Host factors and environmental stimuli that regulate *Schizosaccharomyces pombe* LTR retrotransposons

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Declaration

I certify that this thesis is my own work, except where acknowledged, and has not been previously submitted for a degree for any other qualification at this or any other University.

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Abstract

The fission yeast, *Schizosaccharomyces pombe* is host to the *Tf2* family of long terminal repeat (LTR) retrotransposons. Uncontrolled propagation of these elements is a potential threat to genomic integrity and therefore their expression and mobilization is subjected to strict control. To analyse the host factors and environmental stimuli that influence the propagation of *Tf2* elements, an assay was employed that allows the mobilization frequency of a marked endogenous element (*Tf2-12natAI*) to be monitored. Using this assay it was determined that a high copy number of *Tf1*, a related *Ty3*/gypsy LTR retrotransposon, can stimulate *Tf2* mobilization via a post-transcriptional mechanism that is dependent upon *Tf1*-encoded proteins. Indeed, the data suggest that *Tf2* can hijack the propagation mechanisms of a *Tf1* for its own use. During these studies it was discovered that the composition of the growth medium has a major impact upon *Tf2* activity. Cell culture in minimal (EMM) medium, instead of rich (YE5S) medium, resulted in increased *Tf2* expression and mobilization. The increased level of *Tf2* activity resulted from some specific components of EMM medium, namely ammonium (NH_4^+) and phthalate ions. The finding that the growth medium influences *Tf2* activity also prompted analysis of TORC signalling cascades which are master regulators of cellular responses to environment. Both the expression and mobilization of *Tf2* elements was activated in response to exposure to rapamycin, a drug that forms a complex with the FKBP12 protein (Fkh1) and inhibits the activity of the TORC1 complex. This suggested that *Tf2* activity is under the TORC1 control but surprisingly, the inhibition of TORC1 using a *tor2* temperature sensitive allele or a direct chemical inhibitor (Torin1) did not activate the expression of *Tf2* elements. Therefore, rapamycin influences the expression of *Tf2* elements via a TORC1-independent pathway. This pathway was found to be dependent upon the FKBP12 protein Fkh1, the forkhead transcription factor Fhl1, a putative co-activator protein Crf1 and the Pka signalling pathway.

Abbreviations

ADP	Adenosine di-phosphate
ATP	Adenosine tri-phosphate
bp	Base pairs
CA	Capsid
cDNA	Complementary DNA
ClonNAT	Nourseothricin
dH_2O	Deionised water
DNA	Deoxyribonucleic acid
dNTPs	Deoxynucleotides
dsRNA	Double stranded RNA
Env	Envelope
ERV	Endogenous retrovirus
Gag	Group antigen
HERV	Human endogenous retrovirus
HIRA	Histone regulatory homologue A
IN	Integrase
kb	Kilo base
kDa	Kilo Dalton
LINE	Long interspersed nucleotide element
LTR	long terminal repeat
miRNA	micro RNA
mRNA	Messenger DNA
NC	Nucleocapsid
ncRNA	Non-coding RNA

nt	Nucleotide
ORF	Open reading frame
PCR	Polymerase chain reaction
Pol	Polyprotein
rDNA	Ribosomal deoxyribonucleic acid
RNA	Ribonucleic acid
RNA pol II	RBA polymerase II
RNaseH	Ribonuclease H
RT	Reverse transcriptase
RT-PCR	Reverse transcription polymerase chain reaction
SEM	Standard error of the mean
SINE	Short interspersed nucleotide element
siRNA	Small interfering RNA
ssDNA	Single stranded DNA
SVA	SINE-R, VNTR, *Alu*
TE	Transposable element
Tf	Transposon of fission yeast
tRNA	Transfer ribonucleic acid
TSD	Target site duplication
Ty	Transposon of yeast
U3	Unique in the 3’ end
U5	Unique in the 5’ end
UTR	Untranslated region
VLP	Virus like particle
VNTR	Variable number of tandem repeats

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Chapter 1 Introduction

1.1 Transposable elements

Genomes are not entirely static but undergo constant remodelling by mobile genetic elements which have the ability to move from one location to another in the genome. This was initially discovered by Barbara McClintock though her research in maize (McClintock, 1950). Since then, mobile genetic elements, or transposable elements (TE) have been found in most organisms. Transposable elements have historically been defined as genome parasites or 'junk DNA' as their mobilization was believed only to benefit their own survival (Doolittle and Sapienza, 1980; Orgel *et al.*, 1980; Hickey, 1982). More recently, it has become apparent that transposons have helped to shape the structure and function of genetic material. Indeed, there are numerous examples where host cells have co-opted transposon DNA or proteins for their own use (Voytas, 2008; Mita and Boeke, 2016).

TEs may comprise significant portions of eukaryotic genomes; more than 50% in some plants (SanMiguel *et al.*, 1996), 40% in human (Lander *et al.*, 2001; Deininger and Roy-Engel, 2002), 12% of *Caenorhabditis elegans* (Consortium, 1998), 3.1% in *Saccharomyces cerevisiae* (Kim *et al.*, 1998) and 1.1% in *Schizosacchaomyces pombe* (Bowen *et al.*, 2003). TEs need to locate non-lethal sites for genomic insertion in order that they do not kill their host cell. This is relatively easy in complex genomes, such as human and other higher organisms, where little of the DNA encodes for protein. However, this can be more difficult in compact genomes, for example, yeast and bacteria (Boeke and Devine, 1998).

Eukaryote TEs are classified into three super families according to their structure and transposition mechanism (Slotkin and Martienssen, 2007; Eickbush and Jamburuthugoda, 2008) (Fig. 1.1).

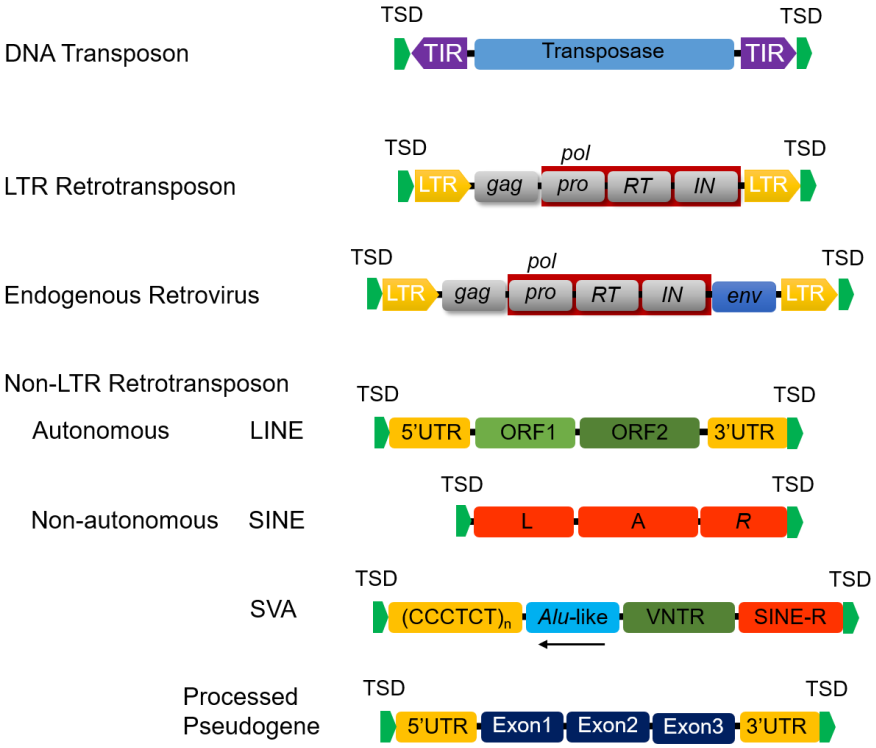

Figure 1.1 The typical structures of the different classes of eukaryote transposons.
DNA transposons encode a transposase enzyme which mediates mobilization through a cut and paste mechanism. LTR retrotransposons are functionally similar to retroviruses but lack the *env* gene. Non-LTR retrotransposons can be subdivided into autonomous elements which can mediate their own mobilization (e.g. LINEs) and non-autonomous element (e.g. SINEs) which rely on expression of a nearby element. Abbreviations: TSD; target-site duplication, TIR; terminally inverted repeat; L, left arm region; A, adenosine-rich region; R, right arm region, LTR; long terminal repeat; UTR, untranslated region; ORF, open reading frame; NTR, variable number tandem repeats; *gag*, virus-like coat protein; *pro*, protease; *RT*; reverse transcriptase; *IN*; integrase; *env*, envelop coat protein. Adapted from (Beauregard *et al.*, 2008; Goodier and Kazazian, 2008; Levin and Moran, 2011)

