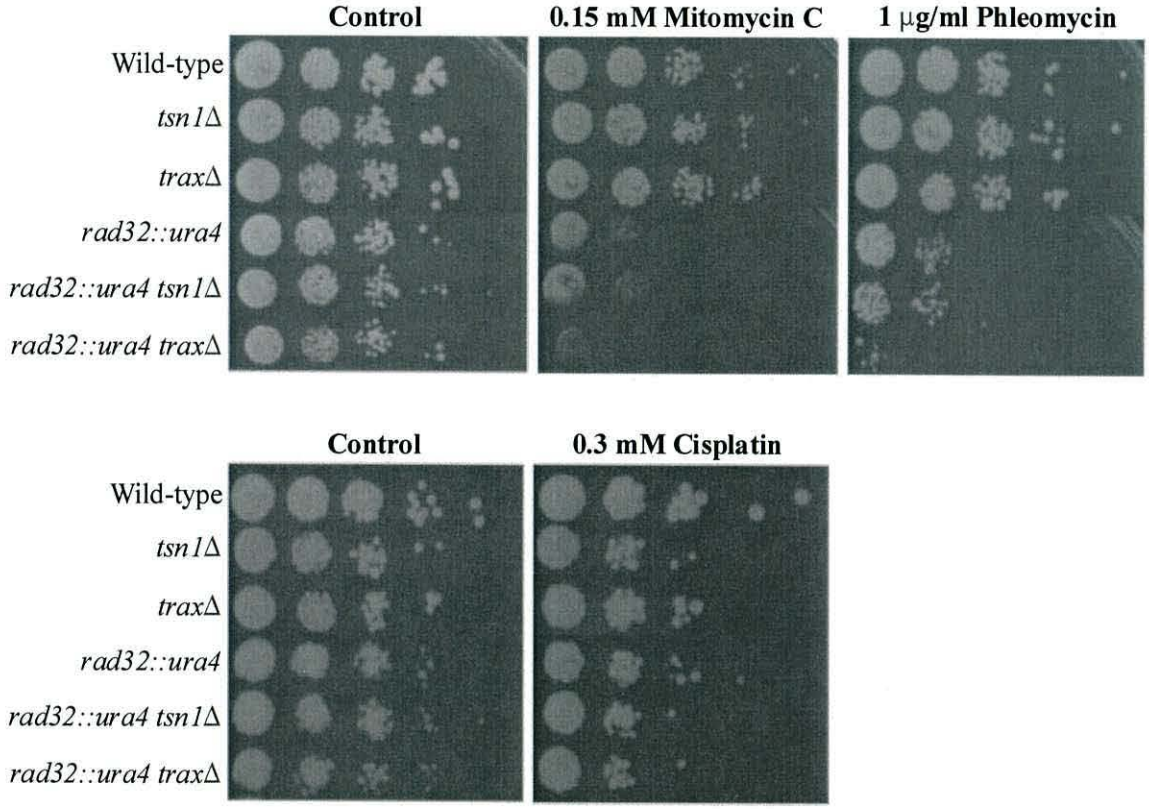


Figure 5.16 Mitomycin C, phleomycin and cisplatin spot tests of *rad32::ura4 tsn1*Δ and *rad32::ura4 trax*Δ double mutants. Cisplatin control plate contains DMF at the same concentration as the test plate. Plates were incubated at 30°C. Serial dilutions were of log phase cultures (10^{-1} to 10^{-5}, from left to right).

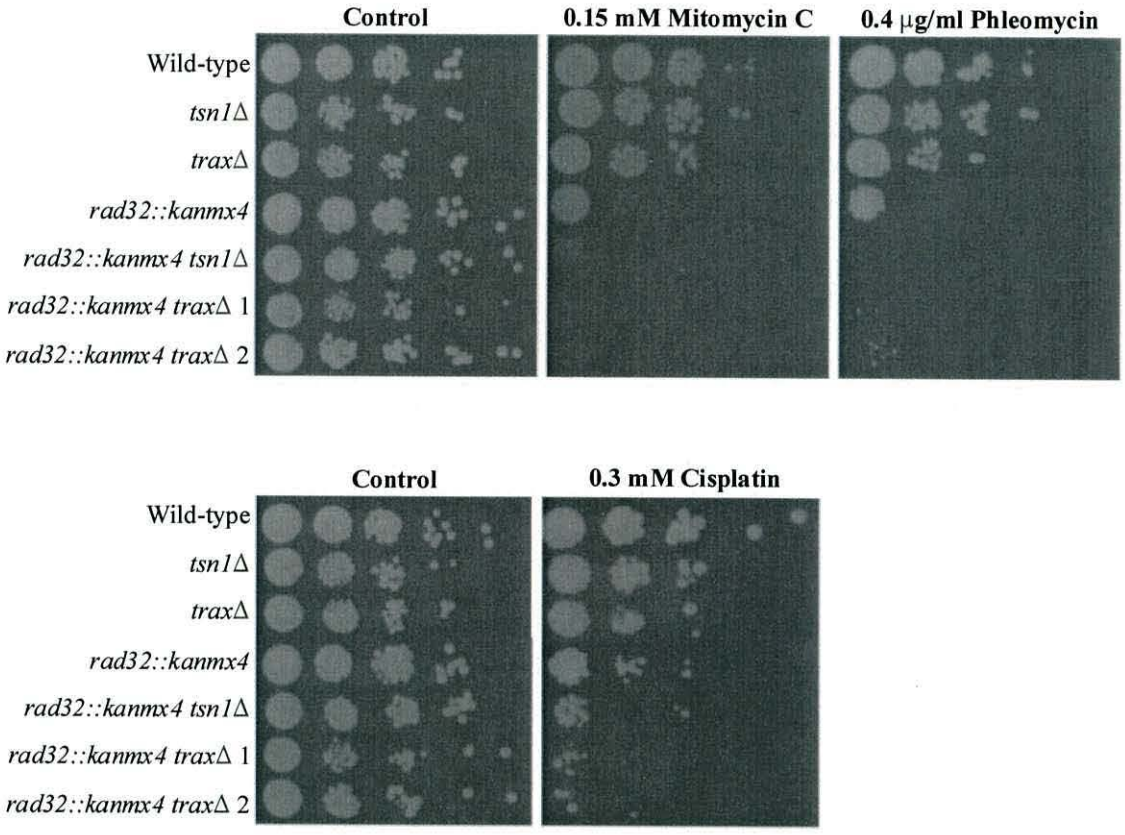


Figure 5.17 Mitomycin C, phleomycin and cisplatin spot tests of *rad32::kanmx4 tsn1*Δ and *rad32::kanmx4 trax*Δ double mutants. Cisplatin control plate contains DMF at the same concentration as the test plate. Plates were incubated at 30°C. Serial dilutions were of log phase cultures (10^{-1} to 10^{-5}, from left to right).

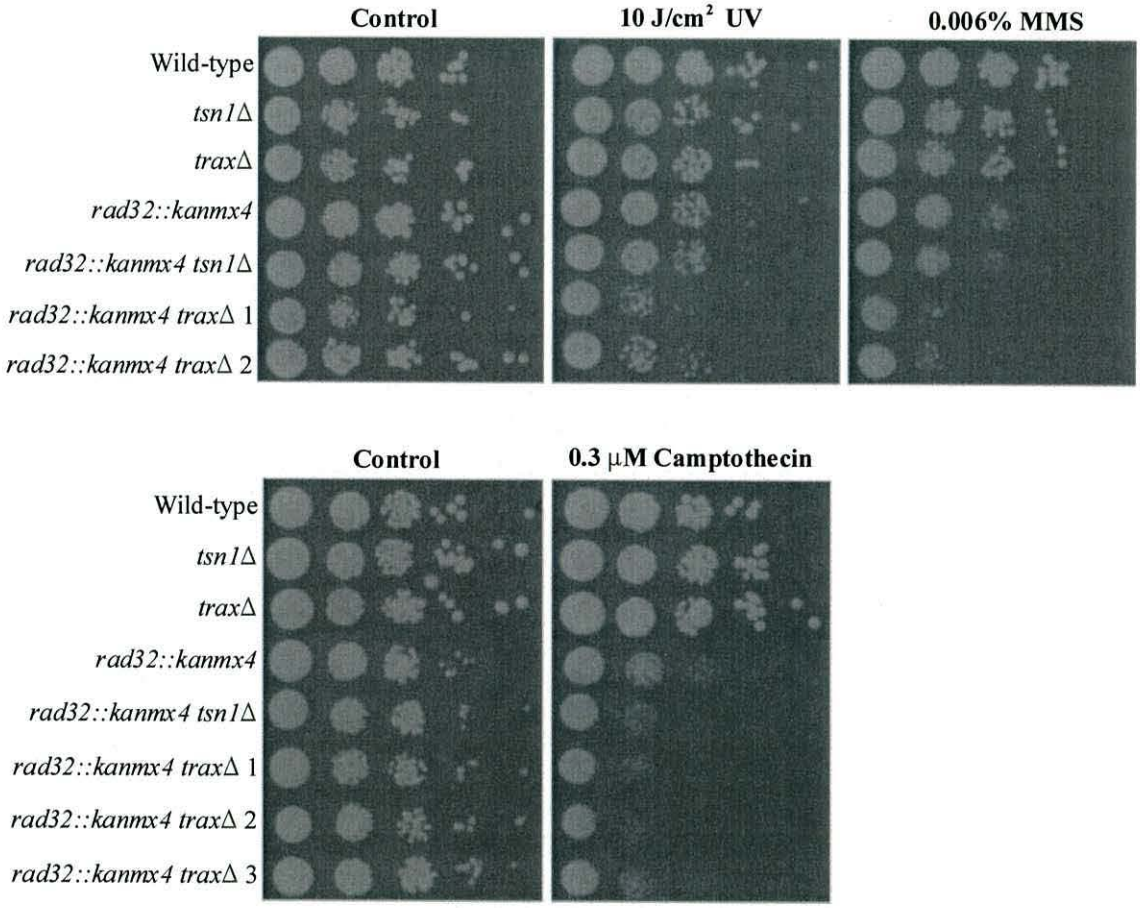


Figure 5.18 UV, MMS and camptothecin spot tests of *rad32::kanmx4 tsn1*Δ and *rad32::kanmx4 trax*Δ double mutants. Camptothecin control plate contains DMSO at the same concentration as the test plate. Plates were incubated at 25°C for the camptothecin test and at 30°C for the rest. Serial dilutions were of log phase cultures (10^{-1} to 10^{-5}, from left to right).

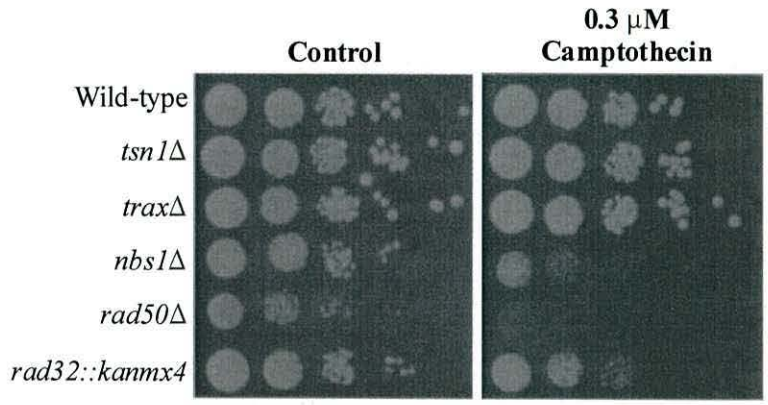


Figure 5.19 Camptothecin spot test of MRN single mutants. Control plate contains DMSO at the same concentration as the test plate. Plates were incubated 25°C. Serial dilutions were of log phase cultures (10^{-1} to 10^{-5}, from left to right).

Table 5.3 Representation of phenotype growth

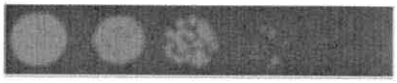
Phenotype	Symbol	Phenotype growth
~100% viable	+++++	
~80% viable	++++	
~60% viable	+++	
~40% viable	++	
~20% viable	+	
Inviable		
No test	-	

Table 5.4 Summary of sensitivity test results completed on MMR, NHEJ and NER Translin and TRAX double mutants. UV, ultra violet radiation; MMC, mitomycin C; MMS, methyl methanesulphonate. (See Table 5.3 for key).

	Sensitivity phenotype				
Strain	100 J/cm^2 UV	0.15 mM MMC	0.4 mM Cisplatin	0.01% MMS	1.5 µg/ml Phleomycin
Wild-type	+++	+++++	++	+	+++++
tsn1Δ	+++	+++++	++	+	+++++
traxΔ	+++	+++++	++	+	+++++
tsn1Δtraxδ	+++	+++++	++	+	+++++
msh2Δ	++	++++	++	+	++++
msh2Δtsn1Δ	+++	++++	++	+	++++
msh2Δtraxδ	++	++++	++	+	+++
lig4Δ	+++	+++++	++	+	+++++
lig4Δtsn1Δ	+++	+++++	++	+	+++++
lig4Δtraxδ	+++	+++++	++	+	+++++
rhp7Δ	+++	++++	++		+++
rhp7Δtsn1Δ	++	++++	++		+++
rhp7Δtraxδ	++	++++	++		+++
	10 J/cm^2 UV	No test	0.4 mM Cisplatin	0.005% MMS	No test
Wild-type	++++	-	++	+++++	-
tsn1Δ	++++	-	++	+++++	-
traxΔ	++++	-	++	+++++	-
rad13Δ	-	-	-	++++	-
rad13Δtsn1Δ	-	-	-	++++	-
rad13Δtraxδ	-	-	-	++++	-
rhp14Δ	++++	-		+++	-
rhp14Δtsn1Δ	++	-		++	-
rhp14Δtraxδ	++	-		++	-

Table 5.5 Summary of tests completed on HR Translin and TRAX double mutants. UV, ultra violet radiation; MMC, mitomycin C; MMS, methyl methanesulphonate; HU, hydroxyurea; CPT; camptothecin. (See Table 5.3 for key).

	Sensitivity phenotype						
Strain	10 J/cm^2 UV	0.15 mM MMC	0.3 mM Cisplatin	0.0006% MMS	3 mM HU	0.3 μM CPT	0.4 μg/ml Phleomycin
Wild-type	++++	++++	+++	+++++	++++	+++++	++++
tsn1Δ	++++	++++	+++	+++++	++++	+++++	++++
traxΔ	++++	++++	+++	+++++	++++	+++++	++++
rhp51Δ	++++			+++	++++	++	
rhp51Δtsn1Δ	++++			+++	++++	+++	+
rhp51ΔtraxΔ	++++			+++	++++	++	
rad50Δ	++		+	+++	+	+	
rad50Δtsn1Δ	++++		++	++++	++	++	+
rad50ΔtraxΔ	++++		++	++++	++	++	+
nbs1Δ	-	+		++++	++	+++	++
nbs1Δtsn1Δ	-	+		++++	++	+	+
nbs1ΔtraxΔ	-	+		++++	++	+	+
rad32::ura4	-	++	++	+++++	+++	-	++
rad32::ura4 tsn1Δ	-	++	++	+++++	+++	-	++
rad32::ura4 traxΔ	-	+	++	+++++	+++	-	+
rad32::kan	++++	+	++	+++	++	++	++
rad32::kan tsn1Δ	++++	+	+	++	++	+	+
rad32::kan traxΔ 1	++		+	+	+	+	+
rad32::kan traxΔ 2	++		+	+	+	+	+
rad32::kan traxΔ 3	-	-	-	-	-	+	-

5.5 Discussion

We have found no evidence that Translin or TRAX have a primary role in DNA damage recovery using a range of different assays. As already mentioned, in HeLa cells there is an increase in nuclear Translin in response to treatment with chemotherapeutic drugs (Kasai *et al.*, 1997), we however found no sensitivity to these agents in *S. pombe* Translin deficient cells. This has also been confirmed in other organisms where MEFs and *Drosophila* embryos defective in Translin show no increase in sensitivity compared to Translin-proficient controls (Yang *et al.*, 2004; Clauβen *et al.*, 2006). The *tsn1*Δ *trax*Δ double mutant also displays no obvious sensitivity to a range of agents. This conforms with the sensitivity tests from a *tsn1*Δ single mutant, which has been demonstrated to lack Translin and exhibit greatly depleted levels of TRAX protein (see Chapter 3). There was still the possibility that they may function in a redundant pathway, and to test this double mutants were made using deletion strains defective in the major DNA repair pathways.

Nine DNA damage sensitive *tsn1*Δ and *trax*Δ double mutants were made and tested against a range of different damaging agents. This produced a large amount of data, which really formed only the initial part of what will become a much larger investigation. At this stage it is difficult to be sure which results are significant and which are a result of strains being non-isogenic. All these tests need to be repeated more rigorously and these results merely provide an indication of possible Translin and TRAX functions. For example the *msh2*Δ *tsn1*Δ rescue on UV exposure and the *msh2*Δ *trax*Δ increased sensitivity on phleomycin are both very slight and were not observed with other agents. The same is true with the *rhp7* double mutants, the sensitivity seen in both the *rhp7*Δ *tsn1*Δ and *rhp7*Δ *trax*Δ double mutants was only slight and was not observed with other reagents that cause damage repaired by the NER pathway. *rad13*Δ *tsn1*Δ and *rad13*Δ *trax*Δ double mutants were only tested for sensitivity to MMS and were found to have no increased sensitivity. Rad13 is the *S. pombe* homolog of human XPG, and XPG has been shown to also function in long-patch BER as well as NER (Bessho, 1999; Klungland *et al.*, 1999; Dianov *et al.*, 2000). It is

possible that Rad13 also functions outside of NER and it has already been shown to be involved in the repair of mismatches generated during meiotic homologous recombination (Kunz and Fleck, 2001). This is also the case for Rhp14, which along with other NER proteins Swi10 and Rad16, functions in *msh2/pms1*-independent mismatch repair (Fleck *et al.*, 1999; Hohl *et al.*, 2001), correcting C/C mismatches that are not substrates of MMR in *S. pombe*. Out of the three sets of NER double mutants made, only *rhp7*Δ appears to function exclusively in NER. There is generally crossover functions of several DNA repair proteins between different pathways. Considering this, it is difficult to rule out a function for Translin and TRAX in NER. The *rhp14*Δ *tsn1*Δ and *rhp14*Δ *trax*Δ double mutants were both more sensitive to UV and MMS than the *rhp14*Δ, *tsn1*Δ and *trax*Δ single mutant, but Rhp14 is believed to have NER-independent functions (personal communication, O. Fleck) and shows a similar level of sensitivity to DNA damaging agents as seen in HR mutants. The later tests that were completed with new *rhp14*Δ *tsn1*Δ and *rhp14*Δ *trax*Δ double mutants were not repeated on UV and MMS exposure, but did show a distinct heterogeneity between isogenic progeny from the same cross (work completed by Lukas Stanczuk). This perhaps points more to a role in development, where Translin and TRAX possibly function in differential regulation and the shutting down of certain genes in a non-mendelian epigenetic manner. These later results do not rule out a function for Translin and TRAX in DNA repair but perhaps moves the focus more towards a function in regulation and development. A second possibility is exposed by a screen in budding yeast where a specific cross produced progeny with varying DNA damage sensitivities, which suggested an additive model to sensitivity (Demogines *et al.*, 2008). Since these *rhp14*Δ *trax*Δ double mutants exhibited a rescue effect when transformed with a *S. pombe trax*$^{+}$ clone, this suggests a possible second factor present within this cross that in conjuction with TRAX plays some role in the DNA damage response. *S. pombe* strains are believed be to isogenic, possess the smallest eukaryotic genome with little redundancy, however this work suggests complex traits that require further investigation.

Interestingly, further sensitivities and rescue effects were seen in all the four different HR Translin and TRAX double mutants studied. On camptothecin and phleomycin plates, the *rhp51Δ tsn1Δ* double mutant showed a slight suppressor effect. RecA is the prokaryotic homolog of Rad51 and is known to have a preferential affinity for GT-rich DNA sequences. Rad51 has also been shown to have a bias for GT-rich DNA sequences, giving an increased rate and extent of homologous pairing activity in GT-rich areas (Tracy *et al.*, 1997). In *S. pombe* homologous pairing of chromosomes was shown to be most frequent near centromeres and telomeres (Scherthan *et al.*, 1994), most telomere repeat sequences are GT-rich, suggesting a role for Rad51 in telomere maintenance in *S. pombe* and other organisms (McEachern and Blackburn, 1996). *S. pombe* Translin also has an affinity for binding GT-rich sequences (Laufman *et al.*, 2005). Since there is a slight rescue effect in the *rhp51Δ tsn1Δ* double mutant, perhaps Translin competes with Rhp51 for GT-rich ssDNA and may possibly inhibit Rhp51 binding and/or repair by HR. Inappropriate recombination can result in genome instability. In yeast inappropriate loading of Rad51 can be reversed by the Srs2 DNA helicase, which dissociates Rad51 nucleofilaments (Krejci *et al.*, 2003; Veaute *et al.*, 2003). RecQ DNA helicases, which include Sgs1 and Rqh1 in budding and fission yeasts respectively and BLM and WRN in humans, can direct the way in which recombination junctions are resolved (Doe *et al.*, 2000; Hickson 2003; Ira *et al.*, 2003; Wu and Hickson 2003). Perhaps Translin and TRAX have a role in HR regulation.

Rescue effects were also seen in the *rad50Δ* double mutants, however the *rad50Δ* single mutant displayed poor viability under normal control conditions. If the pattern of a rescue in both the *rad50Δ tsn1Δ* and *rad50Δ traxΔ* is real then perhaps it is due to the lack of TRAX protein, since in a *tsn1Δ* mutant there is little or no TRAX protein.

In the *nbs1Δ* strains there was no real trend in sensitivities. On camptothecin and phleomycin both the *nbs1Δ tsn1Δ* and *nbs1Δ traxΔ* double mutants were more sensitive than the single mutants. Damage caused by both of these agents is

thought to be targeted by HR. Nbs1 is thought to regulate Rad50 and Rad32, and perhaps Translin and TRAX also have some kind of regulatory function.
Two different *rad32*Δ deletion strains were used to make two sets of *rad32*Δ *tsn1*Δ and *rad32*Δ *trax*Δ double mutants. The *rad32::kanmx4* strains tended to be more sensitive than the *rad32::ura4* strains, but perhaps the latter is not a full deletion mutant and therefore still has some $rad32^{+}$ function. Both the *rad32::kanmx4 tsn1*Δ and *rad32::kanmx4 trax*Δ double mutants showed increased sensitivity to cisplatin, camptothecin and phleomycin, but only the *rad32::kanmx4 trax*Δ double mutant showed increased sensitivity to UV, mitomycin C, MMS and hydroxyurea. Since Translin shows both a rescue effect and increased sensitivity in HR double mutants, it is possible that it has an inhibitory function to HR and also functions in another competing pathway. Translin and TRAX function in NHEJ or NER cannot yet be ruled out. Since it has been shown that, unlike other eukaryotes, the *S. pombe* MRN complex does not function in NHEJ, it is possible that other proteins function in *S. pombe* NHEJ instead. A NHEJ assay using *lig4*Δ *tsn1*Δ and *lig4*Δ *trax*Δ double mutants would give a more conclusive result. Further analysis of NER and MMR double mutants is also required to rule out a function in these pathways.
This work is still in its infancy and opens up new lines of investigation to pin point an exact function for these two enigmatic proteins. Since in a *tsn1*Δ mutant there is no Translin and little TRAX protein, and in a *trax*Δ mutant there is no TRAX protein but Translin is present, it is difficult to attribute a sensitivity to the lack of Translin in a *tsn1*Δ mutant or the lack of TRAX. Also these results show so many sensitivities and rescue effects it requires careful further investigation to establish functional redundancy in a genome maintenance pathway.

5.6 Main conclusions:

- Single Translin and TRAX mutants show no sensitivity to a range of DNA damaging agents and appear to not have a primary role in DNA damage repair

- Sensitivities displayed by DNA damage sensitive Translin and TRAX double mutants suggest a possible role in a redundant function in DNA repair or in regulation or development

Chapter 6 Analysis of the DNA replication fork barrier and recombination potential of a single tRNA gene and 5B-box sequence.

6.1 Introduction

The process of transcription can have two detrimental effects on genome integrity. Firstly, transcription elongation opens up the chromatin structure leaving it more susceptible to genotoxic agents and DSB formation (Gangloff *et al.*, 1994a; Garcia-Rubio *et al.*, 2003). Secondly, if transcription is moving in the opposite direction to DNA replication then an RFB can occur potentially inducing homologous recombination, this is known as transcription-associated recombination (TAR) (Aguilera, 2002; Prado and Aguilera, 2005). For TAR in yeast the level of recombination is related to the level of transcription (Thomas and Rothstein 1989; Saxe *et al.*, 2000; Gonzalez-Barrera *et al.*, 2002). TAR is a natural requirement for the generation of genetic diversity during developmentally regulated processes such as class switching of immunoglobulin (Ig) genes (Jung *et al.*, 1993; Peters and Storb, 1996). Mating-type switching in yeast is another example of a programmed recombination event (Haber, 1998; Klar, 2007). However, there are some sites in the genome that can cause a recombination event in an uncontrolled manner and results in genome rearrangements and instability. These sites have some inherent instability which form DSBs due to a stress event (Aguilera and Gómez-González, 2008; Durkin and Glover, 2007). Naturally occurring chromosomal fragile regions in yeast have been found to be very complex, consisting of various genetic elements including transposons, long terminal repeats (LTRs) of transposons and tRNA genes (Admire *et al.*, 2006; Lemoine *et al.*, 2005). Interestingly, these regions have been found to exhibit higher levels of instability when DNA replication is compromised (Admire *et al.*, 2006; Lemoine *et al.*, 2005, 2008). It is also known that tRNA genes use gene conversion mechanisms to maintain their sequence uniformity, suggesting they have recombination initiating potential (Amstutz *et al.*, 1985; Kohli *et al.*, 1983; Munz *et al.*, 1982). It is known that the transcription of some tRNA genes located

in some DNA replication-related fragile sites can cause an RFP. It is thought that this is caused by DNA replication machinery colliding head-to-head with RNA polIII (Deshpande and Newlon, 1996; refer also to Chapter 1).

Another element that has been shown to cause an RFB in fission yeast is *RTS1* of the *mat* locus, which only allows DNA replication through the *mat* locus to be unidirectional (Codlin and Dalgaard, 2003; Vengrova *et al.*, 2002). The RFB caused by *RTS1* at an ectopic site has been shown to act as a mitotic recombination hotspot (Ahn *et al.*, 2005; Lambert *et al.*, 2005). It has not been demonstrated that RFP formation at tRNA genes can lead to increased recombination. Here we attempt to address this question by using a recombination assay system with strains containing a single tRNA gene within the *ade6* reporter gene.

We also wanted to investigate whether specific elements of the tRNA gene have the potential to cause DNA replication stalling and possibly an increase in recombination. In *S. cerevisiae rrm3*Δ cells, tRNA genes that are unable to assemble a transcription initiation complex do not cause fork pausing (Ivessa *et al.*, 2003). Transcription complex assembly on tRNA genes occurs in three steps; firstly transcription factor TFIIIC binds to the intragenic promoter elements, A-box and B-box, along the whole length of the tRNA gene beginning just upstream of the start site of transcription and extending through the terminator. Then TFIIIC promotes binding of TFIIIB to a region upstream of the transcription start site, and finally the TFIIIB-DNA complex recruits RNA polIII to the initiation site and remains stably bound to the DNA through multiple rounds of re-initiation by RNA polIII (Geiduschek and Tocchini-Valentini, 1988; Kassavetis *et al.*, 1990). Sequences immediately downstream of yeast tRNA genes are thought to only be required for transcription termination (Geiduschek and Tocchini-Valentini, 1988). If TFIIIC binding is eliminated by a point mutation in the B-box, this eliminates transcription and ultimately fork pausing (Kurjan *et al.*, 1980; Allison *et al.*, 1983; Baker *et al.*, 1986; Desphande and Newlon, 1996). So, replication pausing at tRNA genes requires the presence of the transcription complex or the act of transcription itself. Interestingly, tRNA genes have also

been shown to act as heterochromatin barriers (Donze *et al.*, 1999; Donze and Kamakaka 2001; Scott *et al.*, 2006). Heterochromatin can spread *in cis* to adjacent loci, causing epigenetic silencing (Grewal and Moazed 2003). This is prevented by the presence of boundary elements (Sun and Elgin 1999; Donze and Kamakaka 2001; Noma *et al.*, 2001). Heterochromatin around *cenH* and the silent mating-type locus in *S. pombe* is flanked by two inverted repeats, (*IR-L* and *IR-R* for right and left respectively.) Methylation of lysine 9 on histone H3 (H3K9me) associated with heterochromatin structure is highly enriched at the *IR* elements and decreases sharply at a specific site within the *IR* elements which contains a sequence of 5 B-boxes (see Fig. 6.12A) (Noma *et al.*, 2006). Deletion of the B-boxes resulted in the spread of heterochromatin past the barrier, with an increase in levels of H3K9me and a decrease in H3K4me, which is associated with euchromatin (Litt *et al.*, 2001; Noma *et al.*, 2001, 2006). TFIIIC was enriched at this site however RNA polIII was not, suggesting that TFIIIC alone is enough for barrier activity. This chapter addresses whether or not a tRNA gene and/or the presence of B-boxes alone are enough to elicit a RFP or RFB and if there is any associated recombination.

6.2 Construction of single *ade6::*tRNA strains

To determine whether a single tRNA gene is enough to cause a replication block and possibly an increase in recombination at the site of the block, we inserted a single tRNA gene into the *ade6* gene of *S. pombe*. In *S. pombe* there are 170 tRNA genes spread fairly evenly throughout the genome, there are additionally one pseudo tRNA gene and 25 mitochondrial tRNA genes. We amplified a glutamic acid tRNA gene (SPBTRNAGLU.08), which codes for the anticodon CTC and is located on the right arm of chromosome II. Transcription of tRNA genes is dependent on the A and B box. The A-box resides 10-20 bp from the start site of transcription and the B-box is more distal. Many *S. pombe* tRNA genes contain an upstream TATA sequence, which comprises an essential promoter element (Dieci *et al.*, 2000). The termination sequence is located 20-25 bp downstream of the B-box (reviewed by Huang and Maraia, 2001; Fig. 6.1 shows all the positions of the sites where transcription factors bind in the tRNA gene). Thus far no specific differences in function have been found between different tRNAs, however a $tRNA^{Glu}$ gene in *S. cerevisiae* was found to have an RFP effect (Deshpande and Newlon, 1996), and this is partly the reason for selecting this tRNA gene. The tRNA gene was amplified with *Bst*XI sites added to either end which permitted cloning of the PCR product into the *Bst*XI site within the *ade6* open reading frame (ORF; refer to Fig. 6.2). The asymmetry of the *Bst*XI cut site meant that distinct primer sets were used to obtain fragment inserts in both orientations within *ade6*. Clones in both orientations were made as the creation of an RFP is thought to be orientation specific, i.e only when the transcription machinery of RNA PolIII collides head-to-head with DNA replication (see Fig. 6.3). An insert was made in *ade6* so that transformants could be selected as adenine auxotrophs (which grew as red colonies due to pigmentation build up), and also because many well established techniques exist for measuring recombination at the *ade6* locus both meiotically and mitotically.

Plasmids pAJ5 [containing *ade6::*$tRNA^{Glu}$ in orientation 1 (or1) on a pDP9 backbone] and pAJ7 [containing *ade6::*$tRNA^{Glu}$ in orientation 2 (or2) on a pDP9 backbone] were used as template DNA in a PCR with primers Ade6F-Forward

and Ade6E-Reverse to give a fragment of 1063 bp containing the *tRNA^{Glu}* insert flanked by roughly 400 bp of sequence homologous to *ade6*. This was transformed into BP1121, *h^{+} lys1-37 his3-D1 ura4-D18* along with empty vector pFY20 (which contains the selective marker *ura4^{+}* to aid selection) by chemical transformation using the lithium acetate procedure (see Chapter 2). Dark pink *ura4^{+}* colonies were selected and the plasmid was lost following non-selective growth, giving BP1469 *h^{+} ade6::tRNA^{Glu} (or1) ura4-D18 his3-D1lys1-37* and BP1527 *h^{+} ade6::tRNA^{Glu} (or2) ura4-D18 his3-D1 lys1-37*. A *leu1-32* marker was then crossed in and BP1478 *h^{-} ade6::tRNA^{Glu} (or1) ura4-D18 his3-D1 lys1-37 leu1-32* and BP1508 *h^{-} ade6::tRNA^{Glu} (or2) ura4-D18 his3-D1 lys1-37 leu1-32* were selected. These strains were fully sequenced across the insert and the sequence of the inserted DNA was as expected. A control strain was also made (BP1443 *h^{-} ade6::his3' ura4-D18 his3-D1 lys1-37 leu1-32*), which has 283 bp of *his3* sequence inserted into *ade6* (see Chapter 2), following the same procedure as the *ade6::tRNA^{Glu}* strains (this control insert strain was created by D. Pryce).

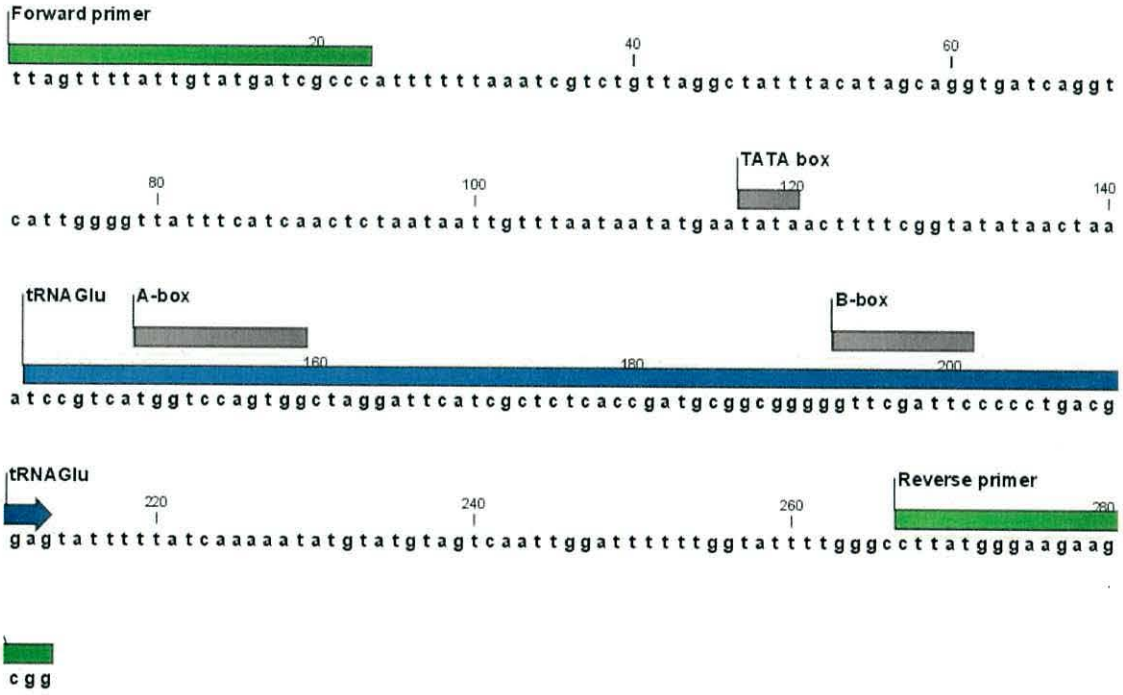
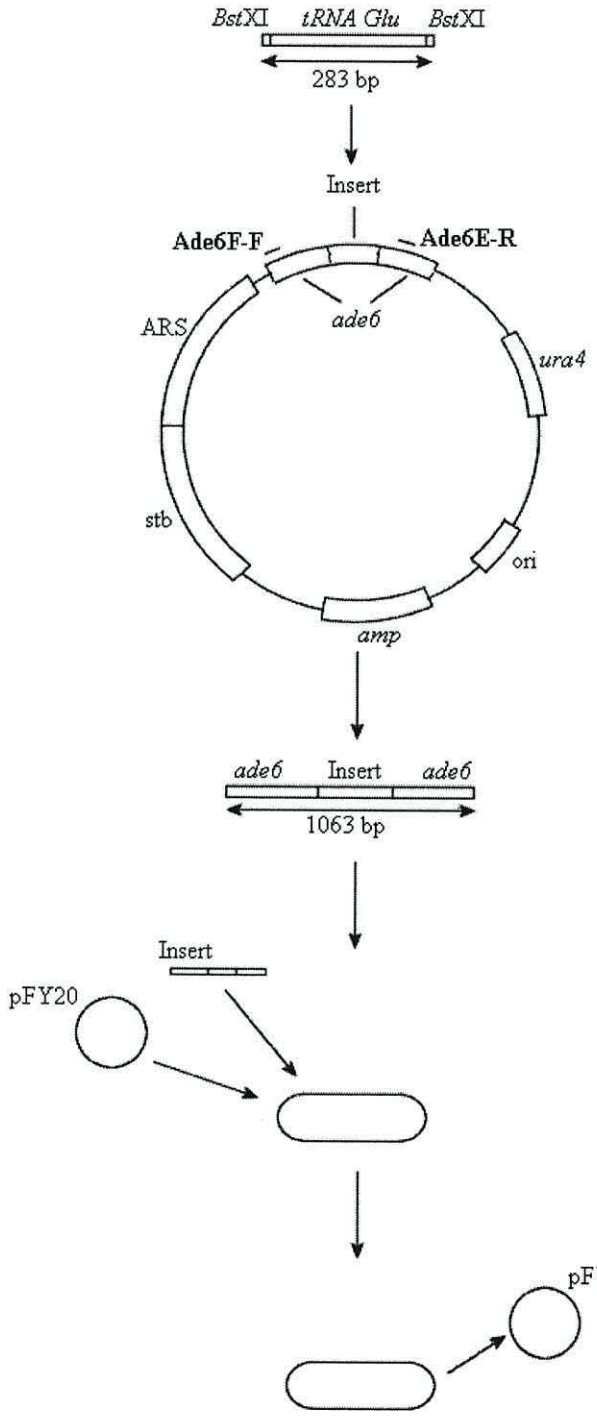


Figure 6.1 Sequence of the inserted DNA containing the tRNA^{Glu} gene. A 283 bp fragment was inserted into *ade6* containing the Glutamic acid tRNA gene SPBTRNAGLU.08. All tRNA genes contain internal A and B boxes for transcription factor binding and an upstream TATA box and these are marked. The termination sequence is located 20-25 bp downstream. The primer sequences used to amplify this genomic fragment are shown.



A glutamic acid tRNA gene (SPBTRNAGLU.08) was amplified and *Bst*XI sites added, giving a product of 283 bp.

The PCR product was ligated into the *Bst*XI site within *ade6* located on plasmid pDP9.

Primers Ade6F-Forward and Ade6E-Reverse were used to produce a 1063 bp PCR fragment containing the insert flanked by roughly 400 bp of *ade6* sequence either side.

The insert along with empty plasmid pFY20 to aid selection were transformed into *S. pombe* cells.

Dark pink ura^+ transformants were selected and the plasmid then lost giving ura^- ade^- colonies.

Figure 6.2 Cloning of a $tRNA^{Glu}$ gene and integration into *ade6*

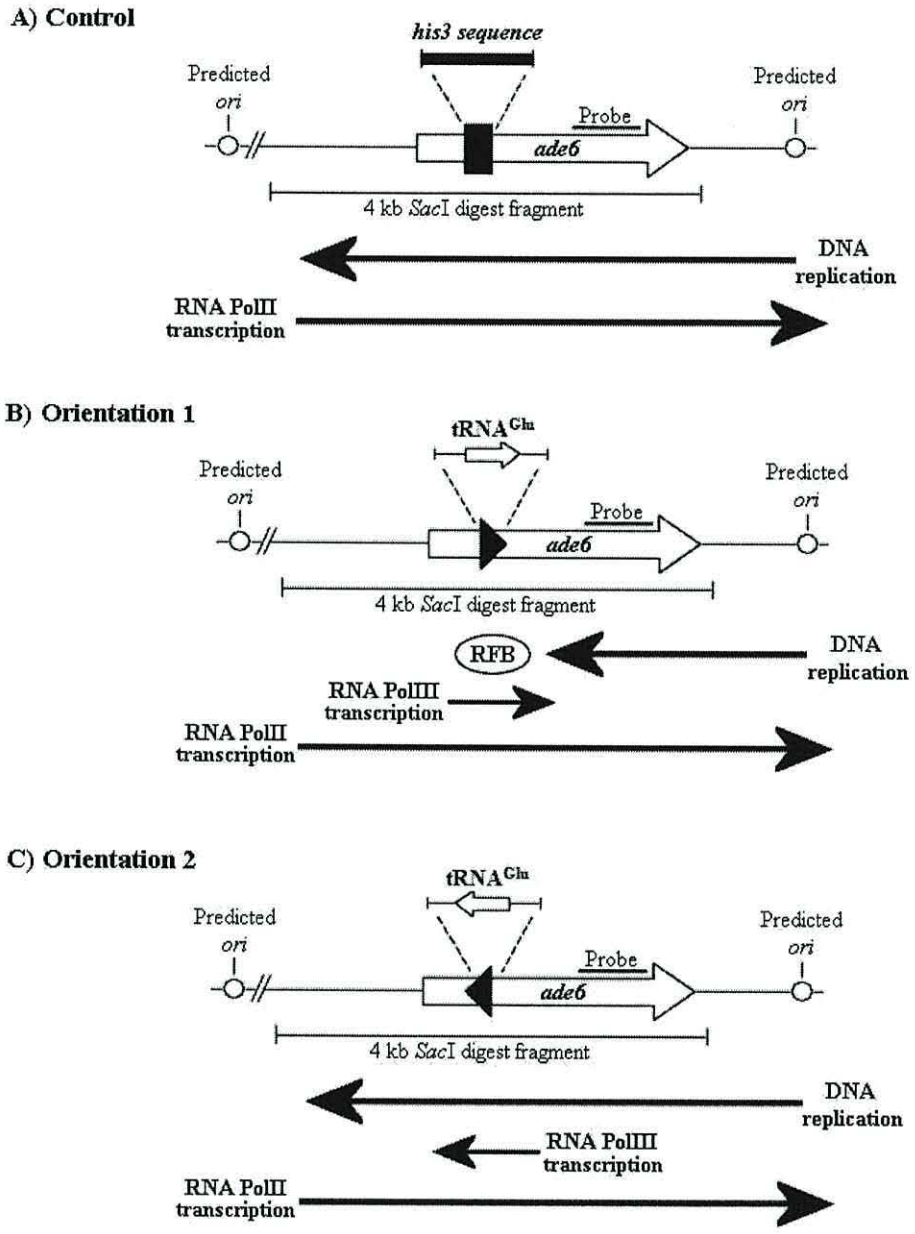


Figure 6.3 Schematic diagram showing the orientations of the *tRNA^{Glu}* insert in *ade6*. (A) A 283 bp fragment of origin free *his3* sequence was inserted for use as a spacer control. (B) Shows *tRNA^{Glu}* (SPBTRNAGLU.08) inserted in orientation 1 where transcription by RNA PolIII is in the opposite direction to DNA replication and is predicted to cause a RFB. The predominant direction of DNA replication is shown (Ahn *et al.*, 2005; Segurado *et al.*, 2003). (C) Shows the same *tRNA^{Glu}* gene in orientation 2 where transcription by RNA PolIII is in the same direction as DNA replication where no RFB is predicted. Genomic digest by *Sac*I results in a 4 kb fragment which can be analysed using 2D gel electrophoreses using the indicated probe.

6.3 RFB and recombination analysis of *tRNA*^{Glu} insert strains

6.3i) RFP analysis

Two-dimensional electrophoreses was used to try and visualize any possible replication fork pause (RFP) in the *ade6::tRNA*^{Glu} inserted strains. Genomic DNA was digested with *Sac*I and separately with *Eco*RI, giving fragments in both instances of approximately 4 kbp containing *ade6* (Fig. 6.3 shows the position of the *Sac*I fragment). This DNA was subjected to 2D gel electrophoresis and Southern blotting and was probed with a PCR product homologous to the 3′ region of *ade6*. If an element in the genome causes a RFP, then this is visualized on a 2D gel as a spot along the Y-arc of the gel, where there is an accumulation of a specific Y-structure. In both orientations of the *tRNA*^{Glu} inserted strains we detected a possible RFP at the inflexion point of the Y-arc in *Sac*I genomic digest and further down the arc in an *Eco*RI genomic digest (Fig. 6.4).

The Swi1-Swi3 complex is known to activate the replication checkpoint kinase Cds1, and is also thought to travel with the replication fork, forming a "replication fork protection complex", stabilizing stalling forks (Noguchi *et al.*, 2004). Loss of Swi1 in *S. pombe* results in an elevation of recombination frequency throughout the genome (Sommariva *et al.*, 2005). So cells with a *swi1*Δ background were thought to be more susceptible to RFB and possibly collapse, giving a higher recombination frequency. However, in fission yeast the RFB activity of the *RTS1* element was shown to be dependent upon Swi1 and Swi3 proteins, and loss of Swi1 resulted in the loss of the *RTS1* RFB-mediated recombination activity (Codlin and Dalgaard, 2003). These differing results suggest that Swi1 functions differentially to control the recombinogenic potential of different sites.

ade6::tRNA^{Glu} strains with a *swi1*Δ background were made and analysed for RFP formation. In both orientations of the *tRNA*^{Glu} gene insert an RFP was detected (Fig. 6.5), to a similar intensity as in the wild-type background, suggesting that Swi1 has no function in RFP formation.

6.3ii) Mitotic plasmid-by-chromosome recombination

To determine whether a single tRNA gene in *ade6* effected recombination at this point, we performed a plasmid-by-chromosome mitotic recombination assay using a plasmid containing the *ade6-469* allele (pade6-469; Szankasi *et al.*, 1988). The mutations in the two alleles used are 1065 bp apart (Fig. 6.6), and will recombine to form adenine prototrophs which can be measured (see Chapter 2). The fluctuation test with pade6-469 showed no change in recombination frequency in the *ade6::tRNA*^{Glu} strains compared to the control strain (Fig. 6.7). The *ade6-469* mutation is not compatible with another tRNA gene insertion strain we made using *sup3-e* (D. Pryce), which is a tandem tRNA gene cassette that encodes a suppressor tRNA which suppresses UGA (opal) stop codons, including the stop codon in the *ade6-469* mutant. Therefore, another plasmid carrying a different *ade6* mutation was created; this was pSRS5 (see Chapter 2). This mutant *ade6* allele has a G deleted at position 2358 in *ade6* if numbered according to Szankasi *et al.* (1988). If A from the ATG start codon is taken as 1, then the mutant allele is at position 1483 and therefore designated G1483 (Fig. 6.6). This deletion causes a frameshift, which results in the creation of four (non-opal) stop codons. The mutated *ade6* is cloned into a pREP42 backbone which has had the *nmt*-promoter and termination sequence removed, leaving a *LEU2* marker, *ARS* and *amp*^{r} genes (see plasmid map Fig. 6.8).

The mitotic recombination frequency was measured with plasmid pSRS5 in both wild type and *swi1*Δ backgrounds. In the wild-type background there was no statistically significant difference between the *tRNA*^{Glu} strains and the control (Fig. 6.9), suggesting that a single tRNA gene inserted in *ade6* does not result in increased hotspot activity in either orientation. In a *swi1*Δ background we found that both *tRNA*^{Glu} strains generate orientation-independent mitotic inter molecular recombination hotspots, which are not apparent in the control strain (Fig. 6.10). This demonstrates that when replication progression is perturbed, tRNA genes become a source of genetic instability.