1.2 DNA transposons

Class II transposons (DNA transposons) mobilize through either single- or double-stranded DNA intermediates (Craig, 2002). These elements can be divided into three subclasses: DNA transposons that use a 'cut and paste' mechanism where double stranded DNA sequences are excised from the genome (Craig, 2002), *Helitrons* that use a rolling-circle replication (Kapitonov and Jurka, 2001), and finally *Mavericks*, where the transposition mechanism is still unclear but is suspected to use a self-encoded DNA polymerase for replication (also known as a self-synthesizing transposon) (Kapitonov and Jurka, 2006). In contrast to the 'cut and paste transposons', the latter two classes are believed to mobilise via a single-stranded DNA intermediate to achieve a 'copy and paste' replication (Feschotte and Pritham, 2007; Bourque *et al.*, 2018). DNA transposons preferentially integrate into gene rich regions, producing genome instability and leave small remnants behind (Feschotte and Pritham, 2007; Bourque *et al.*, 2018).

Although the human genome is dominated by retrotransposons (Lander *et al.*, 2001), it is also the host to 120 families of DNA transposons (Lander *et al.*, 2001; Feschotte and Pritham, 2007). Although DNA transposons exist in prokaryotes and simple eukaryotes, investigation of the genomes and DNA transposons of other mammals such as dog, rat and mouse, suggested that DNA transposons have been inactive during the past 40-50 million years (Craig, 2002; Feschotte and Pritham, 2007). However, the discovery of active DNA transposons in *Myotis lucifigus* (little brown bat) has suggested that DNA transposons have played a great role in mammalian evolution (Feschotte and Pritham, 2007). In the budding yeast *Saccharomyces cerevisiae* and the fission yeast *Schizosaccharomyces pombe*, DNA transposons can no longer be found in their genomes but are still present in the pathogenic fungus *Candida albicans*, suggesting that extinction events occurred in the ancestors of these fungi (Daboussi and Capy, 2003; Johnson, 2019). *C. albicans* hosts multiple families of DNA transposons however the potential genomic interaction of DNA transposons have not been fully characterized (Maxwell, 2020).

1.3 Retrotransposons

Retrotransposons are the second group of mobile elements which relocate within the genome via an RNA intermediate and share a similar structural and functional homology with retroviruses (Beauregard *et al.*, 2008; Goodier and Kazazian, 2008). Basically, retrotransposons replicate themselves by producing a cDNA with reverse transcriptase which cDNA is then inserted back into the host genome. Two major classes of retrotransposons can be separated by the presence or absence of long terminal repeats (LTR) that flank both ends of the coding sequence and play a major role in the replication of the element.

1.3.1 Non-LTR retrotransposons

This class of retrotransposon is highly abundant and widespread in eukaryotes (Lander *et al.*, 2001). The origin of non-LTR retrotransposons can be traced back to the existence of multicellular organisms after the metazoan radiation, 500-800 million years ago (Eickbush and Jamburuthugoda, 2008; Eickbush and Eickbush, 2015). Non-LTR retrotransposons are distinct from LTR retrotransposons in that no virus-like protein coat is involved in the life cycle. The mobilization of non-LTR retrotransposons is believed to be though target-site-primed reverse transcription (TPRT) (Luan *et al.*, 1993). Non-LTR retrotransposons, either encode a single open reading frame (ORF) or two ORFs, which encode the common elements of reverse transcriptase (RT) and endonuclease (EN) (Eickbush and Malik, 2002). The retrotransposition mechanism of non-LTR retrotransposons is not completely understood as they are often absent in model organisms such as *Saccharomyces cerevisiae* (Eickbush and Malik, 2002). The latest models of mobilization are based upon the single ORF R2 element and the two ORF L1 element (reviewed in Christensen *et al.,* 2006 and Eickbush and Eickbush, 2015). First, transcription of template RNA occurs from an internal promoter. Endonuclease encoded by the mRNA then cleaves one strand of the target locus and a 3' hydroxyl break is then generated. The mRNA from the retrotransposon associates with the nicked DNA and primes reverse transcription to produce a cDNA which is inserted into the target site (Fig. 1.2) (Eickbush and Eickbush, 2015).

1.3.2 LINEs

L1 is one of the LINE non-LTR elements and is also the only active element in the human genome. The origin of L1 dates to the last 160 million years of the mammalian radiation and an active sub-family lineage could be traced from 40 million years ago (Khan *et al.*, 2006; Sookdeo *et al.*, 2018). L1 accounts for 17% of human genome with around 500,000 copies. The majority of the L1 elements suffer from 5' truncation, rearrangement, or mutation and therefore cannot mobilize, but around 80-100 elements can still mobilize (Beck *et al.*, 2010).

The L1 element has a length of ~6 kb, contains a 5' UTR promoter and ends with a short 3' UTR and poly(A) tail. To facilitate transposition, an RNA binding protein with nucleic acid chaperone activity is encoded from open reading frame 1 (ORF1). Open reading frame 2 (ORF2) encodes a apurinic/apyrimidinic (AP)– like endonuclease and a reverse transcriptase (RT) (Suarez *et al.*, 2018). Active L1 elements are observed in several human tumours and some specific tissues (e.g. brain), increasing the potential linkage with disease.

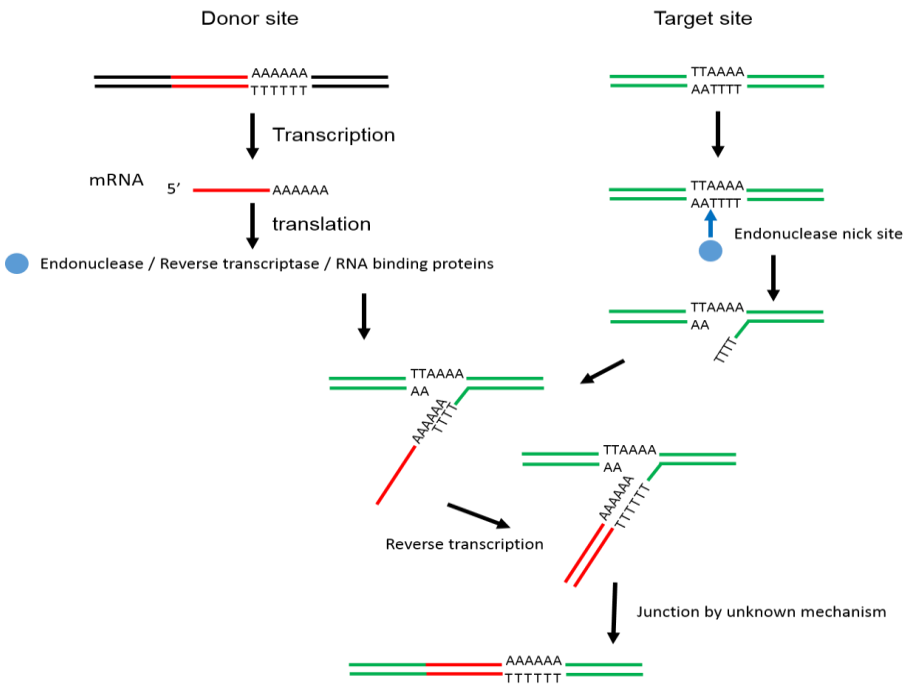

Figure 1.2 Retrotransposition of non-LTR retrotransposons.