6.3iii) Meiotic recombination

Meiotic intragenic recombination was also measured in the *ade6* insert strains by crossing them with a strain carrying *ade6-M216* and *ade6-52* test alleles and the number of adenine prototrophic progeny measured. The physical distances between the *Bst*XI insertion site and *ade6-M216* and *ade6-52* are 366 bp and 553 bp respectively (Fig. 6.6). There was roughly a two-fold increase in recombination frequency in the *ade6-M216* and *ade6::tRNA*^{Glu} crosses compared to the control, and also a two-fold increase in the *ade6-52* and *ade6::tRNA*^{Glu}-Or2 cross compared to the control and Or1 strains (refer to Fig. 6.11). However, because there was such a high variation in all the *ade6::tRNA*^{Glu} crosses, the increase in recombination is statistically insignificant.

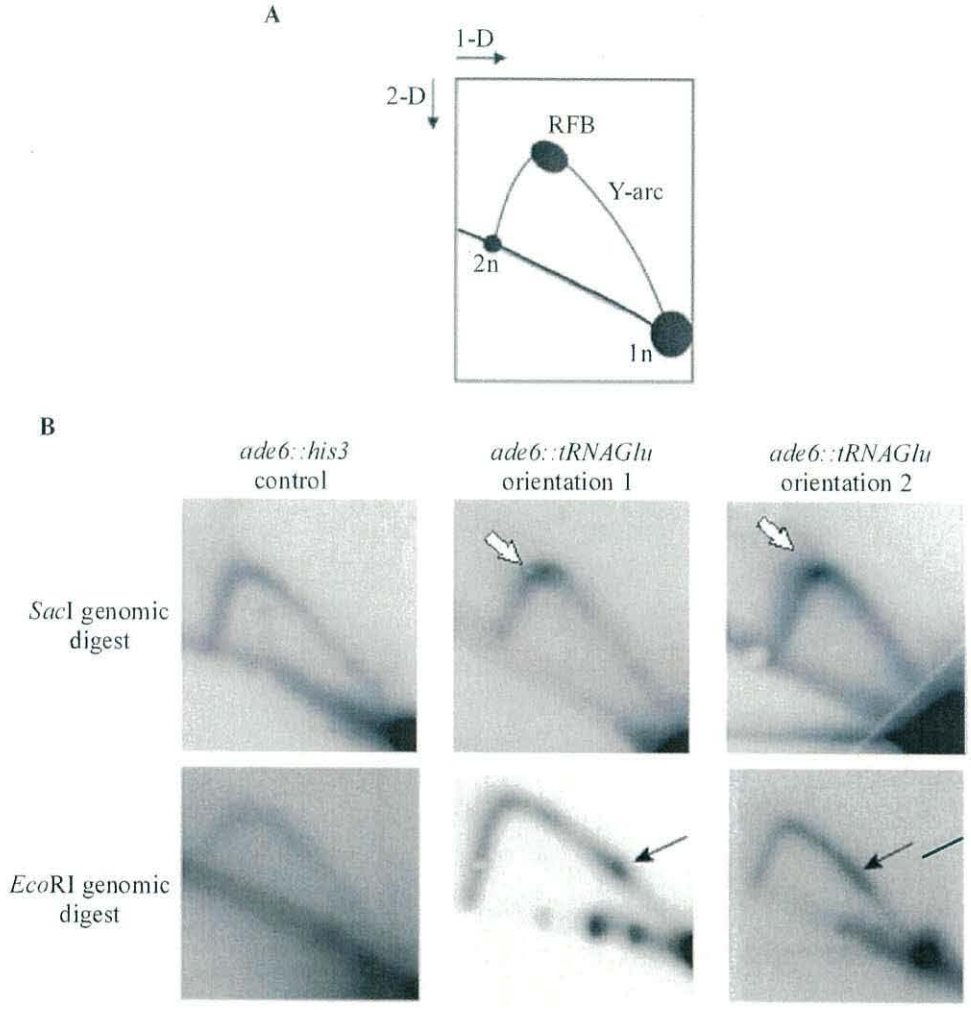


Figure 6.4 2-Dimensional DNA gel electrophoreses analysis of *ade6::tRNA^{Glu}* containing strains. A) Diagram of a 2D gel, showing the Y-arc formed from DNA undergoing replication from 1N to 2N and a RFB spot (adapted from Ahn *et al.*, 2005). In the first dimension DNA molecules are separated according to mass on a low percentage agarose gel at low voltage. The second dimension is run on a high percentage gel and at high voltage so that the mobility of a non-linear molecule is influenced by its shape. B) 2D gels of *Sac*I and *Eco*RI genomic digests of the *ade6::his3'* control strain (left panels) and both orientations of the *ade6::tRNA^{Glu}* strains (middle and right panels), probed with a dsDNA PCR fragment homologous to the 3' region of *ade6*. Arrows indicate an intense build up of Y intermediates at the inflection point (upper panels) and on the Y-arc (lower panels), which are not present in the control strain; indicating a replication pause site.

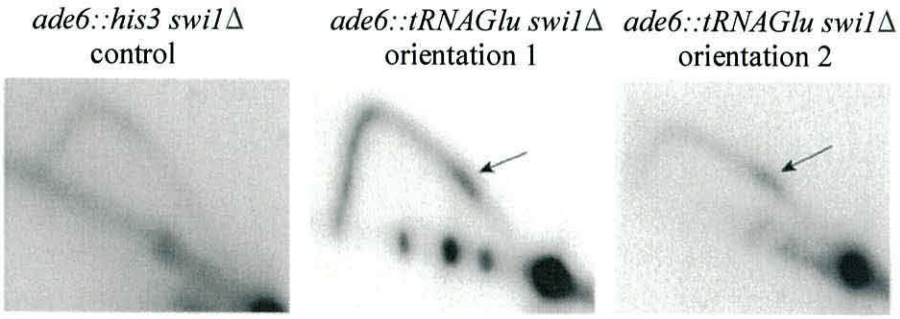


Figure 6.5 2-Dimensional DNA gel electrophoreses analysis of *ade6::tRNA^{Glu}* containing strains in a *swi1*Δ mutant background. 2D gels of *Eco*RI genomic digest of the *ade6::his3'* control strain (left panel) and both orientations of the *ade6::tRNA^{Glu}* strains (middle and right panel) in a *swi1*Δ mutant background were probed with a dsDNA PCR fragment homologous to the 3' region of *ade6*. Arrows indicate a build up of Y intermediates on the Y-arc which are not present in the control strain; indicating a replication pause site.

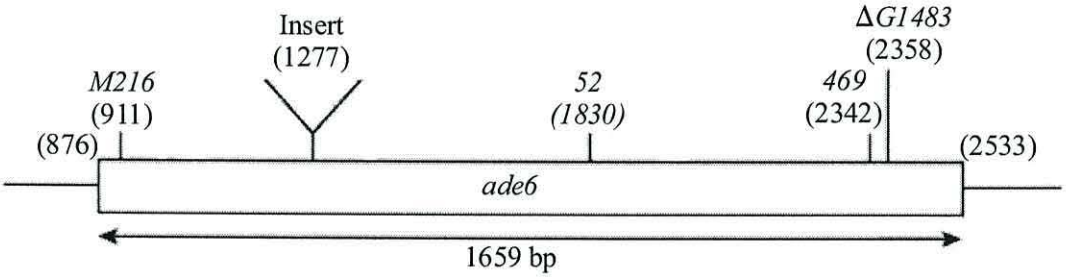


Figure 6.6 Schematic diagram of the *ade6* alleles used. The *ade6* gene is 1659 bp long starting at nucleotide position 876 and ending at 2533 (distance designations are taken from Szankasi *et al.*, 1988). The nucleotide positions of the test alleles and *Bst*XI insertion site are shown in brackets. Δ*G1483* denotes the position where a G nucleotide was deleted to create the frameshift which results in four stop codons being produced after this point. This allele is present on plasmid pSRS5 (see text).

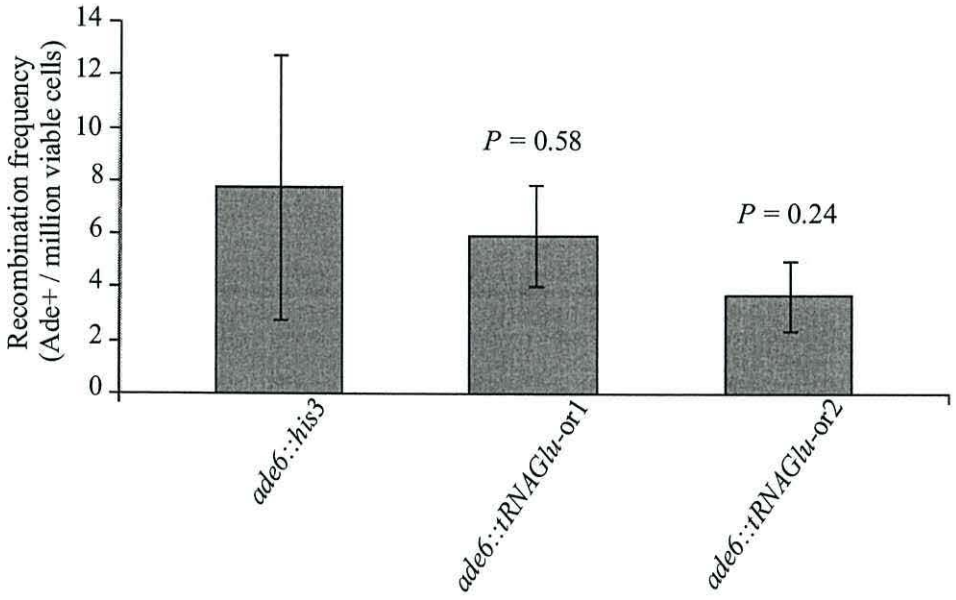


Figure 6.7 Plasmid-by-chromosome recombination assay with pade6-469. The recombination frequency of the *ade6::tRNA*^{Glu} strains with pade6-469 was not statistically different to that of the control strain. Error bars show standard deviation and the *P* values from a Student's *t*-test pairwise comparison between *ade6::his3* control and *ade6::tRNA*^{Glu} orientations 1 and 2, ($n \geq 3$ in all cases).

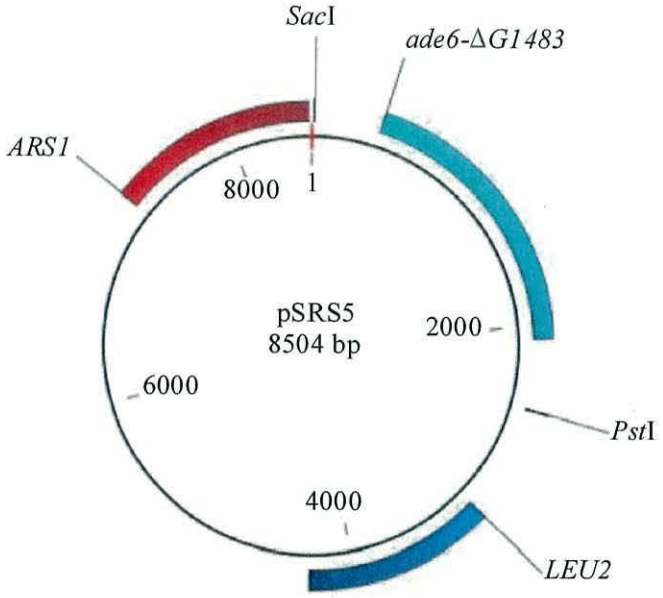


Figure 6.8 Plasmid map of pSRS5. The newly created pSRS5 plasmid contains a mutant *ade6* allele possessing a frameshift which creates four new stop codons. The backbone of the plasmid is based on pREP42.

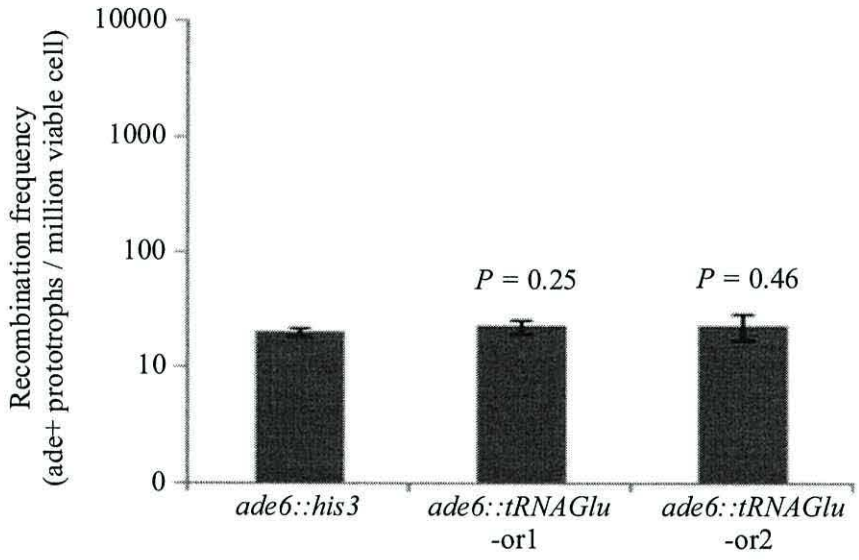


Figure 6.9 Plasmid-by-chromosome assay with pSRS5 and *ade6* inserted strains in a wild type background. Error bars show standard deviation and *P* values from a Student's *t*-test pairwise comparison of *ade6::his3* control strain with *ade6::tRNA*^{Glu} strains orientations 1 and 2 are shown above. ($n \geq 3$ in all cases).

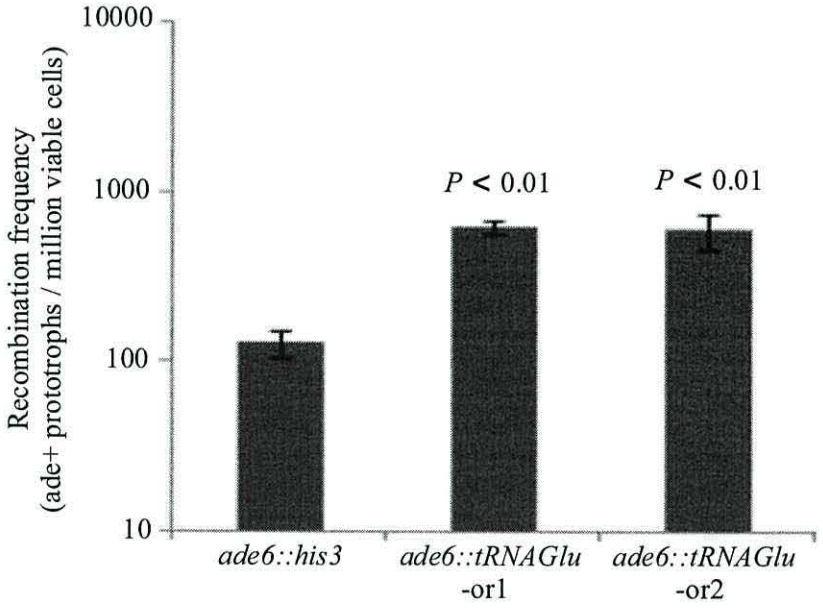


Figure 6.10 Plasmid-by-chromosome assay with pSRS5 and *ade6* inserted strains in a *swi1*Δ mutant background. Error bars show standard deviation and *P* values from a Student's *t*-test pairwise comparison of *ade6::his3* control strain with *ade6::tRNA*^{Glu} strains orientations 1 and 2 are shown above. ($n \geq 3$ in all cases).

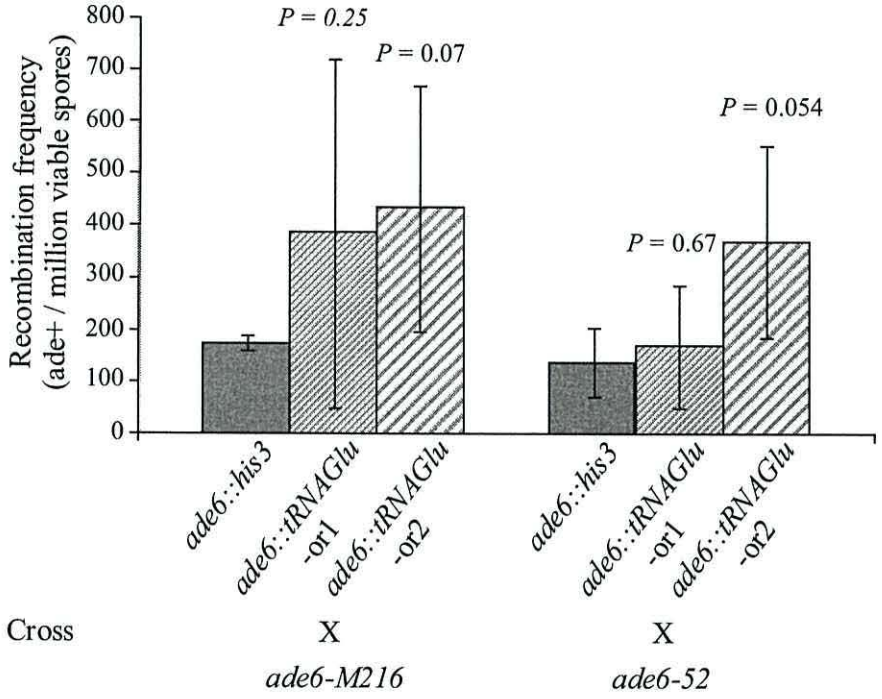


Figure 6.11 Meiotic intragenic recombination assays. The *ade6::his3'* control strain and both orientations of the *ade6::tRNA*^{Glu} strains were crossed with an *ade6-M216* and an *ade6-52* strain and the number of adenine progeny measured. These strains were non-isogenic because of time constraints. As the graph shows, some of the *tRNA*^{Glu} crosses gave a 2-fold increase in recombinants compared to the wild type cross, but in all the cases this was statistically insignificant. Error bars show the standard deviation and *P* values from a Student's *t*-test pairwise comparison of *ade6::his3* control strain with *ade6::tRNA*^{Glu} strains orientations 1 and 2 are shown above ($n \geq 3$ in all cases).

6.4 RFB and fluctuation analysis of 5B-box strains

To determine whether a series of B-boxes could possibly disrupt DNA replication, cause a RFB and serve as a recombination hotspot, the sequence of 5 B-boxes located in the *IR-R* near *mat3M* was inserted into *ade6* following the same construction method as the *ade6::tRNA*^{Glu} strains (see Chapter 2 and Section 6.2 of this Chapter). The 5 B-boxes are contained in a region of roughly 240 bp (see Fig. 6.12). The first B-box is in the forward orientation with the next four in the opposite direction. The whole sequence was inserted into *ade6* in both orientations, where orientation 1 correlates to the first B-box being in the forward direction (see Fig. 6.13).

6.4.1 Detection of a RFB

Replication intermediates were probed for using 2-D gel electrophoreses carried out by probing an *Sac*RI genomic digest with an oligonucleotide homologous for the 3′ region of *ade6*. Interestingly, a strong replication fork block was detected in the *ade6::5B-box*-Or 2 strain, where four of the five B-boxes are in the forward direction, opposing DNA replication. The spot in the 2-D gel is located off the Y-arc in the X-spike region where converging replication forks would be expected from terminator of replication sites or from Holliday junction formation (Fig. 6.14).

6.4.2 Recombination analysis

The *ade6::5B-box* strains were transformed with the plasmid pade-469 (see earlier) and fluctuation analyses were carried out. The results show that there is no statistically significant difference in the recombination rate between the control strain and the *5B-box* insert strain (Fig. 6.15), despite the strong block detected in the 2-D gels. This suggests that the block is processed in a manner that does not result in elevated homologous recombination between the chromosome and the plasmid.

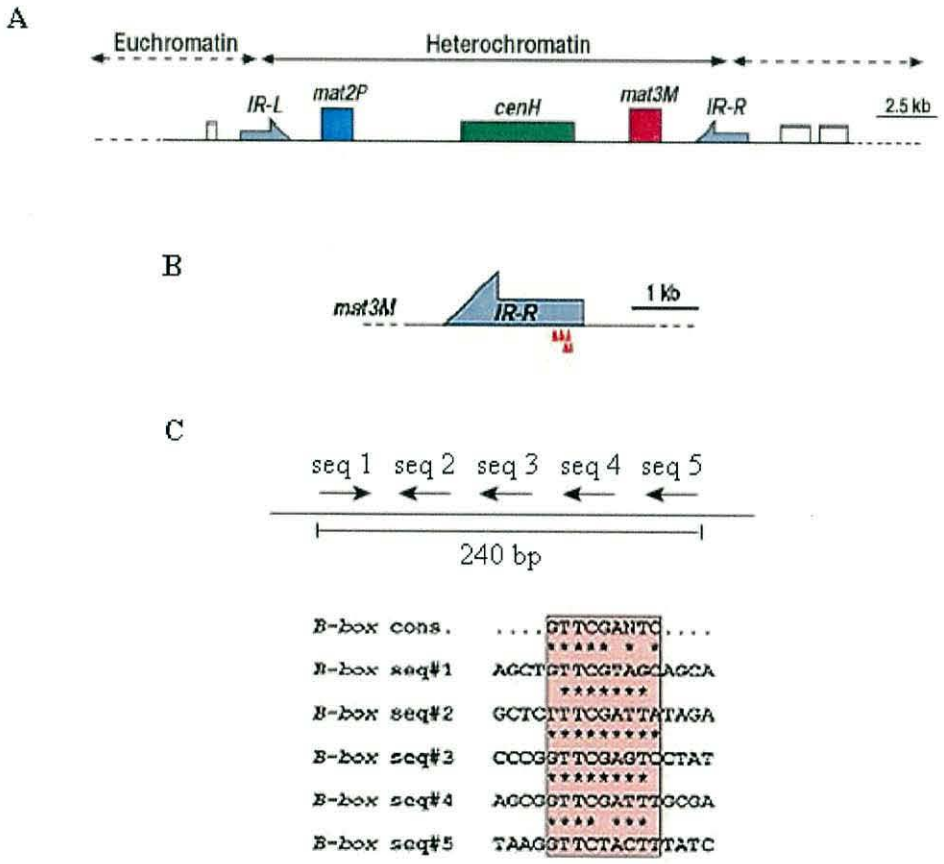


Figure 6.12 B-box sequence and location. (A) Shows a physical map of the mating-type region, where heterochromatin distribution is restricted by the *IR-L* and *IR-R* inverted repeats (light blue arrows) surrounding *mat2/3*. The *cenH* sequence is shown (green box) which shares homology to centromeric repeats. Open boxes correspond to ORFs. (B) Shows the location of the 5 B-boxes within the *IR-R* (depicted by red arrowheads). (C) Shows the direction in which the B-boxes are aligned, and their alignment with the B-box consensus sequence present in tRNA genes (Hamada *et al.*, 2001). Asterisks indicate conserved nucleotides. (Figure adapted from Noma *et al.*, 2006.)

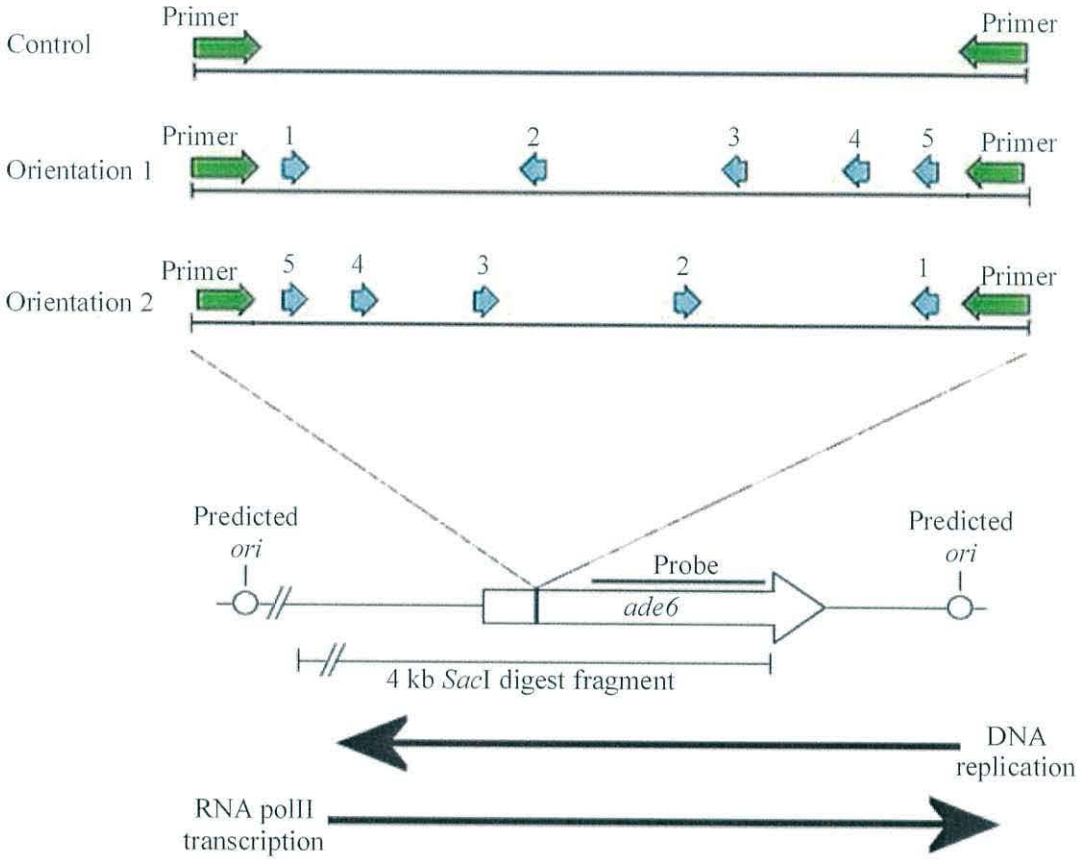


Figure 6.13 Diagram showing insert orientations. The 5B-box sequence located within the *IR-R* near *mat3*m was cloned and inserted into *ade6* following the same method as *ade6::tRNA*^{Glu} strains earlier. The insert is always 283 bp. The control strain contains *his3'* sequence, and the test strains have four of the five B-boxes in the reverse or the forward direction in orientation 1 (Or 1) and orientation 2 (Or 2) respectively. For 2-D gel electrophoreses, genomic digests were made using *Sac*I, giving a fragment of approximately 4 kb which was probed with a dsDNA PCR fragment homologous to *ade6*. The directions of DNA replication and RNA polII transcription are also shown.

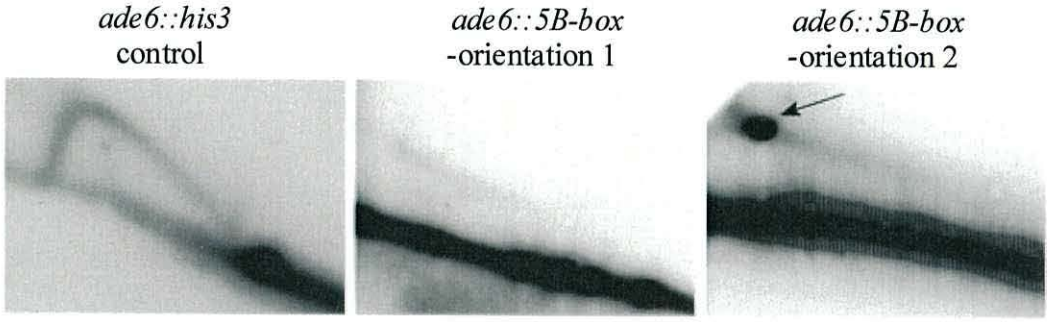


Figure 6.14 2D-gel analysis of 5 B-box constructs. Genomic digests were carried out using *Sac*I and were probed with a PCR product homologous to the 3' region of *ade6*. Both the control strain and *ade6::tRNA*^{Glu}-Or1 strain show no possible RFBs on the arc (left and middle panel), whereas *ade6::tRNA*^{Glu}-Or2 (right panel) shows a build up of X structures in the X-spike, indicating a replication block site (highlighted by the black arrow).

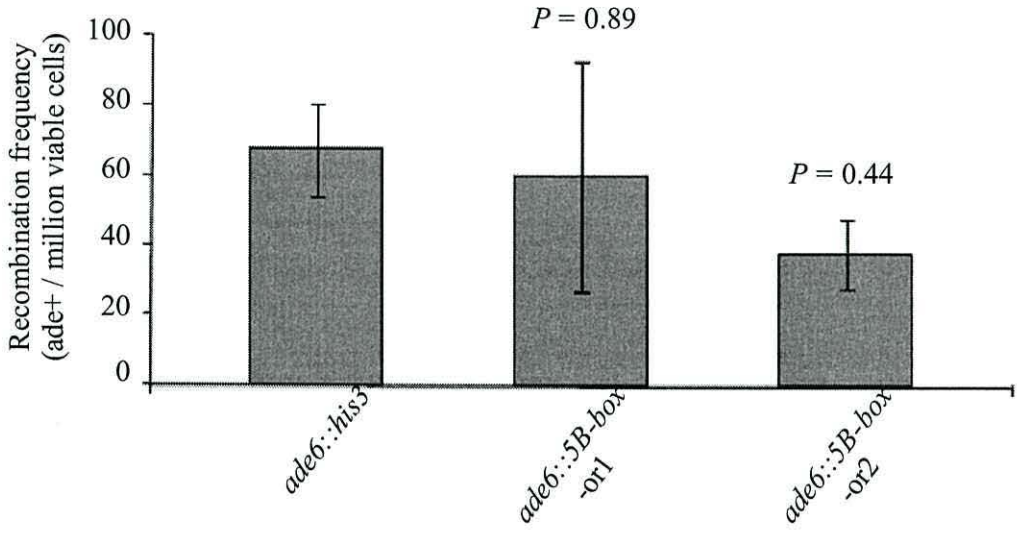


Figure 6.15 Fluctuation analysis with pade6-469 and *5B-box* insert strains. The differences in recombination frequency between the control strain and the test strains were found to be statistically insignificant. Error bars show the standard deviation and *P* values from a Student's *t*-test pairwise comparison of *ade6::his3* control strain with *ade6::5B-box* strains orientations 1 and 2 are shown above. (n $\geq$ 3 in all cases).

6.5 Discussion

6.5i) A single tRNA gene as an inducer of recombination

Previous studies demonstrated that tRNA genes give rise to polar RFBs (Deshpande and Newlon, 1996). From the 2-D gel electrophoreses it can be seen that there is an orientation-independent replication pause generated by a $tRNA^{Glu}$ inserted within *ade6* (Fig. 6.4B). The original hypothesis proposed that orientation 1 would cause a pause as transcription of the tRNA gene by RNA polIII was expected to block DNA replication moving in the opposite direction, such as TAR is thought to be initiated by RNA pol II collisions with the replisome (Aguilera, 2002; Prado and Aguilera, 2005). However, RNA polymerases must commonly encounter the replisome and mechanisms must exist to prevent instability and the generation of recombinogenic lesions. Eukaryotic RNA polymerase II-associated TAR due to head-to-head collisions with the replisome have only been detected on plasmid based transciption, and has not been detected as a normal event on the genome (Brewer and Fangman, 1988; Ivessa *et al.*, 2003; Prado and Aguilera, 2005). Our results suggest that possibly just the association of transcription factors to the inserted tRNA gene is enough to cause a physical block. Unfortunately, we do not know if there is TFIIIC binding at this point or if the tRNA gene is transcribed by RNA polIII. The insert does contain a B-box, A-box and TATA box which are all required for active transcription of tRNA genes, but without carrying out further analysis such as ChIP analysis, to identify what proteins might be binding at this point, it is impossible to conclude that RNA polIII transcription is occurring. The endogenous $tRNA^{Glu}$ used (SPBTRNAGLU.08) is still present in the genome along with several other copies of this tRNA gene. Perhaps a better experimental design would have been to use a suppressor tRNA gene whose transcription can be detected by the suppression of a marker allele. It is also unknown what affect any RNA polII transcription may have in this region. Interestingly, no significant increase in recombination was seen in both a plasmid-by-chromosome or in a meiotic recombination assay, all using different *ade6* alleles, even though an RFP was detected in both orientations

and not in the spacer control. In budding yeast, the checkpoint kinase Mec1 has been shown to be required to prevent 'replication slow zones' from becoming unstable some of which contained tRNA genes (Cha and Kleckner, 2002). Moreover, the loss of the mammalian orthologue ATR, appears to increase fragile site instability (Casper *et al.*, 2002). S-phase checkpoint regulators are also required to stabilise stalled replication forks, indicating that S-phase checkpoint pathways are a fundamental part of the replication-associated instability suppression pathway (Branzei and Foiani, 2007; Labib and Hodgson, 2007; Lambert *et al.*, 2007).

There appears to be no increase in intensity in the RFP in the $tRNA^{Glu}$ *swi1*Δ mutant background strains compared to the wild-type background, suggesting that the increase in recombination in the *swi1*Δ mutant background strains is not related to RFP formation. The RFP in all the $tRNA^{Glu}$ strains is slightly spread out rather than a tight distinct spot, suggesting that the RFP is created over a region of approximately 0.5 kb (the tRNA gene itself being only 75 bp and the insert 283 bp). Binding of tRNA gene transcription factors and RNA polIII may cover this whole region, disrupting DNA replication.

The natural RFB function of *RTS1* is dependent on several trans-acting factors, including Swi1 (Codlin and Dalgaard 2003; Ahn *et al.*, 2005). In other work using the replication terminator sequence, *RTS1* caused a recognisable RFP when inserted into the *Bst*XI site in *ade6* in the orientation that could potentially block DNA replication (orientation 2). Processing of this RFP results in an increase in recombination at this point (personal communication D. Pryce), a *RTS1* Swi1-dependent RFB was also observed by Ahn *et al.*, (2005). Interestingly, this RFP was lost altogether in a *swi1*Δ mutant background, suggesting that the increase in recombination observed in the *RTS1* orientation 2 construct is RFB-dependent, and that the RFB is Swi1-dependent (personal communication, D. Pryce). A second construct using the suppressor tRNA gene *sup3-e*, which is a double tRNA gene with a mutated anti-codon which suppresses opal stop codons, was made. As with the single tRNA constructs, the double tRNA constructs show an orientation-independent RFP in both a wild-type and *swi1*Δ mutant background, and

furthermore, also displayed orientation-independent increase in mitotic inter molecular recombination hotspot activity in the *swi1*Δ mutant background compared to the control strain (personal communication, S. Ramayah). The RFP intensity in the wild-type background compared to the *swi1*Δ mutant background was measured for the single and double tRNA constructs and no significant change in intensity was detected. These findings indicate that the increase in recombination is not concomitant with an increase in pause intensity (Pryce *et al.*, 2008 submitted for publication). This suggests that there is no linear relationship between RFP intensity and recombination in the tRNA constructs. The RFP intensity in the *RTS1* orientation 2 construct incidentally was measured to be nearly 10-fold greater than the RFP intensity measure for the single and double tRNA constructs (Pryce *et al.*, 2008 submitted for publication). Swi1 perhaps functions to permit replication through sites of RNA pol III transcription without generating substrates for recombination, either by stabilising the replication fork or somehow prevents such lesions being processed into recombination products. In bacteria, it is known that RFs arrested in mutants defective in replication processes, UV induced DNA damage or ectopic replication terminator sites (Tus/*Ter*) are processed differently, suggesting different RFBs result in distinct DNA structures at the collapsed fork, which are ultimately processed in different ways (Michel *et al.*, 2004). We have shown that in this work different RFBs have very different recombinogenic potential, and that RFB intensity does not coincide with the level of recombination.

Interestingly, it has been suggested that the protection of stalled replication forks may be essential for the proper establishment of chromosome cohesion. In budding yeast some proteins that are involved in the S-phase checkpoint or in DNA replication are essential in mutants that have defects in the chromosome cohesion pathway (Mayer *et al.*, 2004; Skibbens 2005; Warren *et al.*, 2004).

6.5ii) Replication fork block and recombinogenic potential of B-boxes

It is thought that transcription could impair replication either by increasing the torsional stress of the DNA or due to the binding of sequence-specific proteins

such as transcription factors that could act as physical blocks for replication fork progression. It is expected that the torsional stress would be higher in a head-on than in a codirectional arrangement because a head-on would accumulate positive supercoiling at the converging region. A head-on collision between transcription and replication has been shown to increase the knotting of the sister chromatids behind the fork in *E. coli* (Olavarrieta *et al.*, 2002). The torsional stress generated by converging RNA and DNA polymerase complexes, was increased by eliminating the termination region of a $tRNA^{Y}$ allele in *S. cerevisiae* (Ivessa *et al.*, 2003). This increases the size of transcript and should increase the torsional stress. However, the size of the pause at this site was not increased, suggesting that it is the presence of the transcription complex or other factors that cause RFPs (Ivessa *et al.*, 2003). Here we tried to identify what factors located within tRNAs may elicit an RFP activity.

In fact, RFBs have been found to have some characteristics in common. *S. pombe* RFBs adjacent to the rDNA and *mat1* region, are both polar RFBs (Brewer and Fangman, 1988; Hernández *et al.*, 1993; Dalgaard and Klar, 2001). During eukaryotic rDNA replication, only the fork moving opposite to transcription is arrested close to the 3' end of the coding region (Brewer and Fangman, 1988; Hernández *et al.*, 1993; Linskens and Huberman, 1988; Little *et al.*, 1993; López-Estraño *et al.*, 1998). *RTS1* near the *mat1* locus blocks replication forks moving in the *cenII*-distal direction (Dalgaard and Klar, 2001). Programmed fork barriers have also been found to be recombinogenic (Ahn *et al.*, 2005; Johzuka and Horiuchi, 2002; Kobayashi and Horiuchi, 1996; Lambert *et al.*, 2005; Takeuchi *et al.*, 2003). In fact *RTS1* functions to optimise the replication-coupled recombination event of mating-type switching (Dalgaard and Klar, 2001) *RTS1* placed in an ectopic site will also block replication forks and induce recombination (Ahn *et al.*, 2005; Lambert *et al.*, 2005). RFB regions consist of related *cis*-acting sequences which act as binding sites for *trans*-acting proteins. *RTS1* region B contains four repeated ~60 bp motifs which have been found to bind the replication termination factor Rtf1 (Codlin and Dalgaard, 2003). Fork

arrest in the rDNA requires the binding of the transcription termination protein Reb1 (Sanchez-Gorostiaga *et al.*, 2004).

Here we demonstrate that a sequence of five closely located B-boxes can elicit a block in DNA replication, but only when the orientation of the majority opposes DNA replication.

This feature is in common with other RFBs, however no increase in recombination was detected in these constructs, suggesting that unlike other RFBs, it does not elicit a recombinogenic effect. The sequence cloned is relatively small, only 283 bp, and the 5B-boxes are located fairly evenly throughout the sequence. We have not identified any other known functional sequence in this region and therefore believe that the B-boxes are responsible for the block. Unfortunately it is unknown what proteins may be bound to the 5 B-box sequence in our system, but it has been shown that the same sequence in its endogenous location will bind TFIIIC, and interestingly RNA polIII was not detected (Noma *et al.*, 2006). In the rDNA termination factors have been found to be essential for RFB activity, here it may depend on a transcription factor.

There is only a single B-box located in the *ade6::tRNA*^{Glu} construct, and a pause was detected in both orientations of the insert. In the *ade6::5B-box* construct, a strong block was detected only in one orientation, this suggests that the pause in the tRNA gene constructs is not caused solely by there being a single B-box present, but that perhaps more factors are required.

Interestingly, we now possibly have two separate RFB regions on either side of the mating-type locus. *RTS1* is located between *cen2* and *mat1* and the 5B-box sequence is located after *mat3* on the telomeric side. If the 5B-box sequence acts as an RFB in its endogenous location, then both elements will block DNA replication moving in the *cenII*-distal direction. Perhaps both function to enhance mating-type switching.

It is known in *S. pombe* that both *RTS1* at the *mat* locus and the RFB activity in the rDNA region requires Swi1 and Swi3 factors for activity, and *S. cerevisiae* Fob1 rDNA barrier depends on the homologs Tof1 and Csm3 (Mohanty *et al.*, 2006). However we have found that in both our single and double tRNA gene

constructs, that RFB activity is not Swi1 dependent. It would be interesting to investigate a Swi1-dependence in the 5B-box sequence also.
Even though a strong block is seen in the *ade6::5B-box* Or 2 strain, no increase in recombination was seen, and in fact a slight drop in recombination was measured. This suggests that the block is processed in a non-recombinogenic manner, possibly by translesion synthesis (TLS) past the block, or alternatively it is processed by RecQ helicases which promote non-recombinogenic restart of stalled forks, since a Y-arc is lacking on the 2D-gels this is perhaps not the explanation in this instance. It may be that this element is replicated late in S-phase and that the block is not removed until the end of DNA replication. Identifying what proteins are bound to this location would give in sight into how it is processed.

6.6 Main conclusions:

- A $tRNA^{Glu}$ gene inserted into a*de6* causes an orientation-independent RFP in both a wild-type and *swi1*Δ mutant background
- In a *swi1*Δ mutant background recombination is increased in an RFP independent manner
- A 5B-box sequence shows a strong block in DNA replication when the majority of the B-boxes face DNA replication head-on
- The block caused by the B-boxes is processed in a non-recombinogenic manner

Chapter 7 Final discussion

7.1 Introduction

Genetic rearrangements increase diversity and ultimately drive evolution, however they can also be detrimental to the cell and result in diseases such as cancer (Aplan, 2006). Some rearrangements are initiated at programmed sites but others take place at sites that have an inherent instability. Rearrangements involve both *cis* and *trans* acting factors, and in this work we have looked at both genetic elements which may cause fragile sites and have also attempted to identify the function of proteins such as Translin, TRAX, and Swi1 which may play a role in influencing unwanted genetic changes.

Translin and TRAX are highly conserved, and thus far we can say that in fission yeast *tsn1*Δ and *trax*Δ deletion mutants are viable, have normal cell morphology and show normal growth rates. As in other organisms, TRAX expression is Translin-dependent. We found that the over expression of Translin and/or TRAX does not result in accelerated cellular proliferation. Translin and TRAX appear to have no major role in mitotic and meiotic recombination. We found no deficiencies in any of the major DNA repair pathways in the single mutants. We observed no statistically meaningful alteration to GT repeat stability in Translin-deficient cells. However, in some DNA damage sensitive Translin and TRAX double mutants analysed we observed increased sensitivities to some DNA damaging agents, suggesting a possible role in a redundant pathway in DNA repair, or possibly in regulation or development.

We also studied the RFB potential of a single $tRNA^{Glu}$ gene inserted into *ade6*. In both a wild-type and *swi1*Δ mutant background, we detected an orientation-independent RFP in the *ade6* region, and in the *swi1*Δ mutant background we detected a RFP-independent increase in recombination. We also tried to identify what element present in tRNA genes could elicit a block in DNA replication by inserting a sequence of 5B-boxes into *ade6*, these are located endogenously in the *IR-R* of the *mat* locus. We found that in one orientation this insert resulted in a

strong block but did not show any recombination hotspot activity. This site may act as a natural RFB or DNA replication termination site.

7.2 What might be the biological function of Translin and TRAX?

Translin and TRAX are both highly conserved proteins so are likely to have a fundamental role in the cell, however their biological function remains unclear. Organisms that possess a Translin homolog also possess a TRAX homolog, this suggests that their functions are closely related. We have demonstrated that in fission yeast TRAX protein stability is dependent upon Translin, a function which appears to be conserved.

In a study of the DNA sequence roughly 500 bp upstream from the *Translin* region in both mouse and humans, the sequence was found to be highly GC-rich and contained multiple putative Sp1 factor binding sites (GC boxes, GGGCGG in either orientation; Aoki *et al.*, 1997a), which associate with promoter regions. There were however no TATA and CAAT box sequences typical of eukaryotic promoters (Aoki *et al.*, 1997a). Hence the 5' flanking regions have characteristics frequently observed in CpG islands which are an epigenetic control of transcription (Gardiner-Garden and Frommer, 1987). This would suggest a role as a housekeeping gene in mammalian cells (Aoki *et al.*, 1997a). However, *S. pombe* does not contain CpG methylation sites, and since these proteins are conserved in this unicellular organism it suggests that Translin and TRAX may have another role.

Electron microscopic (Aoki *et al.*, 1997b) and crystallographic studies (Aoki *et al.*, 1997b; Pascal *et al.*, 2002) indicated that Translin possesses an octameric ring shaped structure. *S. pombe* Translin has also been found to exist as an octamer (Laufman *et al.*, 2005). The advantage of a toroidal or ring structure is that they provide an enclosed environment for chemical reactions that may otherwise be unfavourable (reviewed by Hingorani and O'Donnell, 2000). There are many ring shaped proteins involved in DNA metabolism, that manipulate the DNA structure by chemical or physical means so that replication, transcription, repair or recombination machinery can gain access. Translin itself may just recognise

single stranded DNA overhangs and then provide a scaffolding structure for other proteins to bind onto (Hingorani and O'Donnell, 2000). Crystallographic data of mouse and human Translin shows 7 α-helices that constitute over 70% of the amino acid residues (Pascal *et al.*, 2002), which are also predicted in the tertiary structure of *S. pombe* Translin. This structure suggests that the mode of binding is very different to other well known DNA binding proteins such as SSB (*E. coli*), RPA and Cdc13 which all bind by a β-barrel conformation known as an OB-fold. Translin also has 8 potential phosphorylation sites and 1 potential N-glycosylation site (Aoki *et al.*, 1995). These sites may be used to control the conformation of Translin and its activity.

We have shown that Translin and TRAX may have a redundant function in DNA repair. Since our results show DNA damage sensitivities mainly in HR mutant backgrounds it suggests that Translin and TRAX may function in a pathway other than HR, as the sensitivities appear hypostatic. As yet not all the *S. pombe* NHEJ homologs have been identified. Furthermore, in budding yeast the MRX complex has been shown to function in both HR and NHEJ, but in fission yeast the MRN complex does not function in NHEJ, suggesting other proteins must fulfill the role. Many translocation junctions show significant loss of DNA sequence at the breakpoints, suggesting exonucleolytic degradation of DNA ends prior to rejoining. HR does not appear to be a major pathway for translocations associated with DSBs, rather the properties of the junctions are more consistent with the known biochemical properties of NHEJ. However, cells with deficiencies in this pathway still exhibit translocations with very similar junction sequences, suggesting an alternative but less conservative end joining pathway (Burma *et al.*, 2006). Future work could focus on investigating a possible function for Translin and TRAX in NHEJ. We found no deficiency in NHEJ in a *tsn1*Δ single mutant but it would be interesting to investigate any deficiency in *tsn1*Δ *lig4*Δ and *trax*Δ *lig4*Δ double mutants.

We have shown that TRAX protein expression dependency on Translin is conserved in *S. pombe*. We also found that the *trax*Δ mutant in a DNA damage sensitive background is generally more sensitive than a *tsn1*Δ mutant in a DNA

damage sensitive background. If TRAX had the more major role of the two in DNA repair then you would expect both sets of double mutants to be equally sensitive as TRAX protein levels are greatly depleted in a *tsn1*Δ mutant strain. Furthermore in the *tsn1*Δ *rhp51*Δ double mutant we detected a rescue effect. These results may suggest that Translin has an inhibitory effect on Rhp51-mediated repair.

In a later cross of *tsn1*Δ and *trax*Δ with *rhp14*Δ, *trax*Δ *rhp14*Δ progeny showed varying sensitivities to the same DNA damaging agents, and any sensitivities were rescued by a $trax^+$ clone. This may suggest a possible second factor in the cross that in conjunction with TRAX plays some role in DNA repair. This could be determined by transforming these sensitive strains with a cDNA library and testing transformants for a rescue effect.