mRNA of an active non-LTR retrotransposons at the donor site is transcribed by host RNA polymerase and the endonuclease, reverse transcriptase and RNA binding proteins are translated from the mRNA. Endonuclease targets a poly T region at the target site and creates a single strand nick. The poly A sequence from the donor mRNA that is complementary with poly T region directs the single stranded retrotransposon DNA to target site. cDNA is synthesised by reverse transcription. However, the integration process is not fully understood but is thought to be completed by host repair machinery (Eickbush and Jamburuthugoda, 2008; Eickbush and Eickbush, 2015).

1.3.3 SINEs

SINE (short interspersed nuclear elements) are small (<500 bp) retrotransposons that do not encode any proteins. Unlike other retrotransposons, SINE elements are transcribed by RNA polymerase III (RNA pol III). The reverse transcriptase and integrase activities for SINE retrotransposition are provide by other retrotransposons such as LINEs (Tucker and Glaunsinger, 2017). The most abundant SINE in human genome is the *Alu* element which contains an *Alu*I restriction site and also shares homology with the 7SL RNA component of signal recognition particle. One derivative of SINEs are SVA (SINE-R VNTR *Alu*) elements which consist of an antisense direction *Alu*-like sequence, a variable number of tandem repeats (VNTR) and SINE-R region from 3' terminus of the *env* gene and a poly A signal. SVAs are the most recently evolved group of active non-LTR elements in the human genome (Gianfrancesco *et al.*, 2017). More than 100 heritable diseases such as haemophilia, β-thalassaemia, Duchenne muscular dystrophy, cystic fibrosis, Apert syndrome, neurofibromatosis are associated with SVAs (Ostertag *et al.*, 2003; Suarez *et al.*, 2018).

1.4 LTR retrotransposons

LTR retrotransposons, which are often known as extra chromosomally-primed retrotransposons, were the first class of retrotransposon discovered. They exhibit high homology with retroviruses, and propagate in a similar manner (Beauregard *et al.*, 2008; Maxwell, 2020). This type of retrotransposon is highly abundant in eukaryotes, for example, 400 families of LTR retrotransposons make up to 75% of the maize genome (Schnable *et al.*, 2009). In contrast LTR retrotransposons only comprise 1% of the *Drosophila melanogaster* genome with 20 different families (Bowen and McDonald, 2001). In human, LTR retrotransposons comprise less than 8% of genome while the last record of active transposition is suggested to be 59 million years ago (Lander *et al.*, 2001).

The classification and phylogenetic relationship of LTR retrotransposons is basically based on the amino acid sequence and arrangement of the reverse transcriptase. All members of the LTR retrotransposon superfamily contain ORFs encoding a retroviral-like structural precursor protein (gag), reverse transcriptase (RT), integrase (IN),

protease (PR) and RNase H (RH) activities. However, differences in the arrangement of these ORFs separates them into groups, the *Ty1*/*copia* family (*Pseudoviridae*), the *Ty3*/*gypsy/Tf* family (*Metaviridae*), the *BEL/Pao* family and the members of retrovirus and endogeneous retrovirus (ERVs) groups. Little is known of the *Bel/Pao* group, which originate from the silk worm *Bombyx mori* and the giant roundworm *Ascaris lumbricoides*, but this metazoan-only retrotransposon is thought to be the progenitor of both *Ty3*/*gypsy*/*Tf* and retroviruses (reviewed in Eickbush and Malik, 2002). The structural organisation of *Ty1*/*copia* elements, which are absent in vertebrates, is different from all other LTR retrotransposons because the IN domain is located immediately upstream of the RT domain while in the other elements it is located downstream of RT/RH domain (Flavell *et al.*, 1995; Eickbush and Malik, 2002).

1.4.1 Endogenous retroviruses

Retrovirus particles contain RNA copies of the viral genome which are reverse transcribed into a double-stranded DNA molecule upon entry to the host cell and then integrated into the genome. If a retrovirus infects a germline cell then the resulting host gamete will contain provirus genetic material as a novel gene. After natural selection and random genetic drift, permanently endogenized retroviruses are then named endogenous retroviruses (ERV). Many ERVs lose the ability to express their viral genes due to accumulation of mutations during evolution (Johnson, 2019). It was suggested that ERV sequences undergo mutational decay so that the viral sequences no longer encode functional proteins. Frequently, the internal coding sequences are completely removed by recombination events between 5' and 3' LTRs leaving behind a 'solo LTR' (Sverdlov, 1998).

The endogenous retroviruses resident in human genome are termed human endogenous retroviruses (HERVs). The classification of HERVs was originally based on the differences between the primer binding site (PBS). HERV-K (requires lysine tRNA), while HERV-W (requires tryptophan tRNA). However, a universal classification was more recently employed by comparing their similarity to their exogenous counterparts: class I (*gammaretrovirus*- and *epsilonretrovirus*-like), class II (*betaretrovirus*-like) and class III (*spumaretrovirus*-like) (Grandi and Tramontano, 2018). Among these groups, the HERV-K group is the youngest active element especially its subgroup HML-2 (Garcia-Montojo *et al.*, 2018). All the proviruses of this

young HERV group were found to be defective in at least one gene however, many of them contain a complete open reading frame and the proteins encoded were detected in tissues (Subramanian *et al.*, 2011; Garcia-Montojo *et al.*, 2018). It was recently shown that the expression of HERVs could be triggered by *Herpesviridae* (herpes simplex virus type-1, varicella-zoster virus, human herpes virus type 6), causing autoimmune diseases suggesting that such endogenous retroviruses could be involved in the pathologies of diseases (Levet *et al.*, 2019).

1.4.2 LTRs

LTR retrotransposons were classified as an individual family since all the members shared a LTR sequence flanking the both sides. This LTR region contains sequence motifs for gene expression such as core promoter elements, enhancers, transcription initiation (capping), transcription termination and polyadenylation signals. LTR sequences are exclusively recognised by host RNA polymerase II (RNA Pol II) (Bowen *et al.*, 2003; Leem *et al.*, 2008; Esnault and Levin, 2015). Each LTR consists of a central R region, a downstream located U5 region and an upstream U3 region in the order of U3-R-U5. Transcription of LTR retrotransposons is initiated in the 5' LTR and terminates in the 3' LTR. Transcription initiates at the transcription start site (TSS) in the R region from the 5' LTR towards the 3' LTR with the assistance of regulatory motifs in the U3 region. Since the 3' LTR is identical to the 5' LTR it also contains TSS and regulatory regions and therefore, can potentially initiate transcription extending into any adjacent sequences (Grandbastien, 2015).

1.5 The life cycle of LTR retrotransposons

The life cycle of LTR retrotransposons has been extensively studied in *S. cerevisiae* and more lately, also in *S. pombe* (Fig. 1.3) (Curcio *et al.*, 2015; Esnault and Levin, 2015). The transposition event is initiated by the transcription of the element by the host RNA pol II. Retrotransposon proteins are translated primarily as one or two polyproteins. These polyproteins are then processed and matured by the protease (PR) activity. The RNA transcript of the element and the proteins encoded are assembled into a virus-like particle (VLP). Reverse transcription takes place in the VLP and the cDNA transcript is ready for integration into the host genome following import into the

nucleus. The integration site preferences are dependent upon the retrotransposon and every element has its own integration hot spot.