Telomere maintenance is partly achieved by the specialised reverse transcriptase telomerase enzyme, which lengthens shortened telomeres. Many proteins bind to telomeric DNA and protect it from degradation and recombination, as these DNA ends must be maintained, unlike other internal DSBs that must be rejoined (Blackburn, 2001). In both budding yeast and mammalian cells, DNA repair proteins such as Ku70-Ku80 heterodimer and the MRN (MRX) complex are found present at telomeres and are needed for normal function (Nugent *et al.*, 1998; Ritchie and Petes, 2000; Zhu *et al.*, 2000; Tsukamoto *et al.*, 2001). The budding yeast and fission yeast checkpoint proteins Mec1 and Rad3 respectively, also recognize telomeres, but are somehow prevented from activating checkpoints when they interact with them (Dahlén *et al.*, 1998; Naito *et al*, 1998; Matsuura *et al.*, 1999; Ritchie *et al.*, 1999; Longhese *et al.*, 2000). Mutations in *rad1*, *rad3*, *rad17* and *rad26* all cause telomere shortening (Dahlén *et al.*, 1998), showing that checkpoint sensor proteins have a positive role in maintaining telomere length. *S. pombe* Tel1 is a protein kinase related to ATR and ATM. A *rad3*Δ *tel1*Δ double mutant has dramatically shortened telomeres and often circularized chromosomes (Naito *et al*, 1998). The specific role of checkpoint proteins at the telomeres is unknown, they may be required to delay the cell cycle while telomerase completes telomeric DNA synthesis. Epistasis analysis has shown that

Rad3/Rad26 and Tel1/Rad32 form two independent functional pathways required for the maintenance of stable telomeres. HR has also been shown to prevent the *de novo* telomere addition in response to break-induced loss of heterozygosity in fission yeast (Cullen *et al.*, 2007). It would be interesting to investigate a possible role of Translin and TRAX in telomere maintenance, especially since Translin has been shown to have a high affinity for GT-rich DNA sequences and is thought to possibly enhance the activity of telomerase (Cohen *et al.*, 2004).

Protein interaction studies and pull-down experiments have been attempted in *S. pombe* but thus far no significant hits have been obtained (Laufman *et al.*, 2005). Since Translin possesses a multimeric conformation, the addition of protein tags could possibly inhibit the normal protein function. Future work could focus on establishing a functional tagged strain for protein interaction experiments in *S. pombe*. It would also be valuable to determine the cellular localization of Translin and TRAX in both untreated and DNA damage treated cells.

Fragile X mental retardation protein (FMRP) binds and regulates mRNAs. Mice lacking FMRP exhibit multiple abnormal behavioral phenotypes similar to those observed in Translin-deficient mice (Chen and Toth, 2001; Chennathukuzhi *et al.*, 2003; Frankland *et al.*, 2004; Paradee *et al* 1999; Stein *et al.*, 2006). Both FMRP and Translin show the highest expression in the testes and brain (Finkenstadt *et al.*, 2001; Gu *et al.*, 1998; Han *et al.*, 1995; Hergersberg *et al.*, 1995). In the testes Translin binds mRNAs and is involved in their transport between spermatids (Morales *et al.*, 2002). In the brain Translin has been found to bind calcium/calmodulin kinase Iiα and untranslated RNA BC1 (Kobayashi *et al.*, 1998a; Severt *et al.*, 1999; Wu and Hecht, 2000), and dendritically localized RNAs that also associate with FMRP (Zalfa *et al.*, 2003). It is postulated that Translin function is essential in the brain and testes and that mRNA processing in these tissues is the main function of Translin and the reason for its conservation. However, that does not explain why both Translin and TRAX are conserved in fission yeast, a unicellular organism. This would suggest that the function of Translin is not tissue specific and that Translin may also have some other function in the cell.

7.3 *tRNA*^{Glu} gene function as a replication fork barrier

Surprisingly, we have found that a single *tRNA*^{Glu} gene can elicit an orientation-independent RFP, whereas tRNA genes were previously reported to induce polar RFPs (Deshpande and Newlon, 1996). This indicates that even distinct elements possess distinct RFB characteristics. We also found that Swi1, a component of the replication progression complex (RPC), appears to act differentially at different sites. How Swi1 distinguishes between different RFBs is unknown. The fact that recombination is increased in the single and double tRNA gene constructs on loss of Swi1 function, is similar to the observation that the loss of Mec1/ATR checkpoint activity increases instability at fragile sites, some of which contain tRNA genes (Cha and Kleckner, 2002). Swi1/Swi3-independent RFBs in the rDNA locus of *S. pombe* have also been identified (Krings and Bastia, 2004). Swi1 is the fission yeast homolog of the human TIMELESS protein, our findings may indicate an association between DNA replicative stress-induced genome instability and circadian regulation control; furthermore, human TIM has previously been associated with the DNA replication and checkpoint machinery (reviewed in Kondratov and Antoch, 2007). The more wide-spread function of Swi1 may be to suppress unwanted recombination at RFBs, and that the function at *RTS1* is an unique activity at a highly specialized site. This is supported by the fact that intra molecular recombination in a system devoid of RFBs is elevated in the absence of Swi1 (Sommariva *et al.*, 2005; Ahn *et al.*, 2005). There may well be other proteins in the RPC yet to be identified that are crucial in determining the action of Swi1. For example, *RTS1* and tRNA genes have completely different sequences and bind distinct proteins, which may also be a determining factor. For future work it would be valuable to determine what proteins specifically are binding to the RFB region. It would also be interesting to know whether the *tRNA*^{Glu} gene we used elicits a pause at its endogenous locations or at other ectopic locations, as we do not know if there are other factors in the *ade6* region that in conjunction with TFIIIC or RNA polIII for example cause an RFB, or if it is the tRNA gene alone. Repeating the experiment with a suppressor tRNA may

also be informative, and using another mutant background like the RecQ helicase *rqh1*Δ for example. *S. cerevisiae* Rrm3 helicase is known as a 'sweepase', traveling in front of the DNA polymerase and removing obstacles from the DNA, no Rrm3 homolog has been identified in *S. pombe*, but perhaps *rqh1* will have a similar function.

The 5B-box sequence we used appears to be an as yet unidentified natural termination site located in the *IR-R* region on the periphery of the *mat* locus. The *IR-R* where the 5B-box sequence has been found to stop the spread of heterochromatin from the silent mating-type locus, where the last four of the five B-boxes face head-on to the heterochromatin. tRNA genes have also been located at heterochromatic barriers (Partridge *et al.*, 2000; Cam *et al.*, 2005; Scott *et al.*, 2007), perhaps it is the B-boxes within the tRNA gene that has the barrier effect. Heterochromatin domains associated with pericentromeric repeats that contain clusters of tRNA genes have been found to be highly enriched for TFIIIC and RNA polIII. However, there is only one tRNA gene on the left side of *cen1* and none on the right, suggesting that tRNA genes are not the sole factor required to prevent the spread of heterochromatin. The predominant boundary at *cen1* and *cen3* is the inverted repeat element *IRC1*, and TFIIIC binding was not detected at these locations (Noma *et al.*, 2005). Transcripts produced from the *IRC* elements are converted into siRNA which is known to promote heterochromatin and may have a role in functional boundaries (Cam *et al.*, 2005). Interestingly, the production of siRNAs is also essential for the clustering of fission yeast telomeres at the nuclear periphery (Hall *et al.*, 2003; Sugiyama *et al.*, 2005). TFIIIC-associated sites, including the *mat* locus, centromeres and several COC loci (chromosome-organising clamp) were also found to be predominantly associated with the nuclear periphery, and B-boxes were found to be essential for tethering COC loci, suggesting that TFIIIC might have a role in organising the genome (Noma *et al.*, 2005), whether a RFB activity of B-boxes is required for the COC activity remains unknown.

We have now identified a second element on the opposite side of the *mat* locus to *RTS1*, which generates a strong orientation-dependent RFB, and is positioned in

such a way that DNA replication would be block in the same direction at both *RTS1* and 5B-box sequence. However, these are two very distinct sequences, and where the *RTS1* element has been shown to elicit an RFB-dependent increase in recombination, the 5B-box sequence does not. As already discussed the recombinogenic potential of *RTS1* may be unique to this site. It would be interestingly to determine any dependence on Swi1 at this sequence. It is also unknown if the 5B-box sequence causes a RFB at its endogenous location. The *RTS1* element contains two regions, region B which consists of four repeats of a barrier motif, region A which acts as an enhancer for region B activity (Codlin and Dalgaard, 2003). The 5B-box sequence could be broken down into smaller units and the importance of each B-box in RFB formation determined.

7.4 Closing remarks

We have investigated a number of possible functions for *S. pombe* Translin and TRAX and have found no evidence that they are involved in cell cycle regulation or recombination. However, we have found evidence that they may function in a redundant pathway involved in the DNA damage response. We have also found some functional conservation, and believe that these proteins are important in both unicellular and multicellular organisms. Further analysis in this simple eukaryote will give more insight into the importance of these proteins in the cell and in genomic stability.

We have also demonstrated that not all RFBs act as mitotic recombination hotspots, and that limited RFB activity is not sufficient to create a fragile site under normal cellular conditions. It is known that fragile sites in budding yeast are complex and are not the result of a simple RFB element alone. We have also shown that Swi1 functions differentially to regulate the processing of a RFB in a site-specific manner. We have also identified a genetic element that can generate a strong RFB in an orientation-dependent manner, but unlike many other elements that elicit a strong RFB, the 5B-box sequence RFB is processed in a non-

recombinogenic manner. These findings expose a new level of complexity in genome instability associated with DNA perturbation.

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Functional characterisation of the *Schizosaccharomyces pombe* homologue of the leukaemia-associated translocation breakpoint binding protein translin and its binding partner, TRAX

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Abstract

Translin is a conserved protein which associates with the breakpoint junctions of chromosomal translocations linked with the development of some human cancers. It binds to both DNA and RNA and has been implicated in mRNA metabolism and regulation of genome stability. It has a binding partner, translin-associated protein X (TRAX), levels of which are regulated by the translin protein in higher eukaryotes. In this study we find that this regulatory function is conserved in the lower eukaryotes, suggesting that translin and TRAX have important functions which provide a selective advantage to both unicellular and multi-cellular eukaryotes, indicating that this function may not be tissue-specific in nature. However, to date, the biological importance of translin and TRAX remains unclear. Here we systematically investigate proposals that suggest translin and TRAX play roles in controlling mitotic cell proliferation, DNA damage responses, genome stability, meiotic/mitotic recombination and stability of GT-rich repeat sequences. We find no evidence for translin and/or TRAX primary function in these pathways, indicating that the conserved biochemical function of translin is not implicated in primary pathways for regulating genome stability and/or segregation.
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Keywords: Translin; Trax; *Schizosaccharomyces pombe*; Genome stability

1. Introduction

Chromosomal translocations are associated with carcinogenesis [1,2]. Studies of the breakpoints of lymphoma and leukaemia-associated translocation junctions revealed a DNA consensus sequence which binds a novel, highly conserved protein termed translin, implicating this protein in oncogenic translocation formation [3]. Subsequently, translin binding sequences have been identified in other cancer-associated translocation breakpoints [4–8], human male meiotic recombination hot spots [9] and other human chromosomal rearrangement breakpoints [10–12]. Whilst these findings are largely based on *in vitro* gel mobility shift assays they have resulted in proposals suggesting that translin functions to control genome stability.

Translin was independently identified as the testis brain RNA-binding protein (TB-RBP) [13] and has been implicated in mRNA metabolism, particularly in neurones and in the testis [13–21]. In support of a role for translin in neuronal mRNA processing mice and fruit flies defective in translin exhibit a range of neurological and behavioural problems [22–24]. Collectively these observations indicate that translin may function in more than one important biological pathway.

Translin binds to both RNA and DNA [25–29] and forms an octomeric toroidal structure [25,30], which has similarities to the structures of other protein complexes associated with DNA replication, repair and recombination [31]. It has a high affinity for single-stranded microsatellite GT repeats, $d[GT]_n$, and G-strand telomeric repeats, $d[TTAGGG]_n$, but not corresponding double-stranded DNAs [32,33]. This suggests a role for translin in microsatellite repeat/telomere regulation, although 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 simpler eukaryote [34]. In addition to this, translin has RNAse, but not

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DNAse, activity *in vitro* [35]. This is consistent with many lines of evidence suggesting translin regulates mRNA metabolism in higher eukaryotes.

Translin has a binding partner protein termed translin-associated protein X (TRAX) [36]. Loss of translin results in depletion of TRAX from mouse embryonic fibroblasts (MEFs) [22,37] and *Drosophila* cells [38], indicating that translin functions to mediate stable levels of intracellular TRAX in higher eukaryotes. Murine translin and TRAX have functional nuclear export and localisation signals respectively, and nuclear levels of both proteins exhibit interdependence in mouse spermatocytes [39]. TRAX inhibits translin's RNA-binding ability *in vitro*, but enhances its ability to bind specific single-stranded DNAs [40]. Together these findings suggest that TRAX–translin interactions modulate functional specificity.

Both TRAX and translin have been implicated in the regulation of cell proliferation. Translin-deficient MEFs have a reduced cell proliferation rate, although TRAX protein is depleted in these cells, despite TRAX mRNA levels being normal, and so the effect of loss of translin might be indirect [22,37]. Reduction in TRAX levels in HeLa cells resulted in a reduced level 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 [41]. However, over expression of translin in a range of cells resulted in accelerated proliferation indicating that translin may also have a direct influence on cellular proliferation [42].

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. Firstly, murine translin interacts with GADD34, a protein implicated in the DNA damage response [43]. Secondly, treatment of HeLa cells with the chemotherapeutic DNA damaging agents mitomycin C and cisplatin results in an elevation in the levels of nuclear translin [25]. Thirdly, TRAX has been identified as a DNA damage-dependent interacting partner of the C1D protein [44] which regulates homologous recombination (HR) and non-homologous DNA end joining (NHEJ) [45].

Whilst translin and TRAX have been implicated in DNA damage recovery, recent work by Clauβen et al. [38] found that translin and TRAX defective *Drosophila* embryos had no increased sensitivity to ionizing irradiation and that meiotic crossing over was normal. These findings, and the observation that translin-deficient MEFs show no sensitivity to DNA damaging agents [37], argued against a role for translin and TRAX in DNA damage recovery. Here we employ the facile fission yeast model to extensively analyse the role of translin and TRAX in regulating cell growth, DNA damage response, NHEJ, HR and GT repeat stability. We find no evidence to implicate translin or TRAX as primary regulators of these biological processes; which, to some extent, differs to observations in metazoans. We also find that the regulatory controls on TRAX stability by translin are apparent in this lower eukaryote suggesting that this fundamental process is highly conserved in all eukaryotes and is thus unlikely to be related to tissue-specific functions or cell proliferation functions under normal laboratory conditions.

2. Methods

2.1. Schizosaccharomyces pombe strains, plasmids and antibodies

A list of strains employed in this study and their genotypes are shown in Table 1. Culture media, strain storage and *S. pombe* transformation was as described by Moreno et al. [46]. Plasmid pade6-469 is described in detail by Szankasi et al. [47]. Plasmid pFY20 is described in detail by Li et al. [48].

Plasmid pAJ1 was made by cloning a PCR fragment containing the tsn^+ open reading frame (orf) so it was under the regulation of the *nmt* (*no message in thiamine*) promoter of the pREP3X *S. pombe* vector [49]. The tsn^+ gene was amplified using Phusion high fidelity polymerase (Finnzyme). PCR primers contained integral *Bam*HI restriction sites for cloning. The PCR primer sequences were 5′-CGCGGATCCGCGATGAATAAATCAATATTTATTCCG-3′ and 5′-CGCGGATCCGCGTTAAACCAATTTATGTATCCG-3′. *S. pombe* genomic DNA was used as PCR template and the tsn^+ orf within pAJ1 was checked by DNA sequencing.

Plasmid pSRSB3 was made by cloning a PCR fragment containing the $trax^+$ orf so it was under the regulation of the *nmt* promoter on the pREP4X vector

Table 1
S. pombe strains used in this study

Strain	Genotype	Source
BP11	h^- *ade6-M26*	McFarlane collection
BP90	h^- *ade6-M26 ura4-D18 leu1-32*	McFarlane collection
BP420	h^- *pro1-1*	McFarlane collection
BP572	h^- *ade6-3006 ura4-D18 leu1-32 spc1::ura4*$^+$	McFarlane collection
BP621	h^+ *ura1-61*	McFarlane collection
BP685	h^- *ade6-704 ura4-D18 leu1-32 lig4::kanMX6*	McFarlane collection
BP743	h^- *rad3-136*	McFarlane collection
BP1023	h^+ *ade6-52 ura4-D18 leu1-32*	This study
BP1079	h^- *ade6-M26 ura4-D18 leu1-32 tsn::kanMX6*	This study
BP1089	h^- *ade6-M26 ura4D18 leu1-32 trax::kanMX6*	This study
BP1162	h^+ *ura4-D18 leu1-32 bub1::kanMX6*	McFarlane collection
BP1201	h^- *ade6-M26 ura4-D18 leu1-32* (pSRSB3)	This study
BP1205	h^- *ade6-M26 ura4-D18 leu1-32* (pREP4X)	This study
BP1220	h^- *ade6-((GT)$_8$-1397) ura4-D18 leu1-32 trax::kanMX6*	This study
BP1243	h^- *ade6-((GT)$_8$-1397) ura4-D18 leu1-32 tsn::kanMX6*	This study
BP1244	h^- *ade6-((GT)$_8$-1397) ura4-D18 leu1-32*	This study
BP1267	h^- *ade6-M26 tsn::kanMX6*	This study
BP1269	h^- *ade6-M26 ura4-D18 leu1-32* (pREP3X)	This study
BP1271	h^- *ade6-M26 ura4-D18 leu1-32* (pAJ1)	This study
BP1282	h^+ *ade6-52 ura4-D18 leu1-32 tsn::kanMX6*	This study
BP1288	h^+ *arg1-14*	McFarlane collection
BP1390	h^- *ade6-M26 ura4-D18 leu1-32 tsn::kanMX6* (pade6-469)	This study
BP1391	h^- *ade6-M26 ura4D18 leu1-32 trax::kanMX6* (pade6-469)	This study
BP1345	h^- *pro1-1 tsn::kanMX6*	This study
BP1346	h^+ *ura1-61 tsn::kanMX6*	This study
BP1348	h^+ *arg1-14 tsn::kanMX6*	This study
BP1455	h^- *ade6-M26 ura4-D18 leu1-32* (pREP3X, pREP4X)	This study
BP1456	h^- *ade6-M26 ura4-D18 leu1-32* (pAJ1, pSRSB3)	This study
BP1461	h^- *ade6-M26 ura4-D18 leu1-32* (pade6-469)	This study
BP1645	h^- *mts3-1 ade6-M210 arg3 his3 leu1-32 ura4-D18*	McFarlane collection
BP1668	h^+ *tsnΔ mts3-1 ade6 arg3 his3 leu1-32 ura4-D18*	This study

[49]. The fragment was amplified and cloned as described above for *tsn*$^+$ using primers with an integral *Bam*HI restriction site. The primer sequences were 5′-CGCGGATCCATGTGGACCGTAATCGTTTCTCCTCGGGAG-3′ and 5′-CGCGGATCCTTATGGAAGAGGAATTCCTCTCATTTAAAAATTTTTTA-CAG-3′. The *trax*$^+$ gene within pSRSB3 was checked by DNA sequencing.

Anti-Tsn polyclonal antibodies were raised out of house (Eurogentec, Liege, Belgium) in rabbit via inoculation with synthetic peptides EQSRNENLQEKEHGL and LKNDSLRRHFDGLKY corresponding to residues 45–58 and 204–218 respectively of the translation of *S. pombe* gene SPAC30.03c. Anti-Trax polyclonal antibodies were raised out of house (Eurogentec, Liege, Belgium) in guinea pig via inoculation with synthetic peptides SDGFPLPKDFDRTSI and VDTATPPEEK-RLRST corresponding to residues 46–60 and 217–231 respectively of translation of *S. pombe* gene SPCC736.09c. Both serum sets were affinity purified prior to use.

2.2. Gene deletions

Full orf deletions of the *S. pombe tsn*$^+$ and *trax*$^+$ genes were made using the method of Bähler et al. [50]. The primers used for the *tsn*$^+$ orf deletion were 5′-TTATTTGCATACTGAAAACATCATTCGAATATCAACACTACTCAA-CAGCATACATTACAGATTAAGTCGACGGATCCCCGGGTTAATTAA-3′ and 5′-ATATTAAAAAAGCAATTTTATCGGCTCAATTTTAGTCAAGCGTA-CAGCTGGCAAATAAATTGTTAGCAATGAATTCGAGCTCGTTTAAAC-3′. The primers used for the *trx*$^+$ orf deletion were 5′-TATAGACTTATACATT-TATACCTTCCACACGGCTTTGCTGAATTGAGGATATTATAAAACTT-TAACCGAATTTGCCAAATCGGATCCCCGGGTTAATTAA-3′ and 5′-ATTATGATTTTCAAAAGCTGCAAAACAGAAAAACTTTTAA-TAAACTAGTAAGGTGTCTGTCGAGAGCTGTCGATCATATAGAATTC-GAGCTCGTTTAAAC-3′. Correct deletions were confirmed by PCR and Southern blotting.

2.3. Fluctuation analyses

Fluctuation tests were carried out as described by Lea and Coulson [51]. Exponentially growing cultures of the strain to be tested were plated onto appropriate solid medium (selective medium was used for strains carrying plasmids) and incubated at 30 °C until micro colonies were visible. For each test seven or nine micro colonies were picked and inoculated into 5 ml of liquid medium (selective medium was used for strains carrying plasmids). These cultures were incubated with rotation at 30 °C until early stationary phase. They were then subjected to serial dilution and higher concentrations were plated onto solid minimal medium (NBA) lacking adenine to measure for adenine prototroph numbers within the culture; higher dilutions were plated onto identical minimal medium plates with supplementary adenine (100 μg/ml) to measure viable cell numbers within the culture. Plates were incubated at 30 °C and colonies counted. A minimum of three duplicates of each set were carried out and the mean values of the median value for each set were used for statistical analysis.

2.4. Meiotic crosses

Cultures were grown in yeast extract liquid (YEL), supplemented with 100 μg/ml adenine, to a density of approximately 2.5×10^7 cells/ml. 600 μl of each strain to be mated was added to a sterile microfuge tube, pulse centrifuged and aspirated. Cell pellets were washed with 1 ml sterile dH_2O and finally resuspended in 20 μl dH_2O. Suspensions were spotted onto fully supplemented synthetic sporulation media (SPA) plates and incubated at 30 °C for 3–4 days. After incubation, sporulating cells were scrapped into a microfuge tube containing 1 ml of 0.6% β-glucuronidase® (Sigma)/dH_2O solution and incubated for 16 h at 25 °C. After incubation spores were harvested and resuspended in 30% ethanol and incubated at room temperature for no longer than 5 min. Suspensions were then centrifuged and aspirated dry and cell pellets were resuspended in 1 ml sterile dH_2O.

2.5. Determination of recombination frequencies

Intragenic recombination frequencies at the *ade6* locus were determined as previously described [52].

To determine intergenic recombination frequencies using prototrophic markers, serial dilutions of spore suspensions were plated onto yeast extract agar (YEA) plates to a colony density of approximately 50–100 colonies/plate. These were then replica-plated onto nitrogen base agar (NBA) plates with and without appropriate supplements to permit the counting of double auxotrophs and prototrophs. The intergenic recombination frequency is the summed values of double prototrophs and double auxotrophs as a percentage of viable spores.

Recombination frequencies were used to determine the genetic distance (cM) by employing Haldane's mapping function (genetic distance [cM] $= -50\ln[1-2R]$, where R = the total fraction of recombinant spores amongst all spores analysed) [53].

2.6. Whole cell protein extraction and Western blots

Whole cell protein extracts (WCEs) were obtained following the protocol described by Ilyushik et al. [54] with the addition of a specific protease inhibitor set (Roche Molecular Biochemicals, Lewes, UK). The protein concentrations were determined and roughly 30 μg of WCE was run on a 10% polyacrylamide SDS-PAGE gel. The protein was then electroblotted onto PVDF transfer membrane (Amersham Biosciences UK Limited, Little Chalfont, UK). The blot was probed using anti-Translin and anti-TRAX polyclonal antibodies (see above). Donkey anti-rabbit IgG-HRP (Santa Cruz Biotechnology), and donkey anti-guinea pig IgG-HRP (Jackson Immuno Research), were used as secondary antibodies for Translin and Trax blots respectively. The blots were also probed using monoclonal anti-tubulin antibody (Sigma-Aldrich [T5168]) and goat anti-mouse IgG-HRP secondary antibody (Santa Cruz Biotechnology). ECL chemiluminescence technique was used as described by the manufacturer (Roche) to visualize the blot.