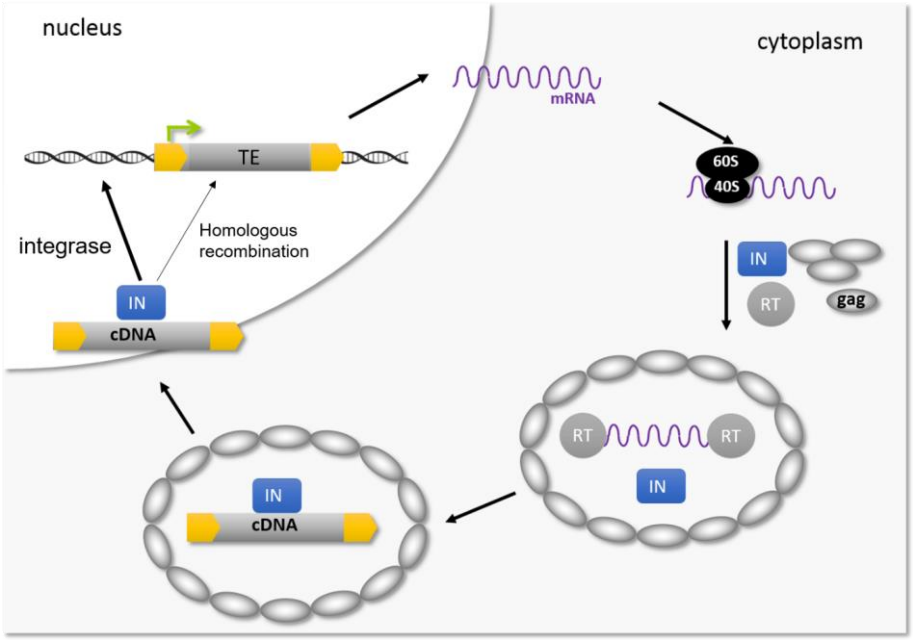

Figure 1.3 The life cycle of LTR retrotransposons.

Host RNA polymerase II initiates transcription at the 5' LTR, producing an mRNA which leaves the nucleus. After translation and polyprotein cleavage by protease (PR), a viral-like particle is assembled by Gag, enclosing mRNA, integrase (IN) and reverse transcriptase (RT). cDNA is then produced by RT and enters the nucleus where it inserts into the genome either by IN-mediated integration or by homologous recombination (adapted from Esnault and Levin, 2015)

1.6 LTR retrotransposon and retroviral encoded proteins

The completion of the LTR retrotransposon life cycle requires the expression of the element as well as the assistance of the proteins encoded by the element's own genome. Moreover, the precursor protein synthesised from retrotransposons is a fused product and processing is required to give mature, functional proteins (Farabaugh *et al.*, 1993). Finally, additional post-transcriptional, co-translational and post-translational mechanisms are required to ensure the correct ratio of proteins is produced for the assembly of VLP.

1.6.1 Protease

Proteolytic processing of precursor polypeptides is mediated by a protease (PR) activity encoded by the *pol* gene (Park and Morrow, 1991; Kirchner and Sandmeyer, 1993; Gao *et al.*, 2003). The *pol* gene is located on the 3' of *gag* and often there is no independent initiation site for its translation resulting in the production of a Gag-pol polyprotein (Gao *et al.*, 2003). The fused Gag-pol polyprotein is cleaved by the PR activity in many types of retroelements including HIV-1, *Ty* and *Tf* (Hoff *et al.*, 1998; Goodenow *et al.*, 2002; Gao *et al.*, 2003). This dimeric enzyme, whose structure and function has been characterised, hydrolyses the peptide bonds at the end of the sequences to cleave between Gag and Pol to release them for additional cleavage events which results in mature reverse transcriptase (RT) and integrase (IN) (Goodenow *et al.*, 2002). However, the order of cleavage of polyprotein varies in different retroelements and results in a variety of intermediate proteins (Hoff *et al.*, 1998).

1.6.2 Gag

A virus-like particle (VLP) is formed by proteins which are encoded by the *gag* gene and is similar to the capsid of infectious retroviruses. The VLP is where reverse transcription takes place and is essential for transposition. In the human infectious retrovirus HIV-1, three proteins are processed from Gag precursor polyproteins including the matrix domain (MA) which interacts with the viral membrane for stability and is important for transport and viral budding, the Capsid protein (CA) which oligomerizes to form the capsid and Nucleocapsid (NC) of which mediates interaction between the RNA and forms a scaffold for genome packaging. These three proteins

combine together to form a structure which is essential for cDNA reverse transcription and facilitates retroviral maturation, budding and infection (Eichinger and Boeke, 1988). In the *Ty3*/Gypsy family retrotransposon, the *gag* gene encodes CA and NC proteins but lacks MA (Sandmeyer *et al.*, 2015) while *Ty1* of the *Ty1*/Copia family does not encode NC (Dodonova *et al.*, 2019). The *Ty3* NC region is essential for *Ty3* mRNA targeting and chaperones the annealing of tRNA primers (Sandmeyer and Clemens, 2010). The latest analysis of the CA protein of *Ty*3 indicates that the capsid structure is highly conserved with HIV-1, suggesting it was already present in an ancestral virus or transposon ~1.6 billion years ago (Dodonova *et al.*, 2019). However, the *gag* region is diverse within LTR retrotransposons, and even closely related family members can show a great divergence in this region (Levin *et al.*, 1990). Nonetheless, the formation of the VLP is thought to be essential for any retroelement to propagate as it provides a protective environment for reverse transcription and prevents degradation by enzymatic proteins from the host defence mechanisms (Teysset *et al.*, 2003; Dodonova *et al.*, 2019).

For *Ty3,* the assembly of the VLP requires a ratio of 10- to 20-fold molar excess of CA protein compared to the *pol*-derived proteins (Hansen *et al.*, 1992; Hatfield *et al.*, 1992). Full length Gag-pol is produced only after the read though of a frameshift region of the termination signal that allows the accumulation of the Gag protein compared to Gag-pol (Hatfield *et al.*, 1992; Farabaugh *et al.*, 1993). However, how other retrotransposons achieve an excess of Gag derived proteins is not clear.

1.6.3 Reverse transcriptase

One of the key proteins that LTR retrotransposons and retroviruses encode is reverse transcriptase which mediates the conversion of transcript mRNA into cDNA for host DNA genome insertion. Even though these proteins are highly divergent in terms of sequence, they share remarkable properties. Reverse transcriptase was therefore used to classify transposons according to the domain arrangement of gene and the sequence of reverse transcriptase (Eickbush and Jamburuthugoda, 2008). Based on this, LTR retrotransposons can be divided into three groups: *Ty1*/copia, Bel and *Ty3*/gypsy. In both *Ty1* and *Ty3* classes of LTR retrotransposons, the reverse transcriptase (RT) and an RNaseH domains are separated by a tethering domain

which shows a three-dimensional structure similarity to an RT RNaseH domain even though there is no sequence similarity (Eickbush and Jamburuthugoda, 2008).

1.6.4 Integrase

LTR retrotransposons and retroviruses encode an integrase (IN) protein to catalyse the insertion of cDNA into host genome once reverse transcription is completed. There is a close relationship between RT and IN in both yeast LTR retrotransposons and retroviruses. IN consists of three conserved domains, the N-terminal Zn finger-like HH-CC motifs for LTR sequence binding (Khan *et al.*, 1991), the central core domain that contains catalytic site (Nymark-McMahon and Sandmeyer, 1999) and the least well conserved DNA binding C-terminus domain (Malik and Eickbush, 1999). The structure of reverse transcriptase in Avian Leucosis Virus (ALV) is a heterodimer formed by fusing a small subunit (α) RT (RT-RH) and a large subunit (β) RT (RT-RH-IN) while the RT of Human T-cell Leukaemia Virus Type-1 (HTLV-1) is in 3α/β formation (Trentin *et al.*, 1998). Meanwhile, some retroviruses, such as Murine Leukaemia Virus (MLV) and Human immunodeficiency Virus Type 1 (HIV-1) separate RT and IN during the maturation process (reviewed by Eickbush and Jamburuthugoda, 2008). Interaction between RT and IN is essential for RT function. Only full length, functional and RT-intact IN supports retrotransposition functions such as initiation of reverse transcription, entry into the nucleus, cDNA 3'-end processing, viral endogenous reverse transcription, RT activity, and proper folding of RT (Wilhelm and Wilhelm, 2005; Wilhelm and Wilhelm, 2006).

1.6.5 Retrovirus *env*

Retrotransposons were originally identified in *S. cerevisiae*. Results from sequencing of *Ty3* elements showed that the amino acid sequence of the proteins (PR RT RH and IN) produced from transposon ORFs are highly homologous to those of infectious retroviruses (Eichinger and Boeke, 1988 and references therein). Extensive research of the phylogeny of vertebrate retroviruses and LTR retrotransposons suggested that retrotransposons are the ancestors of retroviruses, which later acquired an envelope glycoprotein (*env*) gene during evolution (Eickbush and Jamburuthugoda, 2008) (Figure 1.1) On the other hand, a recent study suggested that the mouse intracisternal A particle (IAP) retrotransposons are the off-spring of a retrovirus that infected the

germline (Ribet *et al.*, 2008). Nevertheless, the close relationship and homologous structure allows LTR retrotransposons to function as models for understanding retroviruses. Basically, LTR retrotransposons are regarded as retroviruses without cell-to-cell infection ability although artificially overexpressing *env* can provide extracellular budding ability to an *env*-less VLP (Garoff *et al.*, 1998).

Three key features are associated with *env*: (i) the ability to exit the host cell; (ii) protection of viral genetic material in extracellular environments; (iii) the ability to infect a new cell via cell surface binding and viral-cellular membrane fusion (Kim *et al.*, 2004). The origin of *env* is unclear. The surface component (SU), is different across all viruses while, the membrane-anchored trans-membrane component (TM) is, in contrast, highly conserved across species (Kim *et al.*, 2004). A possibility for the origin of *env* could be the random recombination of two independent genes which encode a receptor-binding protein and membrane fusion protein, respectively to form a *de novo env* gene (Kim *et al.,* 2004 and references therein).

In some cases the acquisition of *env* genes by a transposon has been well studied. The Gypsy retrotransposon in *Drosophila melanogaster* (fruit fly) was found to have acquired an *env*-like third ORF, which is suspected to be captured from a double-stranded DNA insect virus baculovirus (Song *et al.*, 1994; Malik *et al.*, 2000). Homologous sequences were identified between *env*-like genes in *Caenorhabditis elegans* and the gene encoding G2 glycoprotein in Phleobovirus, in terms of the length of protein, cleavage sties and C-terminal transmembrane domain (Bowen and McDonald, 1999; Malik *et al.*, 2000). The viral-host cell membrane attachment and fusion inducting gB glycoprotein encoding sequence are found to be similar between the *env*-like genes of *Ascaris lumbricoides* and herpesvirus (Malik *et al.*, 2000).

1.7 Yeast LTR retrotransposons

Both budding yeast, *Saccharomyces cerevisiae*, and fission yeast, *Schizosaccharomyces pombe*, are employed as model organisms for LTR retrotransposon research. The divergence between the two fungi is believed to be around 420 million years, the same time point mammals separated from yeast (Berbee *et al.*, 1992). At the same time, there are only a few transposons present in both

genomes, making them relatively easy to study and the findings can be applied to understanding LTR retrotransposons of higher eukaryotes and even retroviruses.

1.8 The LTR retrotransposons of *Saccharomyces cerevisiae*

Five families of LTR retrotransposons were identified in *S. cerevisiae*, named *Ty1*-*Ty5* (**T**ransposon of **y**east), which are present in variable copy numbers in different individual lab strains and 'natural' isolates (Borneman *et al.*, 2008). All the *Ty* elements encode PR, IN, RT and RNaseH activities but not *env*, and are therefore not capable of mediating infection. Comparison of the LTR sequences revealed that within a family they share an overall >96% identity (Kim *et al.*, 1998). Three out of the five families, *Ty1*, *Ty3* and *Ty5* are well-studied and are classified into two super-families (*Ty1*/Copia and *Ty3*/Gypsy). *Ty1* and *Ty5* retrotransposons are members of the *Ty1*/copia family while *Ty3* is part of the*Ty3*/gypsy family.

Ty1 is the most abundant retroelement in *S. cerevisiae*, with 32 insertions in the sequenced *S288C* strain and shows a high similarity (~70%) with *Ty2* with almost identical LTR regions (Jordan and McDonald, 1999b; Garfinkel *et al.*, 2003). A hybrid of a *Ty1*/*Ty2* element was identified in *S. cerevisiae* and was suggested to have occurred when two highly related elements were packed into a single VLP and a hybrid cDNA was produced by reverse transcriptase jumping from one mRNA to another (Jordan and McDonald, 1999b). *Ty1* is 5918 bp in length with a 334 bp LTR region on the both ends. *Ty1* has two partially overlapping open reading frames *GAG* and *Pol* (*TYB1*) and they encode a Gag-pol polyprotein using a +1 frameshift which is processed into Gag (CA), PR, IN and reverse transcriptase/RNase H (RT/RH).

Ty3 is the sole member of the *Ty3*/Gypsy family in budding yeast, while one element, *YGRWTy3-1* is considered transpositionally active (Sandmeyer *et al.*, 2015). Full-length *Ty3* elements are 5.4 kb in length with a 340 bp LTR region and two overlapping ORFs *GAG3* and *POL3*. Frameshift of the coding region results in a Gag3-pol3 polyprotein. However, in contrast to *Ty1*, the *POL* encodes PR, RT/RH and IN in the same order, same as in retroviruses (Gao *et al.*, 2003).

Ty5 was identified as the smallest element and lacks functional copies in *S. cerevisiae*. *Ty5*-6p, a full-length functional *Ty5* element was identified in the closely related species, *S. paradoxus* and introduced into *S. cerevisiae* for studies (and named *Ty5*)

(Voytas and Boeke, 2002). *Ty5* is 5.4 kb in length with a 251 bp LTR (*S. paradoxus* omega element) and a single ORF that encodes Gag and Pol homologues (Voytas and Boeke, 2002).