2.7. RNA extraction and Northern blotting

Cells were harvested and washed in 1 ml pre-chilled DEPC-treated water. Cells were harvested and resuspended in 750 μl TES buffer (10 mM Tris [pH 7.5], 10 mM EDTA [pH 8.0], 0.5% SDS), 750 μl of acidic phenol chloroform (Sigma) was added immediately, vortexed and incubated at 65 °C for 1 h. Samples were then placed on ice for 1 min followed by vortexing and centrifugation at 14,000 r.p.m. at 4 °C. Aqueous phase was removed, mixed with a fresh 700 μl of acidic phenol chloroform and recentrifuged. The aqueous phase was removed and mixed in a fresh Eppendorf tube with 1.5 ml pre-chilled (−20 °C) 100% ethanol and 50 μl of 3 M NaAc (pH 5.2). Samples were incubated at −20 °C overnight. RNA was harvested by centrifugation at 14,000 r.p.m. at room temperature. Supernatant was discarded and the pellet washed once with cold (4 °C) 70% ethanol. The pellet was air dried for 5 min. The pellet was finally resuspended in DEPC-treated dH_2O and stored at −20 °C until required.

RNA electrophoresis and Northern blotting was carried out as described by Ilyushik et al. [54].

3. Results

3.1. Regulation of Trax expression by translin is functionally conserved in lower eukaryotes

Homologues of both translin and TRAX have been identified in *S. pombe* [34] (http://www.genedb.org/genedb/pombe/index.jsp; in this report we shall refer to *S. pombe* translin as *tsn*$^+$ and *S. pombe* TRAX as *trax*$^+$). Previously, it has been reported that levels of TRAX in MEFs, and *Drosophila* cells, are dependent upon translin [22,37,38]. Establishing whether this biochemical function for translin is conserved in a unicellular lower eukaryote will contribute to determining whether this activity is tissue-specific, or whether it serves some more fundamental role. To address this we raised polyclonal antibodies against Trax and Tsn proteins (see Methods). Consistent with previous analyses [34], we found that Tsn- and Trax-specific bands migrated with mobility of approximately 23 kDa in a 10% SDS-PAGE gel, which indicates both proteins migrate faster than the

predicted protein size (27.3 kDa for *S. pombe* Tsn and 26.7 kDa for *S. pombe* Trax; http://www.genedb.org/genedb/pombe/index.jsp; Fig. 1 A and B). We used these antibodies to measure the stability of the Trax in the *tsn*Δ strain (see below and Methods for a description of the generation of the null mutants). Fig. 1C shows that Trax cannot be detected in *tsn*Δ cells at moderate chemiluminescent exposure of a Western blot probed with anti-Trax antibodies, demonstrating that the translin function required for the stable expression of TRAX is conserved in the lower eukaryote *S. pombe*. However, on prolonged exposure, a Trax-specific band is observed, indicating that low levels of Trax are present in the *tsn*Δ mutant (Fig. 1D). This species is not detectable in a prolonged exposure of a Western blot of WCEs from the *trax*Δ mutant (Fig. 1D). Whilst a reduction of Trax in translin-deficient cells has been reported for MEFs and *Drosophila* cells, the presence of residual Trax has not been widely observed [22,37,38]. Furthermore, the over exposure revealed three Trax-specific species which migrate with molecular weights in the approximate range of 30–48 kDa (Fig. 1D; black arrows), suggesting that some cellular Trax exists in modified forms (see below).

The regulation of TRAX protein levels has been shown to be post transcriptional in nature in higher eukaryotes [37]; consistent with this we find no change in *trax*$^+$ mRNA levels in the *S. pombe tsn*Δ strain, indicating the post transcriptional regulation is also apparent in a unicellular lower eukaryote (Fig. 1E).

Trax protein levels are restored to TB-RBP (mouse translin)-deficient MEFs when they are treated with the mammalian proteasome inhibitor MG-132 [37]. This indicates that Trax is regulated by ubiquitin-mediated proteolysis and in support of this ubiquitinated Trax has been identified in MEFs [37]. Over exposure of WCE Western blots from wild-type *S. pombe* cells probed with anti-Trax antibodies revealed a ladder of higher molecular weight Trax-specific species (Fig. 1D); whilst the molecular weight shift between these species was less than that expected for ubiquitin monomers, 8.4 kDa, it remains a formal possibility that at least one of these species is a ubiquitinated form of Trax and that inhibition of the proteasome in cells with no translin (*tsn*Δ) might restore levels of Trax in the same fashion observed for MG-132 treated MEFs. To test this we generated a *tsn*Δ mutant which also carried a temperature sensitive allele of the gene coding for the Mts3 protein, which is an essential component of the *S. pombe* proteasome [55]. Whilst no Trax was detectable in the *tsn*Δ strain at the *mts3-1* non-permissive temperature (37 °C), Trax was detectable in the *tsn*Δ *mts3-1* double mutant at this temperature (Fig. 2). Consistent with this there was a slight elevation in Trax levels in the *mts3-1* single mutant at the restrictive temperature (Fig. 2). However, the levels of Trax protein in a *tsn*Δ background are not restored to *tsn*$^+$ levels by the inhibition of proteasome activity, indicating that another Translin-dependent, proteasome-independent pathway is controlling levels of Trax.

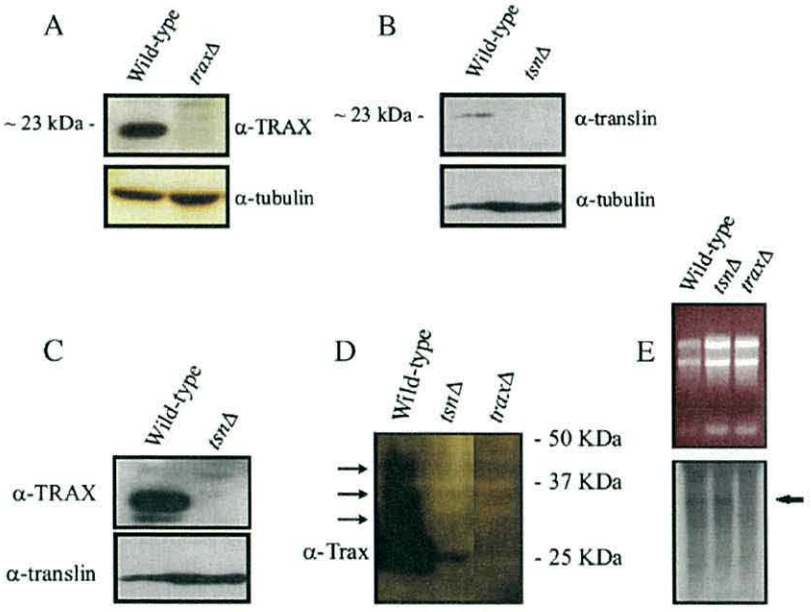

Fig. 1. Trax is depleted in Tsn-deficient cells. A and B. Whole cell protein extracts (WCEs) were made from wild-type, *trax*Δ (A) and *tsn*Δ strains. These were subjected to SDS-PAGE and Western blot analysis with polyclonal antibodies raised against Trax peptides (A) or Tsn peptides (B). Both sets of antibodies give a specific band at approximately 23 kDa. C. WCEs were made from wild-type and *tsn*Δ mitotically proliferating cells were subjected to SDS-PAGE and Western blotting. Anti-Trax antibodies were used to probe the blots and whilst a strong Trax-specific signal was detected with a mobility corresponding to approximately 23 kDa in the wild-type (left hand lane), no Trax-specific signal could be detected in the *tsn*Δ strain WCE following moderate exposure. D. Greatly prolonged exposure results in the detection of a Trax-specific band in the *tsn*Δ mutant which is not apparent in the *trax*Δ mutant. Prolonged exposure also reveals at least three Trax-specific bands which migrate with higher molecular weights in the wild-type (black arrows), indicating modified forms of Trax exist in mitotically proliferating wild-type cells. E. *trax*$^+$ mRNA levels are not altered in a *tsn*Δ strain, indicating that Tsn-dependent regulation of Trax stability is post transcriptional in nature. The lower panel is a Northern blot using a *trax*$^+$-specific probe; it shows a weak single *trax*$^+$-specific band with similar intensity in both the wild-type and the *tsn*Δ strain; this band is lost in the *trax*Δ strain. The upper panel is total RNA stained with ethidium bromide indicating uniform total RNA loading.

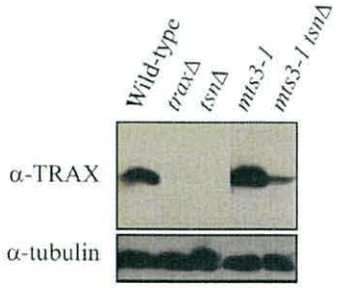

Fig. 2. Loss of proteasome function results in restoration of some, but not all Trax in translin-deficient cells. Stable expression of Trax protein is greatly reduced in the *tsnΔ* mutant (Fig. 1). The Western blot shows levels of Trax protein are elevated in both *tsn*$^+$ and *tsnΔ* backgrounds when the Mts3 proteasome subunit is inactivated by shifting the *mts3-1* temperature sensitive mutant to the restrictive temperature (37 °C). All WCEs are from exponentially growing cells shifted to 37 °C for 4 h.

3.2. Tsn and Trax do not play a primary role in regulating cell proliferation

Translin-deficient MEFs have a slow growth phenotype, suggesting that translin functions in controlling mitotic cell proliferation in metazoans [37]. To explore whether this was the case in lower eukaryotes we generated deletion mutants of both the translin (*tsn*$^+$) and TRAX (*trax*$^+$) genes in haploid *h*$^-$ *S. pombe* cells (see Methods). As with other deletion mutants previously generated [34] we found null mutants of both *tsn*$^+$ and *trax*$^+$ to be viable, with cell proliferation rates similar to the wild-type at a range of temperatures (20 °C, 25 °C, 30 °C, 33 °C and 37 °C) and no differences in cell morphology or size were noted between mutants and wild-type (data not shown). Both mutants appeared to recover from stationary phase with similar kinetics to the wild-type and mating and meiosis also appeared to proceed normally, with timing and asci morphology being indistinguishable from the wild-type for both *tsnΔ* and *traxΔ* strains (data not shown).

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 [37,41,42]. However, loss of *S. pombe* translin and Trax appears to have little overt effect on rates of cell proliferation (see above). To investigate whether over expression would alter proliferation kinetics in a simple eukaryote we cloned the *S. pombe tsn*$^+$ and *trax*$^+$ genes under control of the inducible, high expression promoter, *nmt* (see Methods). Elevated expression of *tsn*$^+$ did not significantly alter the cell proliferation rate over a 10 h period of logarithmic growth following growth in the absence of thiamine for a period of 16 h (Fig. 3 A and C). When the plasmid pSRSB3 was induced to over express *trax*$^+$ (in the absence of thiamine) Northern blotting revealed a large increase in expression of the *trax*$^+$ mRNA (Fig. 3D); however, no corresponding increase was observed in the Trax protein levels (Fig. 3E) and no change in growth rate was observed (data not shown). Two prominent mRNA bands of approximately 1.0 and 1.5 kbs were observed to hybridise with a *trax*$^+$-specific probe when *trax*$^+$ is over expressed (Fig. 3D), in keeping with previous observations for murine and human TRAX mRNA [56,57]. However, when endogenous *trax*$^+$ mRNA levels were analysed by Northern blot only a single unique mRNA species was observed at relatively low levels (high exposures are required to identify this band; Fig. 1E). 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 Tsn is required for regulation of Trax levels (Fig. 1), we explored the possibility that elevated Tsn was required for over expression of Trax. We co-transformed wild-type cells with plasmids over expressing *trax*$^+$ (pSRSB3) and *tsn*$^+$ (pAJ1). These strains exhibited no change in growth rate (Fig. 3B). However, despite Tsn being over expressed, no measurable over expression of Trax could be detected in these cells (Fig. 3F); this suggests that over expression of Tsn does not result in the stimulation of higher levels of Trax protein when mRNA containing the *trax*$^+$ orf is expressed at high levels.

3.3. tsnΔ and traxΔ mutants exhibit no overt defect in response to a range of DNA damaging agents, salt or TBZ

Previous works in higher eukaryotes have, to some degree, implicated both TRAX and translin in the response to DNA damage (see Introduction). 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. We took advantage of the fact that subtle sensitivities to DNA damaging agents can be readily tested using *S. pombe*. We assayed the sensitivity of the *tsnΔ* and *traxΔ* mutants to an extensive range of DNA damaging agents [ultra violet irradiation, mitomycin C (MMC), camptothecin, methyl methane sulfonate (MMS), phleomycin, cisplatin] and the DNA replication inhibitor hydroxyurea (HU); this enables us to explore a range of DNA damage recovery pathways, which have previously gone untested or uncorroborated. Sensitivity was tested at a range of temperatures [20 °C, 30 °C and 37 °C]. No sensitivity to any of these agents was observed at any temperature tested. Fig. 4C shows an example of the sensitivity test for mitomycin C and cisplatin for *tsnΔ* at 30 °C.

To date no studies have explored the possibility that these proteins have a role in a wider response to cellular stress. To explore this we determined whether or not *tsn*$^+$ and/or *trax*$^+$ have a role in the stress response and/or osmotic stress response pathways. We tested the *tsnΔ* and *traxΔ* strains for sensitivity to salt (10 mM KCl) and sorbitol (1.5 M). Neither strain was sensitive at any temperature tested (20 °C, 30 °C and 37 °C) (see Fig. 4A, for example).

In higher eukaryotes translin localises to centrosomes and microtubles, possibly suggesting a role in regulating the spindle apparatus [17,42,58]. Many *S. pombe* mutants defective in spindle and kinetochore function are sensitive to the microtubule depolymerising drug thiabendazole (TBZ). To test whether Tsn and Trax have a direct role to play in regulation of the spindle apparatus we tested *tsnΔ* and *traxΔ* cells for sensitivity to TBZ. However, we detected no sensitivity to this agent for either mutant (for example, see Fig. 4B).

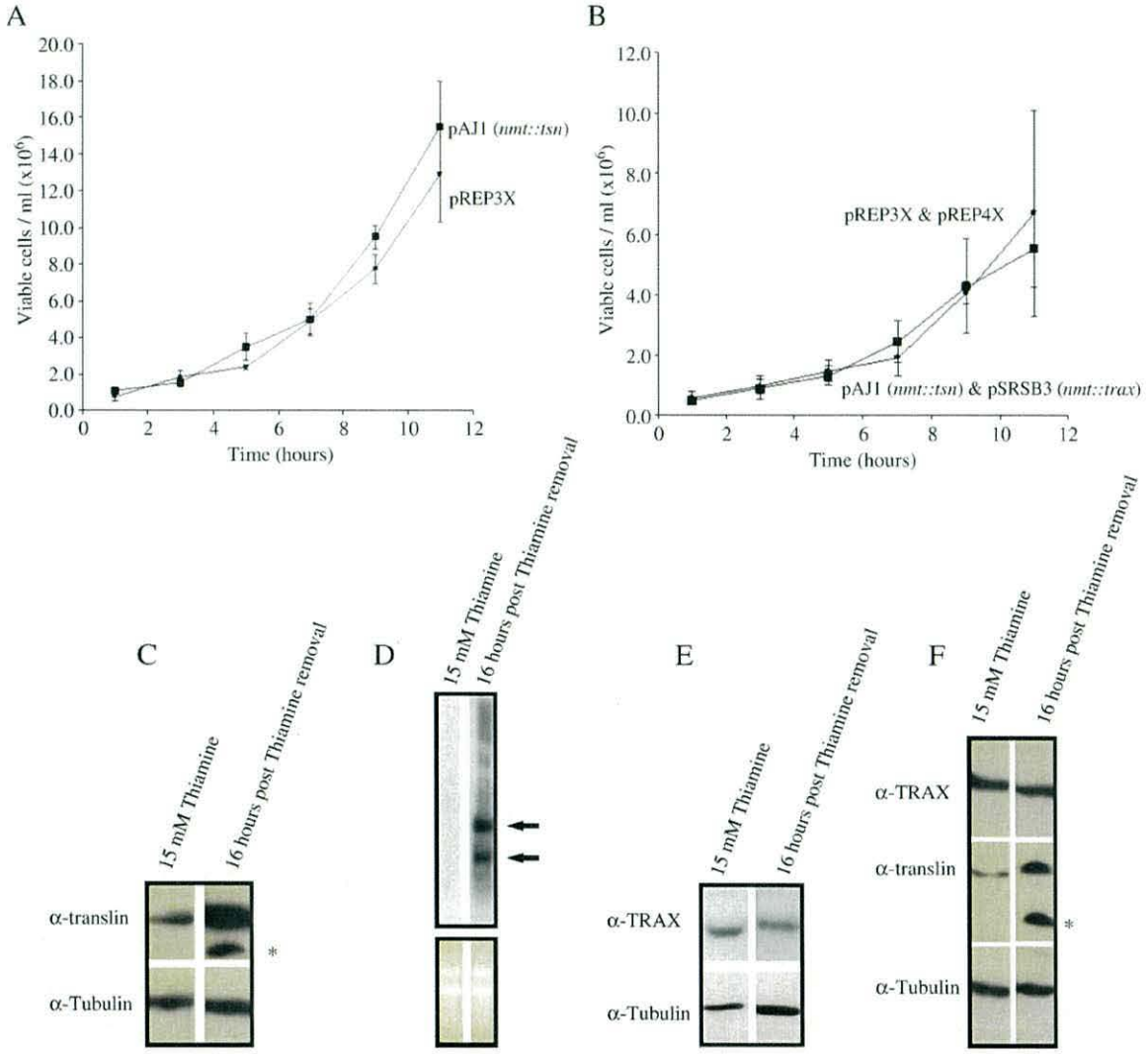

Fig. 3. Over expression of tsn^+ and $trax^+$. A. tsn^+ was over expressed on the plasmid pAJ1 under the regulation of the *nmt* promoter, which is induced after 16 h in the absence of thiamine. No difference could be detected in growth rate between pAJ1 (tsn^+) and the vector control (pREP3X). No measurable difference was noted in cell size or morphology (data not shown). Western blot analysis was carried out to demonstrate that the Tsn protein was over expressed from pAJ1 in the absence of thiamine (C; asterisk indicates a Tsn-related band which we believe to be a Tsn degradation product detectable in some WCEs). B. Plasmids with tsn^+ (pAJ1) and $trax^+$ (pSRSB3) under the regulation of the *nmt* promoter were transformed into a wild-type strain (both plasmids have different selectable markers). Under expression conditions (no thiamine) no major difference in growth rate was observed relative to the control strain containing empty vectors (pREP3X and pREP4X). No difference in cell size or morphology was noted (data not shown). Western blot analysis of WCEs from strains carrying both plasmids with regulatable tsn^+ (pAJ1) and $trax^+$ (pSRSB3) show that whilst Tsn protein is over expressed Trax is not (F). $trax^+$ expression under the regulation of the *nmt* promoter did not result in any measurable over expression of the Trax protein following 24 h in the absence of thiamine (E) when in the absence of the tsn^+ over expression (pSRSB3 alone); however, the levels of $trax^+$-specific mRNA species increases dramatically in this period without thiamine (D), indicating the *nmt* promoter is being induced at the transcriptional level (lower panel is total RNA stained with ethidium bromide, showing equal loading of total RNA). Two species of mRNAs predominate which migrate with an approximate size of 1.0 kb and 1.5 kp in cells over expressing $trax^+$ (black arrows). All growth data shown were from cultures grown at 30 °C.

3.4. The tsnΔ mutant exhibits no defect in non-homologous DNA end joining (NHEJ)

Due to the possible role of translin in generating chromosomal translocations, it has been suggested that translin might function in NHEJ. *S. pombe* mutants defective in NHEJ, such as *lig4Δ*, are similar to *tsnΔ* and *traxΔ* strains as they exhibit no measurable sensitivity to DNA damaging agents [59]. The proposal that suggests translin functions in a NHEJ pathway has not previously been formally tested. To do so we employed a NHEJ plasmid recircularisation assay. In brief, plasmids are linearised with restriction endonucleases which generate 5′, 3′ or blunt termini; these linear plasmids are then transformed into cells and the transformation frequency is used as a metric for the efficiency of plasmid recircularisation. Given that the plasmid (pFY20 in this case; 48) has no extensive internal homology, then recircularisation is either by perfect religation or a non-homologous recombination reaction, resulting in deletion of some plasmid DNA (both are referred to as NHEJ). A value for NHEJ efficiency is represented by an *L/C* ratio which is generated by dividing the transformation frequency obtained with linear plasmid DNA (*L*) with that obtained with covalently

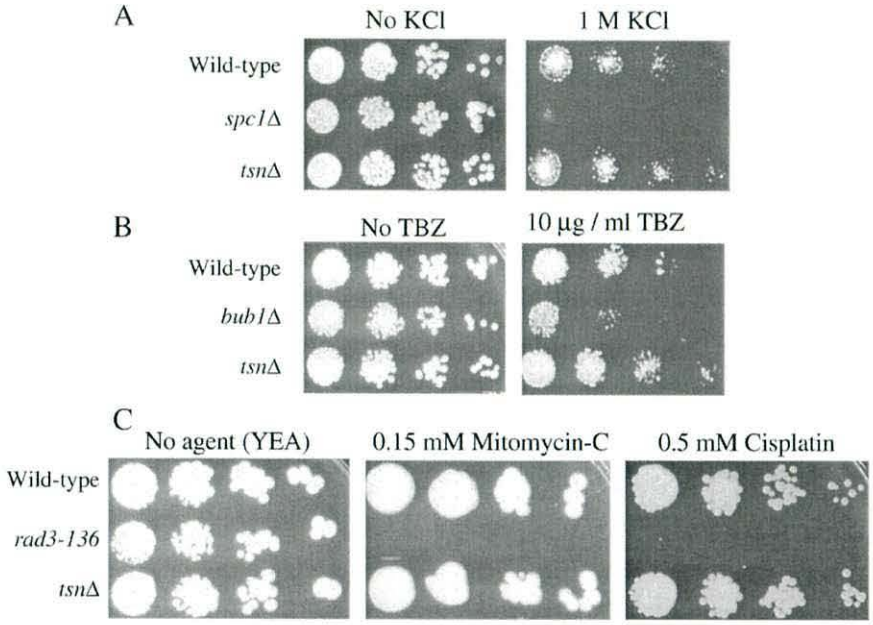

Fig. 4. Example of robust response to cellular insult by *tsn1Δ* cells. A. *tsn1Δ* cells exhibit no defect in salt (1 M KCl). *spc1Δ* cells, which are highly sensitive to 1 M KCl, are shown as a control. B. *tsn1Δ* cells exhibit no sensitivity to the microtubule depolymerising agent thiabendazole (TBZ; 10 μg/ml). The *bub1Δ* strain exhibits sensitivity to this agent and this strain is used as a positive control. C. Examples of the lack of sensitivity of the *tsn1Δ* strain to DNA damaging agents (cisplatin and mitomycin C); *rad3-136* cells are used as a positive control. All images were from plates incubated at 30 °C and all show serial dilutions of log phase cultures (10^{-2} to 10^{-5}, from left to right).

closed circular DNA (*C*) (uncut pFY20), which normalises for any strain to strain variance in transformation efficiency (rather than NHEJ efficiency). Table 2 shows that whilst the *lig4Δ* strain (known to be defective in NHEJ; [59]) has greatly impaired NHEJ, the *tsn1Δ* strain was similar to the wild-type, irrespective of the structure of the linear DNA termini. Given the depletion of Trax in the *tsn1Δ* strain (Fig. 1) the *traxΔ* strain was not tested.