Reverse transcription, and thus cDNA production, follows a similar overall scheme to that of retroviruses and is described in Figure 1.4. For *Ty1*, reverse transcription is initiated from the 3' OH of the $tRNA_i^{Met}$ primer that binds to the primer binding sequence (PBS) immediately adjacent to the 5' LTR(Fig. 1.4) (Chapman *et al.*, 1992). The minus-strand strong-stop DNA is first reverse transcribed and contains the U5 and R regions that link to $tRNA_i^{Met}$ (Muller *et al.*, 1991). The RNA portion of the RNA:DNA hybrid is then degraded by RHaseH after RT reaches the 5' end of the template. This degradation event triggers the minus-strand strong-stop cDNA to translocate from the 5' end to 3' end of the RNA template and binds to R region via Crick-Watson base pairing. Using the R-U5-tRNA minus strand as the primer, the cDNA minus-strand is then extended and synthesized until the PBS region. The retroelement RNA is then degraded by RHaseH. Only the polypurine tract (PPT) region is an exception and it serves as the primer for the synthesis of the plus-strand until the 5' end of the minus-strand (Lauermann and Boeke, 1997). The synthesized plus-strand is then translocated to the 3' end of the minus strand and serves as the primer for itself and the template for the minus-strand. Finally, the double-stranded cDNA with complete 5'-U3-RU5-ORF-U3-R-U5-3' is synthesized. Although *Ty1*, *Ty3* and retroviruses employ polypurine tracts (PPT) for the plus-strand primer site, differences in the PPT are observed. Indeed the region of the PPT is specific, and *Ty3* RNaseH fails to recognise retrovirus PPTs such as HIV-1 (Rausch *et al.*, 2000; Nair *et al.*, 2012).

The synthesized cDNA is required to integrate into the host genome in order to complete the LTR retrotransposon life cycle, however, the selection preference of each individual retroelement and retrovirus are different. Since retrotransposons cannot horizontally spread from cell to cell, it is especially crucial for a retroelement to integrate into a new location to avoid harming the host. Therefore, integration of these elements is not completely random but exhibits a strong bias towards specific genomic regions to prevent disruption of essential genes or control/regulation regions (Goodier and Kazazian, 2008). Studies in *S. cerevisiae* showed that each of the element (*Ty1*, *Ty3* and *Ty5*) has employed different mechanisms to target genomic regions that are non-essential/gene-poor (Bushman, 2003; Sandmeyer, 2003).

Ty1 retrotransposon targets an integration window of between 80-700 bp upstream of RNA pol III transcribed genes (Devine and Boeke, 1996; Bachman *et al.*, 2004). The mechanism of *Ty1* integration has not been extensively studied, however, the site selection was demonstrated *in vivo* and showed that the integration machinery recognizes as little as 4 bp of the conserved inverted dinucleotides 5'-TG…CG-3' at the ends of the double stranded cDNA (Friedl *et al.*, 2010; Cheung *et al.*, 2018). Meanwhile, the integration process was described as a strand transfer step (reviewed by Wilhelm *et al.*, 2005). Integrase cleaves phosphodiester bonds near the ends of the cDNA to produce staggered ends. IN then cuts the chromosomal target DNA and the joins both cDNA ends to target DNA 5'-phosphates. Gap repair is not carried out by *Ty1* IN but an unidentified host protein and creates a target site duplication (TSD) that flanks the integrated *Ty1* cDNA.

In contrast, integration site selection of *Ty3* is not dependent on genetic sequences but transcription factors. *Ty3* targets the specific transcription factors TFIIIB and TFIIIC that are required for transcriptional initiation by RNA pol III of tRNA genes (Chalker and Sandmeyer, 1992; Sandmeyer *et al.*, 2015). TFIIIC associates with the 10 bp region of the tRNA gene promoter elements box A and box B which are located 20 bp and ~100 bp downstream of transcription start site (TSS). TFIIIC directs the assembly of TFIIIB to a region upstream of the transcription initiation site which in turn recruits RNA pol III. The subunits of TFIIIB, Brf1 and Bdp1 are responsible for the recognition of *Ty3* IN and the TFIIIC-TFIIIB-IN complex targets the 3' end of the cDNA into the TSS region in the presence of Mn^{2+} (Qi and Sandmeyer, 2012).

The *Ty5* element shows a different insertion preference in that ~95% of the transposition events are carried out in either telomeres or at the silent mating *HM* loci (Zou *et al.*, 1996). These loci are associated with a heterochromatic structure, and it was suggested that the biochemical signal recognised by *Ty5* is chromatin structure (Boeke and Devine, 1998). The targeting domain (TD) of *Ty5* IN interacts with a protein component of heterochromatin called, Sir4 (silent information regulator 4). This interaction is dependent upon phosphorylation of the TD by host kinase(s). Interestingly, under stress conditions (such as low nitrogen) phosphorylation is reduced allowing *Ty5* IN to target cDNA integration throughout the genome (Dai *et al.*, 2007). Therefore post-translational modification by the host determines the effect of *Ty5* towards the integrity of its genome.

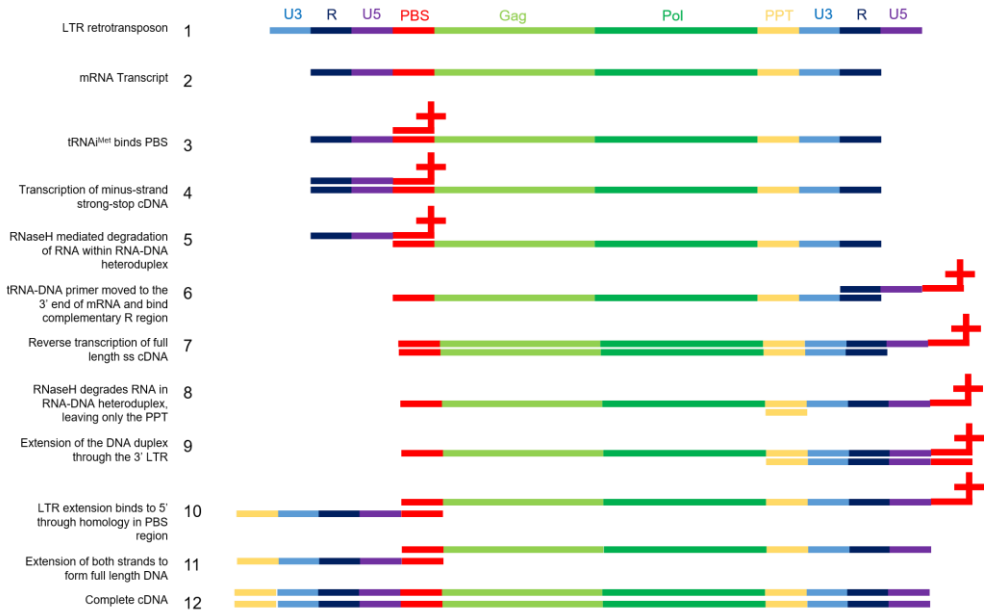

Figure 1.4 Reverse transcription of LTR retrotransposon mRNA

1) The structure of LTR retrotransposon DNA in the genome. 2) The mRNA transcript of the LTR retrotransposon. 3) The tRNA, with the complementary to primer binding site (PBS) binds in the 5' LTR. 4) tRNA primes the reverse transcription, copying the R and U5 region to give a minus-strand strong-stop DNA. 5) RNaseH degrades the R and U5 region of mRNA from the RNA:DNA hybrid. 6) The tRNA:DNA hybrid is relocated to the 3' LTR region and binds to complementary R region. 7) Reverse transcription of the full length of the open reading frame. 8) RNaseH degrades the RNA of the hybrid and only poly purine tract (PPT) left. 9) PPT primes the initiation of the plus strand DNA. 10) Reverse transcriptase translocate the template of the plus strand DNA to the 5' LTR and binds to PBS by complementary binding. 11) Reverse transcription completes both plus and minus strand with using each other as template. 12) Complete full length ds cDNA is produced for retrotransposition.

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1.9 The LTR retrotransposons of *Schizosaccharomyces pombe*

Following the discovery of the *Ty* family, LTR retrotransposons were identified in *S. pombe*, a yeast that diverged from *S. cerevisiae* 10^9 years ago. A repeat DNA sequence isolated from *S. pombe*, was later found to be a LTR and was used to identify two retrotransposons, *Tf1* (**t**ransposon of **f**ission yeast 1) and *Tf2* (Levin *et al.*, 1990). Both *Tf1* and *Tf2* families belong to the *Metaviridae* family and are closely related to animal retroviruses (Levin *et al.*, 1990; Esnault and Levin, 2015).

1.9.1 The architecture of Tf elements

Tf1 and *Tf2* elements are 4.9 kb in length and consists of a single ORF that encodes Gag (CA), Pol (PR, RT/RNaseH, IN) flanked by 358 and 349 bp LTR regions in *Tf1* and *Tf2* respectively (Levin *et al.*, 1990; Weaver *et al.*, 1993). Analysis of the LTRs of *Tf1* and *Tf2* showed that the TATA box regions are identical to the TATA box of other *S. pombe* genes such as housekeeping alcohol dehydrogenase *adh1* (Levin *et al.*, 1990). The structure of *Tf* LTRs are also conserved with other retrotransposons and retroviruses and contain U3, R, and U5 regions (Levin *et al.*, 1990; Weaver *et al.*, 1993). However, the *Tf1* and *Tf2* LTR sequences are less than 30% identical and the region extending from just the downstream of 5' LTR to the middle of the CA domain in the *gag* gene is drastically different (Levin *et al.*, 1990). Indeed, the *gag* regions of *Tf1* and *Tf2* are only 36% identical while the other regions have a 98% homology (Teysset *et al.*, 2003). Initial searches for retrotransposons in the common laboratory strains, Leupold strain *972* (h^-) and *975* (h^+) were unsuccessful. However, both *Tf1* and *Tf2* were subsequently identified in an alternative wild type isolate, *NCYC132* (Levin *et al.*, 1990). This wild-type strain contains between 30-40 active *Tf1* elements. In contrast, no full length *Tf1* elements are present in the *972* background but there are 13 full length copies of *Tf2* (Wood *et al.*, 2002). Analysis of the *972* strain revealed that there are also 249 solo LTR or LTR fragments (>200 bp) derived from nine clades of LTR retrotransposons (Wood *et al.*, 2002). Solo LTRs are the remains of previous insertions that have been lost through homologous recombination. Some solo LTRs have been reported to be transcriptionally active and can influence the transcription level of neighbouring genes (Sehgal *et al.*, 2007; Anderson *et al.*, 2009).

1.9.2 *Tf1* and *Tf2* polyprotein processing

The *Tf* family contains only a single open reading frame which is translated as single polyprotein and then processed. This polyprotein contains Gag (CA), PR, RT/RNaseH and IN activities. The order of these proteins is conserved within the *Metaviridae* family and processing is mediated by the encoded PR activity. The major steps in polyprotein processing for *Tf1* and *Tf2* are outlined in Fig. 1.5. The order of the proteolytic processes is different between the two elements, resulting in an accumulation of PR-RT fused species for *Tf2* and a lower level of mature CA (Gag), IN and thus cDNA (Fig. 1.5) (Levin *et al.*, 1993; Atwood *et al.*, 1996; Hoff *et al.*, 1998). The differences in processing has been suggested to result from the divergence in the sequence of the CA (gag) and PR proteins (Weaver *et al.*, 1993; Dodonova *et al.*, 2019) .

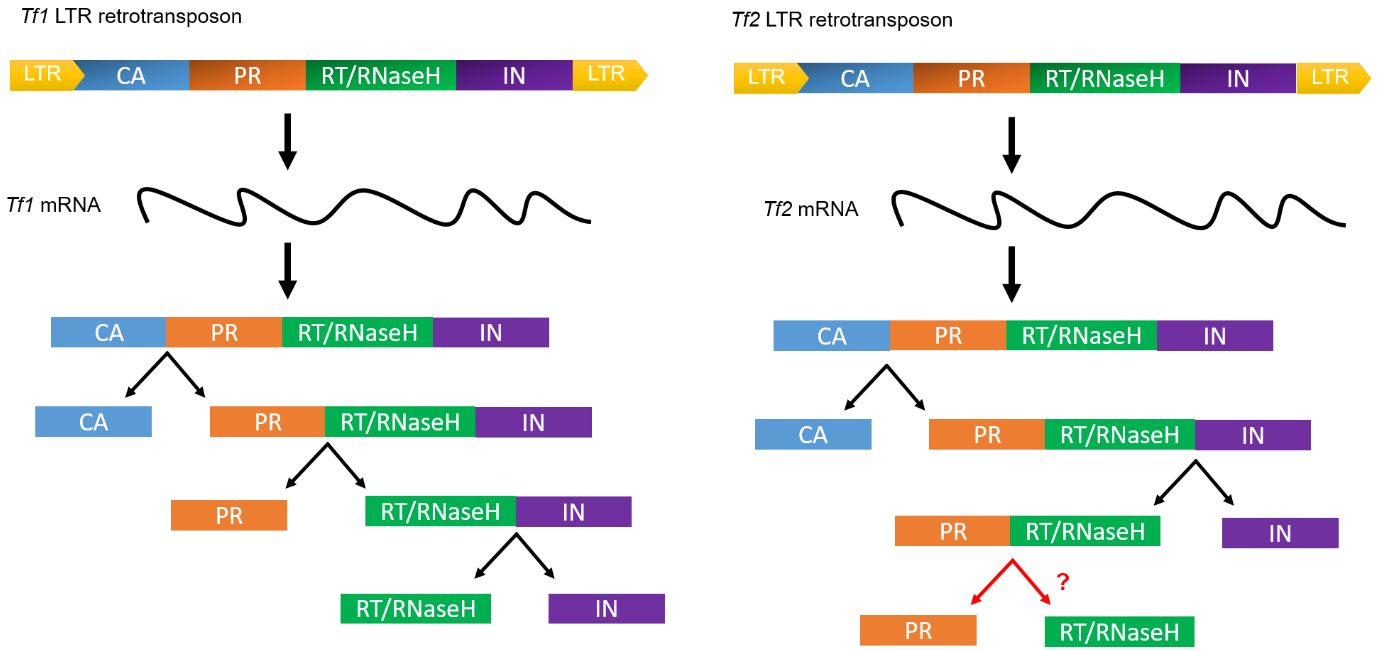


Figure 1.5 Processsing of *Tf* element polyproteins.

The translated polyprotein of *Tf1* and *Tf2* requires processing to produce functional proteins and this is mediated by protease (PR). Cleavage sites are indicated by black bi-directional arrows and a possible cleavage site to form *Tf2* PR and RT-RNaseH proteins that was not observed *in vivo* is indicated with red bi-directional arrows. Abbreviations: Long terminal repeat (LTR), Capsid (CA), Protease (PR), Reverse transcriptase (RT), Ribonuclease H (RAseH), Integrase (IN). Adapted from (Hoff *et al.,* 1998).

1.9.3 Reverse transcription of *Tf* elements

The reverse transcription of the *Tf1* mRNA into full-length cDNA follows the same overall scheme as other retroelements (Esnault and Levin, 2015). However, unlike other LTR retroelements, *Tf1* does not use a tRNA to prime cDNA synthesis as the PBS region lacks the conserved UGG motif that is required for association with tRNA. Instead, *Tf1* reverse transcription initiates by a self-priming process. The first 11 bp of the *Tf1* mRNA acts as the primer by folding back and annealing to the PBS region though complementary, based pairing. This represents a novel mechanism that is called 'self-primed' reverse transcription (Levin, 1995). Following the formation of the looped mRNA, the loop is cleaved by the RNaseH activity of the *Tf* encoded RT which produces a 3' end competent for extension by the RT polymerase activity (Levin, 1996).