3.5. The tsn1Δ mutant exhibits no defect in mitotic or meiotic homologous recombination

It has been suggested that translin functions in regulating a mitotic recombination pathway, but this remains untested. To determine whether *S. pombe* Tsn has a primary function in mitotic recombination we employed a sensitive plasmid-by-chromosome recombination assay. In brief, cells carrying the *ade6-M26* mutant allele at the *ade6* chromosomal locus are transformed with a circular plasmid carrying a second *ade6* mutant allele, *ade6-469*; the *ade6-M26* and *ade6-469* alleles were generated by point mutations which are 1332 base pairs apart and so recombination between these alleles can generate conversion of either mutation to the $ade6^+$ sequence, which can be measured genetically as adenine prototrophy. Using this system, fluctuation analyses were carried out on wild-type, *traxΔ* and *tsn1Δ* strains [51]. Fig. 5A shows that the frequency of adenine prototroph production was not significantly different between wild-type, *tsn1Δ* and *traxΔ* strains.

Translin binding sites have been detected in male meiotic recombination hot spots, suggesting a possible role for the regulation of meiotic recombination [9]. Whilst crossing over (CO) levels have previously been tested in *Drosophila* and were normal for the intervals tested [38] a role for translin in regulating gene conversions (GCs), which can occur via mechanistically distinct

Table 2
Non-homologous DNA end joining of linear DNA with 3′, 5′ and blunt termini is not impaired in translin-deficient cells

	*Pst*I (3′ over hang)		*Sma*I (blunt ended)		*Xma*I (5′ over hang)	
	Mean L/C[a, b]	% of wild-type	Mean L/C[a, b]	% of wild-type	Mean L/C[a, b]	% of wild-type
Wild-type	0.21 (0.20)	–	0.37 (0.37)	–	3.0×10^{-3} (1.0×10^{-3})	–
lig4Δ[d]	$<3.1\times10^{-4}$[c] (3.0×10^{-4})	<0.15	4.7×10^{-4} (4.7×10^{-4})	1.3	$<2.2\times10^{-4}$[c] (3.0×10^{-4})	<7.3
tsn1Δ	0.2 (0.15)	95.2	0.49 (0.27)	132.4	6.0×10^{-3} (6.0×10^{-3})	200.0

[a] The *L/C* ratio is the ratio of the number of transformants per viable cell obtained using linearised pFY20 plasmid DNA to the number of transformants per viable cell obtained for covalently closed circle pFY20 plasmid DNA.

[b] $n=3$ in all cases. Standard deviation is given in parentheses. Pair wise comparison of wild-type and *tsn1Δ* values using Student's *t*-test gave *P*-values>0.05 in all cases.

[c] No transformants were obtained; the value of 3 was used as the number of transformants obtained for the calculation of the *L/C* ratio.

[d] This strain carries the *ade6-704* allele, whereas the wild-type and the *tsn1Δ* strain carry the *ade6-M26* allele.

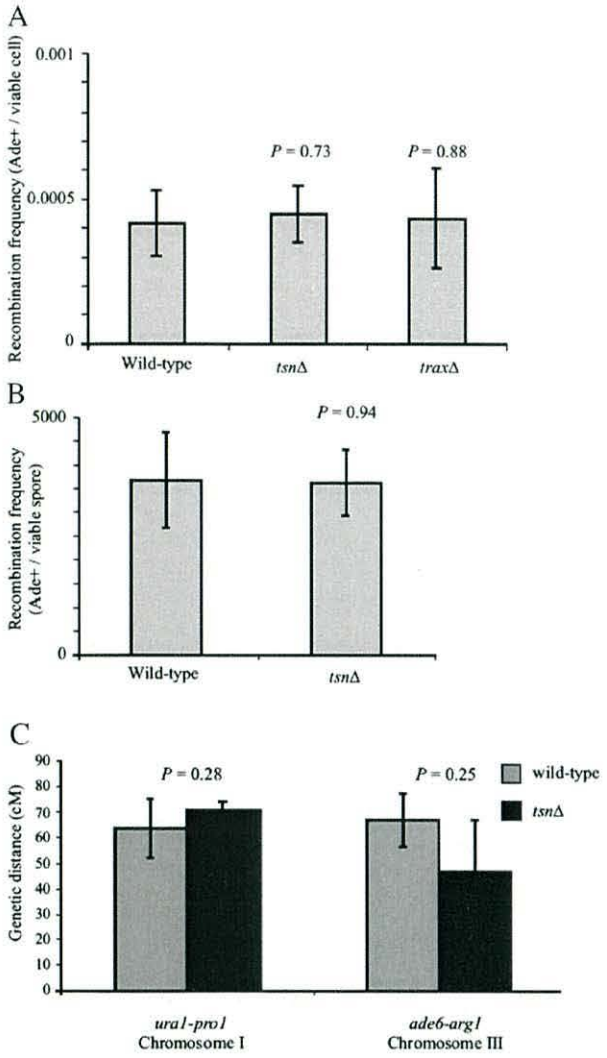

Fig. 5. *tsn1Δ* cells are not defective in mitotic or meiotic recombination. A. Mitotic plasmid-by-chromosome recombination is not reduced in *tsn1Δ* and *trx1Δ* mutants. See the main text for a brief outline of the assay system. B. Meiotic intragenic recombination is not altered in the *tsn1Δ* mutant. C. Intergenic meiotic recombination was measured at two intervals, *ura1–pro1* on chromosome I and *ade6–arg1* on chromosome III, in both wild-type and *tsn1Δ* strains. Genetic distances were calculated by use of Haldane's mapping function as described in [for example, see 53]. In all cases (A–C) error bars show standard deviations and pair wise comparison of wild-type and *tsn1Δ* genetic distances using Student's *t*-test give *P*-values which are shown within the plot.

pathways [60,61], has not been tested in any system. We tested meiotic genetic recombination, both intragenic (GC), at the *ade6* locus, and intergenic (CO), at two separate intervals. Two factor crosses using the *ade6-M26* hot spot allele and the *ade6-52* marker allele show no differences between the wild-type and the *tsn1Δ* strains (Fig. 5B); this shows there is no loss of meiotic intragenic recombination or *ade6-M26* hot spot activation.

Intergenic recombination, crossing over, was measured at the *pro1*$^+$–*ura1*$^+$ intervals on chromosome I and *ade6*$^+$–*arg1*$^+$ interval on chromosome III. These intervals were chosen to represent one locus for the different meiotic recombination regions in *S. pombe* [62], and because they are adjacent to different prominent meiotic double-strand break sites (*mbs1* for *pro1*$^+$–*ura1*$^+$; *ade6-M26* for *ade6*$^+$–*arg1*$^+$). Fig. 5C shows that genetic distance for these intervals are statistically indistinguishable for the *tsn1Δ* mutant relative to the wild-type.

3.6. GT microsatellite repeats remain stable in tsn1Δ and trax1Δ strains

S. pombe translin binds selectively to single-stranded d[GT]$_n$ and d[GTT]$_n$ DNA repeats [34]. Alterations to the stability of microsatellite repeat sequences can result in human genetic disease, many associated with neurological disorders [for example, see 63] and cancer [for example, see 64]. Microsatellite repeat instability has been linked to impaired mismatch repair (MMR) systems and, in *S. pombe*, GT microsatellite repeat stability is dependent upon MMR pathways [65]. We employed the system of Mansour et al. [65] to study [GT]$_8$ repeat stability in Tsn- and Trax-deficient cells to determine whether the selective binding of Tsn to d[GT]$_n$ repeats [34] reflected a function in regulation of microsatellite repeat stability. This system is

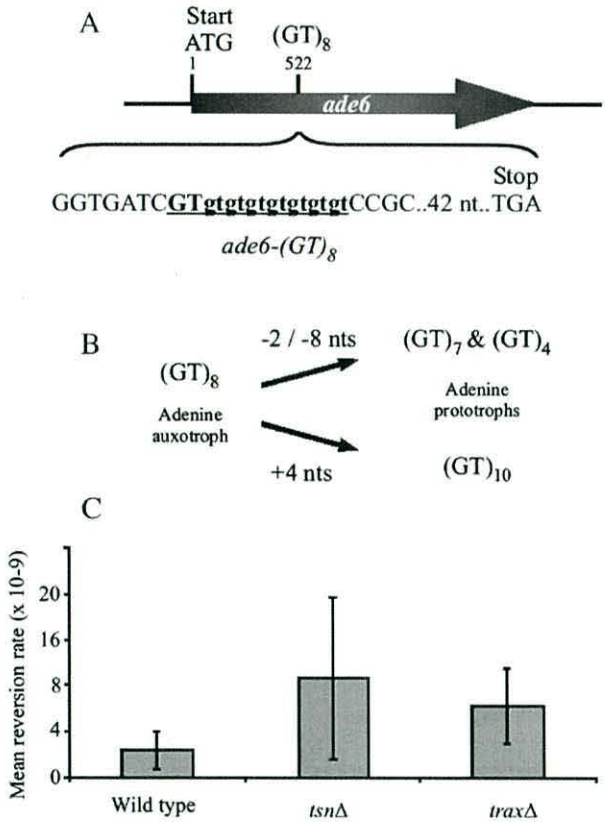

Fig. 6. *tsn1Δ* cells exhibit a small increase in GT microsatellite repeat instability. A. [GT]$_8$ repeat is integrated at position 522 within the *ade6* open reading frame [65]. B. Frame shifts, due to loss or gain of GT repeats, can result in expression of a functional Ade6 protein capable of conferring adenine prototrophy; loss of one [GT] or four [GT]$_4$ repeats or gain of two [GT]$_2$ repeats results in adenine prototrophy. C. *tsn1Δ* and *trax1Δ* cells exhibit a small, but insignificant, increase in the rate of GT microsatellite instability (Student's *t*-test gives *P*-values > 0.05 in pair wise comparisons of mutants with wild-type). Fluctuation analyses were carried out and mean reversion rates are calculated from at least three independent median values obtained from independent experiments (error bars are standard deviations).

described in detail elsewhere [65], but, in brief, it consists of eight GT repeats inserted within the *ade6* open reading frame resulting in inactivation of the *ade6* gene (Fig. 6A); change 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) (Fig. 6B).

The rate of adenine prototrophy formation for *tsn*Δ and *tsn*$^+$ cells carrying $[GT]_8$ repeats was carried out by fluctuation analysis [51]. A small rise in $[GT]_8$ instability was observed for *tsn*Δ and *trax*Δ cells (Fig. 6C), but these are not statistically significant, although a broader range of values was observed for the mutants, particularly the *tsn*Δ strain, possibly indicating a subtle underlying effect.

4. Discussion

4.1. Translin function is conserved in lower eukaryotes

The high level of conservation of the TRAX and translin proteins seems to suggest that they play an important biological role which provides a selective advantage. In both mouse and *Drosophila* the translin protein is required for the stable expression of TRAX protein [22,37,38]. We have demonstrated that whilst amino acid sequence identity between *S. pombe* murine and *Drosophila* translin proteins is 35% and 31% respectively, there is functional conservation as *S. pombe* Tsn, like murine and *Drosophila* translin, is required for stable expression of the *S. pombe* Trax protein. 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 [37,38]. Our data indicate that such regulatory interaction is highly conserved. In MEFs chemical inhibition of the proteasome results in a full restoration of TRAX levels to levels seen in translin-proficient cells [37]. By mutating an *S. pombe* proteasome sub-unit we restore some Trax, consistent with the proposal that translin functions to protect Trax from proteasome degradation. However, we do not observe restoration of wild-type levels of Trax in the *S. pombe* proteasome mutant. This might be due to the fact that the *mts3-1* conditional mutant is functionally leaky and that residual proteasome activity is sufficient to mediate some Trax degradation in the absence of translin, possibly indicative of Trax being highly labile. Alternatively, we cannot dismiss a second role for translin in positively regulating the translation of *trax*$^+$ mRNA.

Whatever the function of translin in mediating Trax levels, our data indicate that this biological process is important for both unicellular eukaryotes and metazoans. This might suggest that the conserved function does not relate to tissue-specific biological processes in metazoans as it is conserved in unicellular eukaryotes.

In this study we over expressed the *trax*$^+$ mRNA. However, this did not result in a measurable increase in the Trax protein. This suggests that the levels of Trax protein in the cell are determined by post transcriptional regulation, consistent with the proposal that translin is required for efficient translation of the *trax*$^+$ mRNA (see above). If Tsn is responsible for Trax stabilisation, then this regulation is more complex as co-over expression of Tsn in the presence of higher levels of *trax*$^+$ mRNA did not increase the measurable Trax in the cell. It might be possible that we 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 Tsn. The system we have developed here will provide a tool for further exploration of this possibility.

4.2. What is the biological function of Tsn and Trax?

In this report we have extensively explored the possibility that Trax and Tsn play a fundamental role in the maintenance of the genome integrity, meiotic/mitotic recombination and the cell growth process. We find no defects in any of these processes, other than a small increase in GT repeat instability. This can be explained in one of two ways; firstly, 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 Tsn function. It has been suggested that the neurological defects in mice and flies defective in translin [22–24], and the binding of translin to neuronal mRNAs [for example, see 13], 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. A simple explanation for this incongruity is that *S. pombe* Trax and Tsn may function in the regulation of other subsets of mRNAs and that higher eukaryotes have evolved a tissue specificity for this function. If *S. pombe* Trax and Tsn are involved in mRNA metabolism loss of function appears to result 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 findings that *S. pombe* Tsn has a greater affinity for RNA repeat sequences than DNA supports a role in RNA metabolism [34].

We have extensively studied the sensitivity of both *tsn*Δ and *trax*Δ cells to DNA damaging agents and we find no indications that either protein is required for recovery from DNA damage, dispelling the suggestion that these proteins are evolutionarily conserved due to a fundamental role in the DNA damage response. In HeLa cells mild increases in nuclear translin were observed in response to the chemotherapeutic agents mitomycin C and cisplatin [25], but here we have demonstrated that in *S. pombe* *tsn*Δ and *trax*Δ cells are as resistant to both of these agents as wild-type cells, indicating that any increase in nuclear amount of Tsn is not essential for recovery from the DNA damage these agents generate. This is consistent with later findings that both MEFs and *Drosophila* embryos defective in translin exhibit no increase in sensitivity to some DNA damaging agents relative to their translin proficient isogenic controls [37,38]. 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 [44,45]; however, we found no evidence to substantiate that proposal.

4.3. Do Trax and Tsn regulate GT repeat stability?

Whilst we observe no measurable loss of recombination function, we did note a small, increase in the instability of a $[GT]_8$ repeat sequence artificially introduced into the *ade6* locus in both mutants. Whilst this was not reproducibly statistically significant, this is largely due to the large ranges in values we obtained for the *tsn1* mutant, which exhibited a greater mean increase in instability than the *trax1* strain. This might reflect some heterogeneity within the population of *tsn1* cells with respect to the way GT repeat sequence stability is maintained. Further analysis into the nature of this broad range of GT instability in *tsn1* cells may yet uncover a role for these conserved proteins in genomic regulation.

4.4. Closing remarks

We have extensively analysed the phenotypes of *S. pombe tsn1* and *trax1* cells. We find no strong evidence to indicate that these proteins function in cell cycle regulation, recombination or DNA damage recovery. However, we do find functional conservation which suggests that these enigmatic proteins play a role in a biological process which is important for both multicellular and unicellular eukaryotes, suggesting it is of fundamental biological importance. The finding that TRAX and translin seem to regulate cell proliferation in higher eukaryotes, but not in *S. pombe*, where the biochemical function is conserved, indicates that there is not a clear correlation between the conserved biochemical function and regulation of cell proliferation, suggesting the two are not linked. Further analysis in this simple eukaryote will provide insight into the nature of this process.

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Recombination at DNA replication fork barriers is not universal and is differentially regulated by Swi1

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DNA replication stress has been implicated in the etiology of genetic diseases, including cancers. It has been proposed that genomic sites that inhibit or slow DNA replication fork progression possess recombination hotspot activity and can form potential fragile sites. Here we used the fission yeast, *Schizosaccharomyces pombe*, to demonstrate that hotspot activity is not a universal feature of replication fork barriers (RFBs), and we propose that most sites within the genome that form RFBs do not have recombination hotspot activity under nonstressed conditions. We further demonstrate that Swi1, the TIMELESS homologue, differentially controls the recombination potential of RFBs, switching between being a suppressor and an activator of recombination in a site-specific fashion.

fission yeast | genome stability | TIMELESS

Evolution is driven, in part, by genetic events that result in global changes to genomic structure. However, gross genetic rearrangements in human cells can result in genetic disease states, including cancers (1, 2). Such potentially harmful rearrangements can be mediated by distinct pathways, including homologous recombination, creating new chromosomal structures (3, 4).

Some processes, such as the conjoining of homologous chromosomes during meiosis I (5, 6), V(D)J recombination in mammals (7), and mating type switching in yeast (8, 9), require the initiation of recombination in a highly programmed fashion, where the sites of recombination and the partner choice are governed to produce specific functional outcomes. However, sites that aberrantly mediate gross chromosomal rearrangements are not programmed to initiate recombination; rather, they have some inherent instability or become unstable because of exogenous factors, generating an unscheduled recombinogenic potential (3, 10). Studies in yeast aimed at identifying naturally occurring fragile chromosomal regions have found that such sites can be complex in nature and can consist of aggregates of distinct genetic elements, including transposons, LTRs of transposons, and tRNA genes (11, 12). Interestingly, these sites exhibit higher levels of instability when DNA replication is compromised (11–13), suggesting that the inherent instability of these sites is related to DNA replication. This hypothesis is consistent with the proposal that perturbations in DNA replication may be among the primary oncogenic stresses in tumor formation (14). It is proposed that these regions provide particularly poor substrates for the replication machinery, making them more susceptible to failures of DNA replication fork progression that 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 replication fork barrier (RFB) activity that is thought to arise from the replication machinery, the replisome, colliding head-to-head with RNA polymerase III, which mediates tRNA gene transcription (15).

Transcription-associated recombination (TAR) initiated via collisions of RNA polymerase with the replisome has also been suggested as a potential source of instability for genes transcribed by RNA polymerase II (16, 17); however, such encounters between RNA polymerases and the replisome are relatively common occurrences within S-phase of the cell division cycle, so highly effective mechanisms must exist to prevent the generation of recombinogenic lesions. The facts that replisome stalling is not normal when RNA polymerase II collides head-to-head with the replisome in the genome and that eukaryotic RNA polymerase II-associated TAR has been reported only for plasmid-based transcription support the existence of such mechanisms (17–19). In budding yeast, Mec1 checkpoint kinase activity has been shown to be required to prevent so-called "replication slow zones" from becoming unstable (20). This function of the Mec1 signaling pathway seems to be conserved, because loss of mammalian Ataxia Telangiectasia and Rad3 related (ATR) (the mammalian Mec1 orthologue) increases the instability of fragile sites (21). Mec1 and, by extension, ATR are required to promote replication fork progression, indicating that these proteins are a fundamental part of genome duplication during normal proliferation (22–24).

In the fission yeast, it has been demonstrated that a defined RFB, the *RTS1* element from the *mat* locus (25, 26), can serve as a mitotic recombination hotspot in a RFB-dependent fashion (27, 28), providing firm evidence that RFBs in mitotically dividing cells can drive genetic change. The RFB activity of the fission yeast *RTS1* element has been shown to be dependent on a number of *trans*-acting proteins, 2 of which, Swi1 and Swi3, are the homologues of human TIMELESS and TIPIN, respectively (25). When Swi1 function is lost, so too is the RFB activity of the *RTS1* element (25). Loss of Swi1 also results in the loss of the *RTS1* RFB-mediated recombination activity, consistent with the existence of a direct link between RFB activity and recombination (27, 28). Although this observation might suggest that Swi1 serves as a mediator of recombination, this view is countered by the fact that loss of Swi1 function results in an elevation of recombination in an assay system devoid of a specific, strong RFB (29).

The Swi1 and Swi3 homologues in *Saccharomyces cerevisiae*, Tof1 and Csm3, respectively, have been shown to be intimately associated with the replisome in a complex known as the "replisome progression complex" (RPC) (30). This association suggests a model in which Swi1 (Tof1/TIMELESS) functions within the RPC to monitor the status of the chromosomal traffic ahead of the replisome so as to modulate by some means the response of the replisome to potentially problematic factors. Consistent with this view, Swi1/Swi3 have been shown to maintain replication fork stability and S-phase checkpoint activation (29, 31, 32).

In this study we used the fission yeast model to demonstrate that different eukaryotic RFBs have different recombinogenic potential and to show that the RPC component, Swi1, functions

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GENETICS

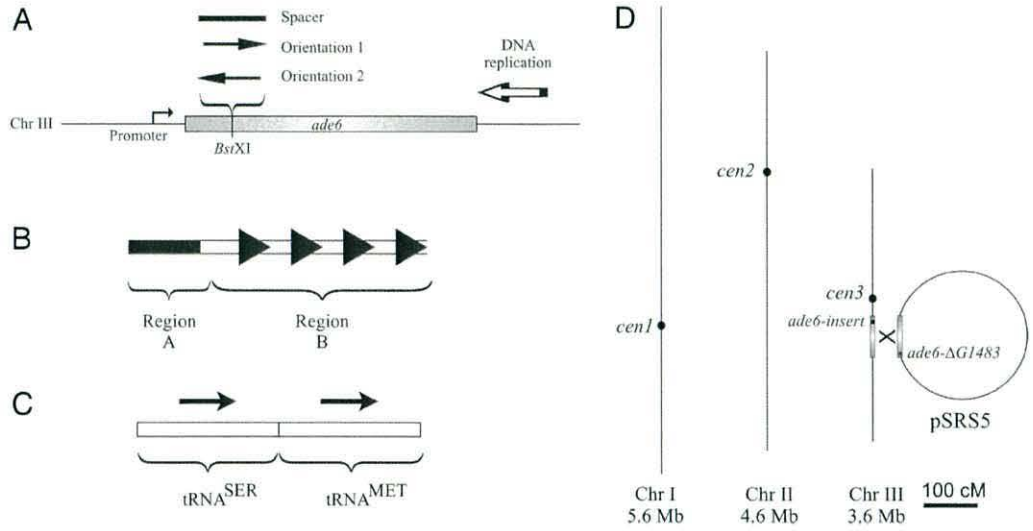

Fig. 1. Schematic representation of the systems used to monitor the recombination potential of distinct genetic elements. (*A*) Genetic elements *RTS1*, *sup3-e*, or $tRNA^{GLU}$ were inserted into the *ade6* ORF (*open rectangle*) at the BstXI site. Elements were inserted into this site in both orientations independently, as indicated by the black arrows above the BstXI site. Two distinct spacer controls, consisting of origin-free stretches of the *his3* ORF, were inserted independently at this site. The *ade6* ORF is expressed from left to right; the angular arrow indicates the promoter. The large open arrow indicates the predominant direction of DNA replication. (*B*) A schematic representation of the *RTS1* element (25). The element consists of 2 regions, region A, which interacts with Rtf2 protein, and region B, which is made up of 4 repeats (*black arrowheads*) and interacts with Rtf1 proteins. Both Rtf1 and Rtf2 are required for RFB activity (25). The direction of the black arrowheads indicates the polarity of the *RTS1* barrier. An RFB is generated when the replication fork approaches region A first (i.e., from left to right in the diagram). (*C*) A schematic representation of the *sup3-e* element. This element is made up of 2 tandemly arranged tRNA genes, $tRNA^{SER}$-$tRNA^{MET}$. They are co-transcribed using the regulatory elements of the $tRNA^{MET}$ gene, and a mature suppressor, $tRNA^{SER}$, is produced. Black arrows indicate the direction of transcription. Orientation 1 would be expected to generate a head-to-head collision between the replisome and RNA polymerase III. (*D*) Plasmid-by-chromosome intermolecular recombination assay. The 3 chromosomes of *S. pombe* are represented by the thin vertical lines. The wild-type *ade6* locus is located at a centromere (*cen1*) proximal position on chromosome III. The inserts generated in the *ade6* ORF (depicted in *A*) are located at this position on the chromosome in distinct strains. A second *ade6* allele, *ade6-Δ1483*, was created within the plasmid (pSRS5). This *ade6* allele has a mutation at a 3′ position within the gene distal to the BstXI site into which the test elements were inserted (see *Materials and Methods*). Gene-conversion events between the plasmid borne *ade6* allele and the chromosomal borne *ade6* allele (the genetic element being tested) result in adenine prototrophs. The frequency of prototroph production represents recombination frequency.

differentially to control the recombinogenic potential of different sites in a site-specific fashion.

Results

Distinct RFBs Have Different Recombination Potentials. The fission yeast *RTS1* element is a polar RFB, and previous work has demonstrated it can function as a polar, intrachromatid mitotic recombination hotspot (27). To test whether a single *RTS1* RFB can drive intermolecular homologous recombination, we established a plasmid-by-chromosome recombination reporter system based on a system we have used previously (33). Briefly, we introduced the *RTS1* element into the ORF of the *S. pombe ade6* gene at the BstXI site (Fig. 1*A*), thereby rendering the strains auxotrophic for adenine. The *RTS1* element (Fig. 1*B*) was inserted in both orientations in distinct strains. A third strain was created with a spacer control that comprises an origin-free stretch of DNA. The predominant direction of DNA replication through the *ade6* locus is from a centromere-distal origin, so the majority of passive DNA replication of this locus is from this direction (Fig. 1*A*) (34–36). Consequently, only *ade6::RTS1-orientation-2* should result in an *RTS1*-mediated RFB, because of the polar nature of *RTS1* (Fig. 1*B*), which we shall refer to as the "barrier orientation." Second, we transformed these strains with a plasmid, pSRS5 that carries an *ade6* allele with a mutation engineered at the 3′ end of the gene, distal to the site into which the *RTS1* element was inserted (Fig. 1*D*). (pSRS5 also has an *ars1* element enabling it to replicate autonomously in fission yeast.) The 3 strains generated were then subjected to fluctuation analysis to obtain a recombination frequency (adenine prototrophs per million viable cells).

Fig. 2*A* shows that the spacer control and *RTS1* inserted in the orientation that does not create a RFB (orientation 1) gave similar levels of intermolecular recombination. However, *RTS1* inserted in the barrier orientation (orientation 2) resulted in considerable stimulation of intermolecular recombination. We then carried out 2D gel electrophoresis on total cellular DNA to determine whether we observe the expected RFB for *RTS1* in the barrier orientation (orientation 2) but not for *RTS1* in the opposite orientation or for the spacer control. Fig. 2*D* (*Left pair*) shows that a strong RFB is observed for the barrier orientation (orientation 2) but not for the opposite orientation (orientation 1). Quantification of triplicate gel sets demonstrated that *RTS1* in the barrier orientation generated a RFB with a pause intensity of more than 1 order of magnitude greater than the nonbarrier orientation (orientation 1) (Fig. 2*E*, *2 Left columns*).

Previously, it has been shown that tRNA genes can generate strong polar RFBs (15). To test the universality of RFB-mediated recombination hotspot activity for other RFBs, we generated new strains with a tRNA gene cassette, *sup3-e*, in place of the *RTS1* element within the *ade6* gene (Fig. 1*A*). *sup3-e* is a double-tRNA gene that consists of tandemly arranged serine and methionine tRNA genes that can suppress UGA (opal) codons by insertion of a serine residue (Fig. 1*C*) (37), and it has been used previously to demonstrate that tRNA genes maintain genomic sequence conservation via intergenic gene conversion (38–40). The use of the suppressor tRNA gene permitted us to check for tRNA gene expression by monitoring the suppression of a stop codon in a marker gene. The *sup3-e* cassette was inserted in both orientations into the BstXI site within the genomic *ade6* locus, as done for the *RTS1* constructs (Fig. 1*A*).

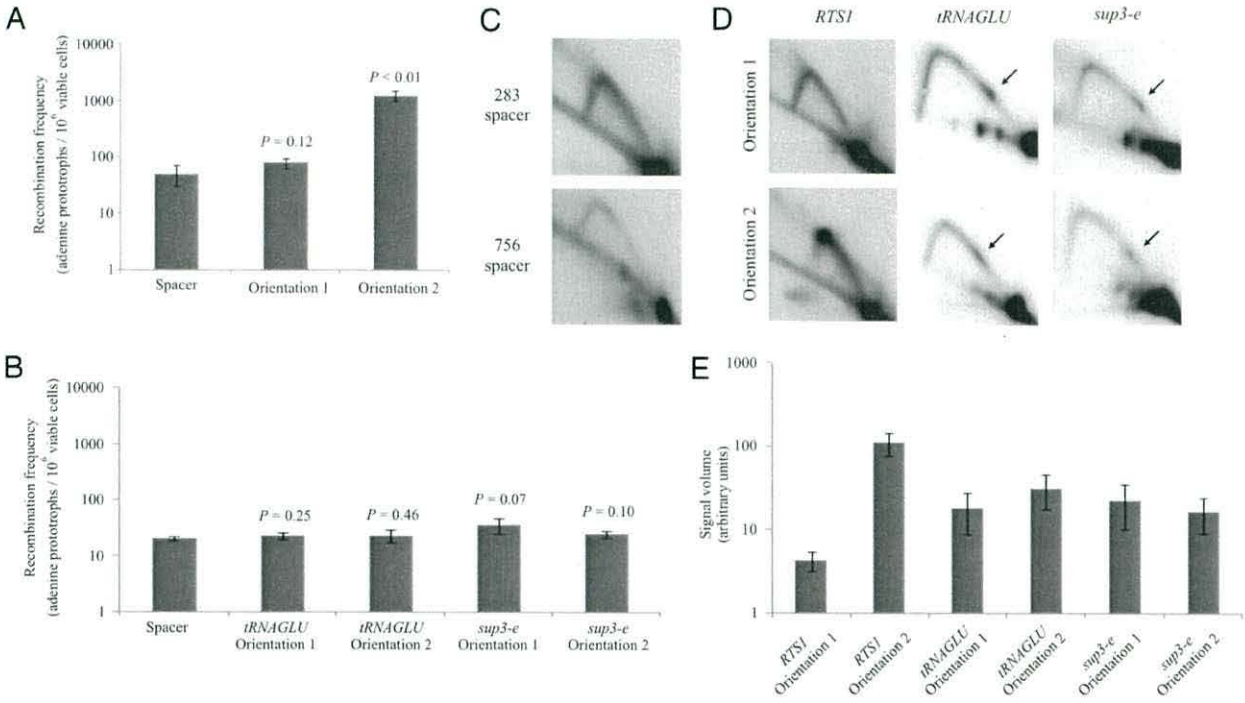

Fig. 2. Differential mitotic intermolecular recombination hotspot activity of DNA replication fork barriers. (*A*) *RTS1* is an orientation-dependent intermolecular mitotic recombination hotspot. *RTS1* in orientation 2 generates a recombination frequency almost 2 orders of magnitude higher than in either orientation 1 or the space control. (*B*) tRNA genes do not generate mitotic intermolecular recombination hotspots. Mean recombination frequencies for $tRNA^{GLU}$ and *sup3-e* in both orientations are indistinguishable from the mean recombination frequency obtained for the spacer control. *P* values are derived from Student's *t* test of pairwise comparisons of the spacer control and the individual elements. (*C* and *D*) RFB activity of *RTS1* and tRNA genes in a *swi1*$^+$ background. Two-dimensional DNA gel electrophoresis and Southern blotting were used to analyze DNA replication intermediates for the *ade6* locus of strains with the genetic elements inserted. (The strains used for this analysis did not carry the plasmid pSRS5.) Neither control element (*his3*$'_{283}$/*his3*$'_{756}$) generates an RFB. Both *RTS1* (orientation 2) (*D, Bottom left*) and the tRNA gene elements (*D, Middle* and *Right*) generate RFBs (*arrows*). (Note: the restriction enzymes used in the analysis of the *RTS1* and tRNA gene elements differed because of the different sequences of the element. This difference results in the RFB being located at distinct positions on the replicative Y arc, as is also the case in Fig. 3*D*.) *P* values are obtained from Student's *t* test of pairwise comparisons between the values for the spacer control and individual elements ($n \geq 3$ in all cases). (*E*) Quantification of RFB intensity. *RTS1* barrier orientation (orientation 2) results in an RFB of significantly greater intensity than the nonbarrier control (*Extreme left*). All tRNA gene elements generate RFBs of uniform intensity that are significantly less intense than the *RTS1* orientation 2 RFB. Values are obtained from 3 independent gels. Error bars represent SD.

A new spacer control insert was generated also, because the *sup3-e* element is smaller than *RTS1*. These strains were also transformed with the plasmid carrying a recombination marker allele of *ade6*, pSRS5, and were subjected to fluctuation analyses (Fig. 2*B*). Unlike *RTS1, sup3-e* does not generate an intermolecular recombination hotspot (Fig. 2*B*). Because this observation is distinct from that observed for *RTS1*, and to dispel the possibility that *sup3-e* is unique and not representative of more general RNA polymerase III transcribed elements, we created a further strain pair in which a single tRNA gene, $tRNA^{GLU}$, was inserted individually in both orientations into the BstXI site within the *ade6* ORF. (The spacer control is the same as that used for *sup3-e*, because the single tRNA gene was inserted within an identically sized DNA fragment.) The $tRNA^{GLU}$ gene behaved in a fashion identical to *sup3-e*, with no measurable recombination hotspot activity in either orientation. Next we subjected these strains to 2D gel electrophoresis and found that we could detect RFBs of uniform intensity (as quantified from triplicate gels) for both *sup3-e* inserts and both $tRNA^{GLU}$ gene inserts (Fig. 2 *D* and *E*), indicating that the tRNA genes generate nonpolar RFBs, although the intensity of these RFBs was significantly less than that of *RTS1* in the barrier orientation (Fig. 2*E*).

Swi1 Is an Element-Specific Regulator of DNA Replication-Associated Intermolecular Recombination. *RTS1* RFB activity is dependent on Swi1. To determine whether Swi1 is required for RFB-associated intermolecular recombination, we tested plasmid-by-chromosome recombination levels in a Swi1-deficient strain. When Swi1 function is lost, the orientation-dependent stimulation of intermolecular recombination by *RTS1* is lost (Fig. 3*A*), consistent with the data for intramolecular recombination (27). Also, as reported previously (25), the strong RFB activity of *RTS1* is lost in the Swi1-deficient strain (Fig. 3*D*, *left-hand pair*).

We then constructed *ade6*::*sup3-e* and *ade6*::$tRNA^{GLU}$ strains that were Swi1-deficient and carried the test plasmid. We carried out fluctuation analyses on these strains to quantify the recombination frequency. Fig. 3*B* shows that when Swi1 function is lost, *sup3-e* and $tRNA^{GLU}$ generate orientation-independent mitotic intermolecular recombination hotspots that are not apparent in the wild-type strain. This observation demonstrates that, when the RPC is perturbed, tRNA genes become a source of genetic instability.

No Correlation Between Recombination Levels and RFB Intensity. The observation that Swi1 functions to suppress tRNA gene-induced recombination hotspot activity led us to conclude that Swi1 functions to permit the replisome to replicate through sites of RNA polymerase III transcription without generating substrates for recombination. We hypothesized that in the absence of Swi1 function(s), DNA replication would be disrupted significantly when RNA polymerase III collision with the replisome results in a significantly enhanced RFB. To test this hypothesis, we carried out 2D gel electrophoresis analysis of DNA extracted from

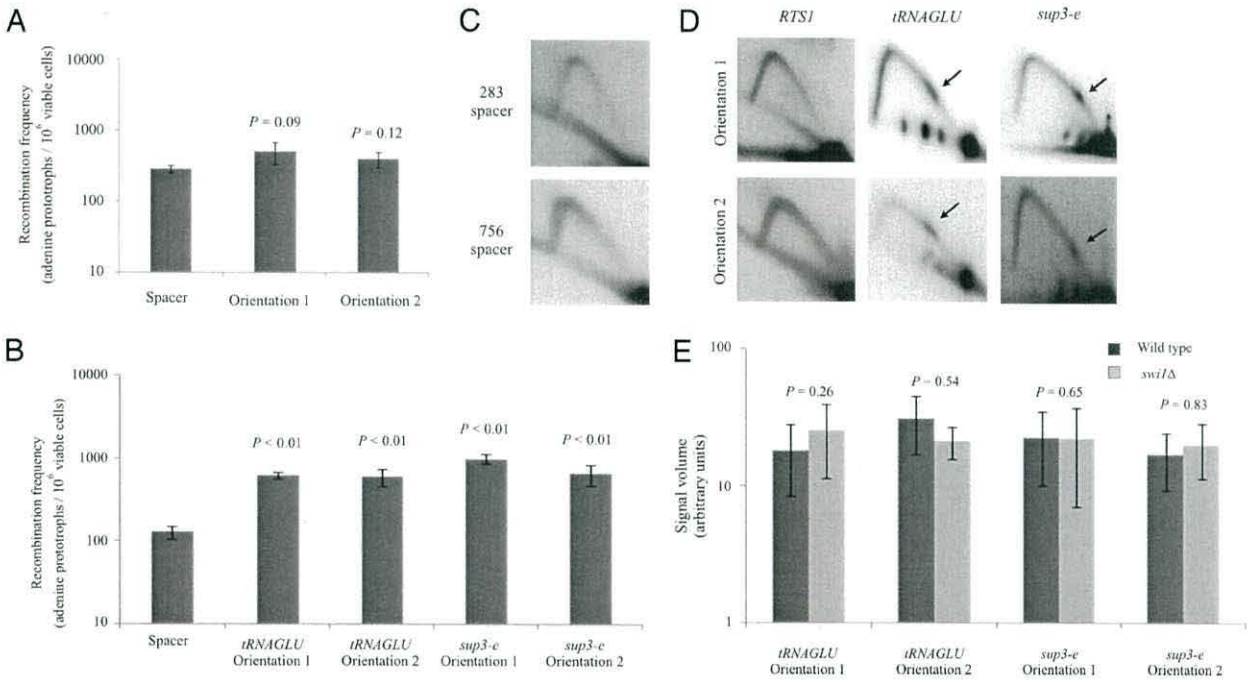

Fig. 3. Swi1 differentially regulates the recombination potential of distinct RFBs. (*A*) Intermolecular recombination frequencies for *RTS1* in a *swi1*Δ background. The orientation-dependent recombination hotspot activity for *RTS1* is lost when *swi1* is mutated, indicating that *swi1* is required for RFB-associated hotspot activity. (*B*) Loss of Swi1 function results in tRNA genes becoming orientation-independent, intermolecular mitotic recombination hotspots. In a *swi1*Δ mutant background all tRNA gene elements become mitotic recombination hotspots. *P* values are obtained from Student's *t* test of pairwise comparisons between the values for the spacer control and individual elements ($n \geq 3$ in all cases). (*C*) Spacer controls do not exhibit any RFB activity in a *swi1*Δ mutant background. (*D*) The polar RFB activity of *RTS1* is lost in the *swi1*Δ mutant (*Bottom left*). Conversely, tRNA gene-containing elements retain RFB activity in the *swi1*Δ mutant (*arrows*). (*E*) RFB intensities for tRNA genes do not alter in the *swi1*Δ mutant. Quantification of RFB activity for all tRNA gene elements demonstrates no significant difference between *swi1*$^+$ and *swi1*Δ strains. All values have bee derived from 3 independent gels. *P* values are obtained from Student's *t* test of pairwise comparisons between *swi1*$^+$ and *swi1*Δ for each element. Error bars represent SD.

Swi1-deficient strains containing *sup3-e* and *tRNA*^{GLU} elements (Fig. 3*D*, *Middle and Right pairs*). Quantification of the replication pauses did not identify any significant change in RFB intensity for either *sup3-e* or *tRNA*^{GLU} in a *swi1*Δ mutant background (Fig. 3*E*), indicating that the increase in recombination is not concomitant with an increase in pause intensity (Fig. 3 *B* and *E*) and suggesting that there is no direct relationship between RFB intensity and recombination.

Discussion

Eukaryotic chromosomes do not have uniform levels of stability along their length, and there are fragile sites that are more susceptible to initiating genetic change, a feature that is, paradoxically, conserved (10). However, a uniform feature of genomes that directly affects their stability is that they must be replicated correctly. To ensure this outcome, the replication machinery must be capable of responding to different features of the genomic landscape. In this study we have demonstrated that in fission yeast the TIMELESS homologue, Swi1, a component of the RPC, functions differentially to regulate genome stability, switching between a stimulatory and inhibitory role for replication-associated recombination in a site-specific fashion. We demonstrate that DNA replication-associated fragile sites are not determined simply by replication perturbation alone.

Different Genomic Elements Possess Different Recombinogenic Characteristics. Previous studies have shown that DNA replication-associated fragile sites can contain tRNA genes, implicating these elements in the generation of recombinogenic lesions (11, 20). Also, tRNA genes use gene-conversion mechanisms to maintain their genome-wide sequence uniformity, suggesting they have recombination-initiating potential (38–40). Because tRNA genes have been demonstrated to generate RFBs (15), it is tempting to speculate that collisions of RNA polymerase III and the replisome result in instability of the replication fork. In the case of *sup3-e* and *tRNA*^{GLU}, however, we observed no stimulation of recombination in the wild type, despite clearly measurable RFB activity (Fig. 4 *C* and *D*). Because RNA polymerase III is responsible for the transcription of many different species of RNA from the genome template (41), this finding is consistent with a model that proposes that RNA polymerase III generally does not serve as a significant recombination-initiating factor during S-phase.

This observation is in stark contrast to the high levels of recombination observed for the *RTS1* element (Fig. 4*A*) and indicates that different RFBs have very different recombinogenic potential. Moreover, these data demonstrate that there is not a simple, linear relationship between RFB intensity and recombination.

The Replisome Progression Complex Is a Regulator of the Relative Fragility of a Genomic Locus. Here we have demonstrated 2 fundamentally opposing activities of the RPC in response to distinct RFBs (Fig. 4). On the one hand, in *RTS1* the RPC component Swi1 is absolutely required for RFB activity and the associated recombination (Fig. 4 *A* and *B*). For *sup3-e* and *tRNA*^{GLU}, however, Swi1 functions either to prevent the RFB being processed into recombination-initiating lesions (i.e., stabilization of replication forks) or to prevent any such lesions being processed into recombination products (Fig. 4 *C* and *D*). The function of Swi1 that determines this distinction between different RFBs remains unknown, but this protein has been

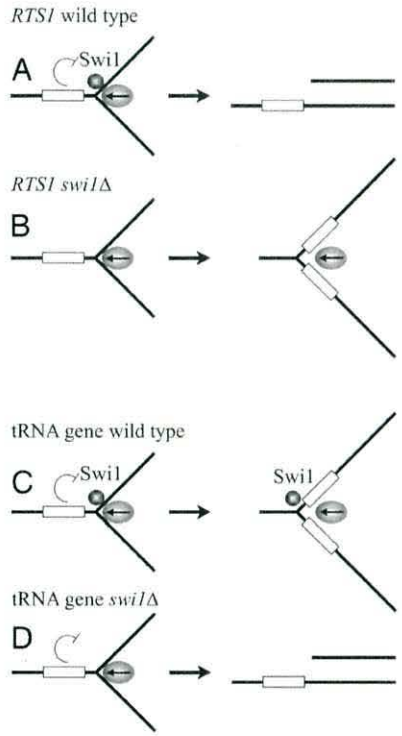

Fig. 4. Model for differential regulation of distinct RFBs. (*A*) *RTS1* barrier orientation requires Swi1 function (*small sphere*) for barrier activity (*half circle*) and the generation of recombinogenic lesions (most likely 1-sided double-stranded break; ref. 27). The open rectangles represent the appropriate *cis* element. The ovals containing an arrow represent the replisome, with the arrows indicating the direction of replisome progression. (*B*). On loss of Swi1 function, *RTS1* barrier activity is lost, and recombinogenic lesions are not stimulated. (*C*) tRNA genes generate a lower-intensity Swi1-independent RFB. RFB activity is transient, and no stimulation of recombinogenic lesions is apparent. Swi1 is required to prevent the RFB from creating recombinogenic lesions. (*D*) Loss of Swi1 function does not impair the replisome's ability to pause in response to the tRNA gene, but it does result in the pause becoming genetically less stable, with elevated levels of recombinogenic lesions being generated. (The nature of these lesions remains unknown; a 1-sided double-stranded break is shown for illustration.)

implicated in the maintenance of replication fork stability and S-phase checkpoint function. The observation that recombination is increased at tRNA genes on loss of Swi1 function is similar to the observation that loss of Mec1/ATR checkpoint activity increases instability at fragile sites (20), perhaps suggesting that there is a link to a Swi1 checkpoint function (29, 31, 32). Links between genotoxic stress regulation and circadian control have been known for some time (42). Given the homology between Swi1 and TIMELESS, our findings indicate an association between genome instability induced by DNA replicative stress and circadian regulation control, consistent with the fact that human TIMELESS has previously been associated with the DNA replication and checkpoint machinery (reviewed in ref. 42).

The fact that Swi1 functions very differently when the replisome encounters tRNA genes might suggest that the more widespread function of Swi1 is to suppress unwanted recombination at RFBs throughout the genome and that the function at *RTS1* is a unique activity that has evolved to mediate events at a highly specialized site. An alternative view is that *RTS1* generates such a strong RFB that a tipping point has been reached beyond which Swi1 no longer has the ability to maintain replication fork stability, a proposal supported by the relative intensities of the distinct RFBs (Fig. 2*E*). This idea might provide an attractive explanation of why some *S. cerevisae* fragile sites contain multiple elements capable of blocking DNA replication, including tRNA genes (11). Some of these sites, however, become fragile only when the checkpoint pathways are perturbed (20), indicating that complex sites can be endured during normal S-phase and that the relationship between the level of the replicative blockage and fragility is not simple. A more general role for Swi1 in suppressing recombination at milder RFBs is supported by the observation of elevated intramolecular recombination in the absence of specified RFBs in a *swi1*Δ mutant (29).

Previous studies have demonstrated that tRNA genes provide a polar RFB (15). Here we show that both *sup3-e* and $tRNA^{GLU}$ function as orientation-independent, nonpolar RFBs (Figs. 2*D* and 3*D*). This finding again indicates further complexity, because distinct elements have distinct RFB characteristics, and chromosome context may influence this difference.

Finally, others have previously reported Swi1-/Swi3-independent RFB in the rDNA locus of *S. pombe* (43). Here we show that *sup3-e* and $tRNA^{GLU}$ located within the *ade6* ORF provide further examples of Swi1-independent RFBs that function as recombination hotspots in the absence of Swi1 function.

Closing Remarks. Previously, it has been reported that RFBs can serve as mitotic recombination hotspots. Here we demonstrate that this function is not a universal feature of all eukaryotic RFBs. Indeed, we 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. Consistent with this idea, natural fragile sites in the budding yeast genome are complex and are not generated by simple RFB elements alone. Last, we demonstrate that a key component of the RPC, Swi1 (*Sc*Tof1/*Hs*TIM), functions differentially to regulate the outcome of an encounter between a RFB and the replisome in a site-specific fashion. These findings expose a new level of complexity to the study of genome instability associated with perturbation of DNA replication.

Materials and Methods

Yeast Strains and Plasmids. Table S1 lists the strains used in this study. Culture media, strain storage, and *S. pombe* transformation were as described in by Moreno et al. (44). For a detailed description of the construction of the modified *ade6* alleles and the plasmid pSRS5, see SI *Materials and Methods*.

Determination of Recombination Frequency. Recombination frequency was determined using fluctuation analysis. Briefly, cells were plated on appropriate solid medium at a dilution that gave well-dispersed single colonies. Colonies were permitted to grow to no greater than 1 mm in diameter. At this stage the whole colony was placed in 5 ml of appropriate liquid medium and incubated with shaking at 30 °C until the early stationary phase. Serial dilutions of cultures were made and plated out onto yeast extract agar (YEA) and YEA containing 20 mg/ml guanine (pH 6.5), which prevents the uptake of adenine because of purine antagonism (45), or onto selective pombe minimal glutamate medium (with and without adenine). Plates were incubated at 33 °C for 3 days before counting. Recombination frequencies were determined from colony counts. For each strain to be tested, the recombination frequencies of 7 independent cultures were measured, and the median value was used. This process was repeated a minimum of 3 times for each strain to be tested, and mean values of the median values were generated. *P* values were generated by pairwise comparisons using Student's *t* test.

Analysis of DNA Replication Intermediates. For a detailed description of DNA purification and 2D gel electrophoresis protocols, see SI *Materials and Methods*.

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