The *Tf1* retrotransposon is not the only retroelement species that employs self-priming mechanisms in the *Metaviridae* family, other LTR retrotransposons (including the closely related *Tf2* of *S. pombe*, *Maggy* elements of the rice infectious fungus *Magnaporthe grisea*, *Skippy* elements of the plant pathogenic fungus *Fusarium oxysporum*, *Cft-1* elements of the mold fungi *Cladosporium fulvum*, *Boty* elements of the plant necrotrophic fungus *Botrytis cinerea* , and *sushi* elements of the Japanese puffer fish *Fufu rubipes*) are all now known to do so (Malik and Eickbush, 1999; Butler *et al.*, 2001; Craig, 2002). It was therefore proposed that the above elements belong to a single lineage of *Metaviridae*. In contrast, in retroviruses and *Ty* retrotransposons in *S.cerevisiae*, reverse transcription initiates by the binding of tRNA primer anticodon domain to the complementary viral PBS and the cleavage of the primer to expose a 3'-OH for DNA synthesis.

1.9.4 Insertion of *Tf* elements into host genome

As mentioned above, the two retrotransposons *Tf1* and *Tf2* in *S. pombe* show similarities and differences in the genomic architecture, suggesting they share some biochemical processes while others are different. *Tf1* employs IN and performs cDNA integration into the *S. pombe* genome in a similar manner to the *S. cerevisiae Ty* elements. However, the mechanisms by which *Tf1* avoids integrating into essential coding regions is different to *Ty* elements. Mapping studies revealed that *Tf1* prefers insertion sites in a window of 100-400 bp upstream of RNA Pol II transcribed genes,

generally upstream of the TATA box (Behrens *et al.*, 2000; Singleton and Levin, 2002; Bowen *et al.*, 2003). Insertion upstream of TATA box reflects a strategic advantage that reduces the possibility of disrupting the transcription of genes (Kelly and Levin, 2005). It was observed that the targeting of *Tf1* integration is dependent on the recognition of the RNA pol II promoter without IN binding directly to DNA (Ebina *et al.*, 2008; Leem *et al.*, 2008). In addition, *Tf1* has a strong bias for in integration into stress-responsive genes as IN interacts with the stress activated transcription activator, Atf1 as a navigator (Leem *et al.*, 2008). Nonetheless, insertion site location and selection of *Tf1* occurs throughout the *S. pome* genome but clusters in promoter regions upstream of ORFs (Singleton and Levin, 2002; Cherry *et al.*, 2014).

For *Tf2* a decreased level of cDNA and IN is produced relative to *Tf1* (Hoff *et al.*, 1998). Furthermore, unlike *Tf1*, the majority of integration events of *Tf2* (~70%) are IN-independent and occur via homologous recombination (HR) with an already existing elements (a process which has been termed integration site recycling) (Hoff *et al.*, 1998). The frequency of mobilization via HR varies between elements and species but is usually relatively low (<10%) compared with IN-dependent mobilization events (Hansen and Sandmeyer, 1990; Levin, 1995; Zou *et al.*, 1995). This unique strategy not only recycles integration sites maintaining their integrity but also ensures host encoding genes are not being disrupted. Experimental results revealed that the IN of *Tf2* is functionally active and accounts for the remaining 30% of mobilization events in a new integration site (Hoff *et al.*, 1998). The reasons that *Tf2* utilizes HR instead of integration is not well understood since the IN of *Tf2* is fully functional. Furthermore swapping *Tf2* IN with the *Tf1* sequence does not change the preference for HR mediated integration (Hoff *et al.*, 1998). It has therefore been suggested that the low frequency of *Tf2* integration results from the sequence differences in CA (gag) and PR.

Regardless, both *Tf* elements in *S. pombe* showed a unique phenomenon in that either solo LTRs or new integration sites are close to RNA pol II promoters that are stimulated by environmental stress and are potentially capable of influencing nearby gene expression (Leem *et al.*, 2008; Esnault *et al.*, 2019). As discussed below, it is possible that the retrotransposition of retrotransposons helps to improve host fitness for survival in stress conditions.

1.10 The impact of retroelements on host genome and evolution

Retrotransposons have developed strategies to integrate into gene poor or gene free regions in order to minimize harmful effects on the host genome. However, unrestricted mobilization can result in over-accumulation, potentially reducing genome stability. Before the discovery of the regulatory effects of host genes, retroelements were classified as selfish parasites in the host genome without any function or evolutionary role (Doolittle and Sapienza, 1980; Orgel *et al.*, 1980; Hickey, 1982). However it was later suggested that transposons may have a role in responses to stress and shaping the genome of organisms (McClintock, 1984). It is now accepted that retrotransposons have been involved in changing the genomes in many multicellular eukaryotes including plants (Bennetzen *et al.*, 2005; Vitte and Bennetzen, 2006), fruit fly *Drosophila* family (Boulesteix *et al.*, 2006), salamanders (Marracci *et al.*, 1996), avian species (Organ *et al.*, 2007), primates (Liu *et al.*, 2003) and humans (Sen *et al.*, 2006).

In addition to changing the size of genome, retroelements are also suggested to mutate the host genome by insertions where 50-80% of mutations in fruit flies are due to insertion of transposable elements (Biemont and Vieira, 2006). Transposons affect host genome evolution in various ways including the breakage and re-joining of different chromosomes, gene and segmental duplication, functional domain shuffling in exons (Fig. 1.6). An example is the Mammalian L1 element that affects human and other mammals in adverse ways. Human, mouse and gorilla all suffer from disease-producing mutations due to L1 insertions into the genome. Indeed haemophilia B in dogs is caused by the disruption of factor IX caused by a L1 insertion (Ostertag and Kazazian, 2001; Brooks *et al.*, 2003; Kazazian, 2004).

By moving across chromosomes, transposons can reshuffle the genome and alter gene expression in a number of different ways (Fig. 1.6) (Kazazian, 2004; Goodier and Kazazian, 2008; Rebollo *et al.*, 2012). Insertion of mobile elements into novel sites in the genome can lead to disruption of host gene or genetic region. Elements either full-length or truncated carry novel sequences and so can introduce regulatory sequences at new loci. Similarly, transposon insertions can also leads to deletions in the host genome and cause disease. Indeed, removal of part of a gene was identified to be the cause of pyruvate dehydrogenase complex deficiency and leukaemia in

human (Mine *et al.*, 2007; Takasu *et al.*, 2007). Retroelements insertions either side or within a gene can also result in the production of novel transcripts (thus proteins). As such, the transposition into a new position can shuffle exons and potentially create a new gene, and this process is known as 3' or 5' transduction. For instance, the transduction of SVA in humans resulted in multiple copies of the gene encoding acyl-malonyl condensing enzyme 1 (AMAC1) (Moran *et al.*, 1999). The similarity between the genomes of mobile elements allows homologous recombination events and mis-alignment and mis-pairing causes rearrangement events of the retrotransposons and in-turn, affects the host genome. Retrotransposons can provide regulatory sequences (e.g. enhancers, promoters and terminators) that can influence the expression level of adjacent genes, alter the splicing of introns or the induction of antisense transcripts. An examples is the B1 element in mouse where binding of transcription factors to retrotransposon sequences affects the transcription of nearby genes (Roman *et al.*, 2008).

Retrotransposons can also influence higher order genome architecture. Retrotransposons can contribute to heterochromatin formation, and therefore alter nearby gene expression and the physical structure of chromosomes (Grewal and Jia, 2007; Slotkin and Martienssen, 2007). *Tf2* elements in *S. pombe* are clustered in the nucleus into structures called '*Tf* bodies' therefore retrotransposons can influence the spatial organisation of the genome (Cam *et al.*, 2008; Mikheyeva *et al.*, 2014). Another important property of retroelements is that the reverse transcriptase of an active autonomous element such as human L1 can mobilize non-autonomous elements such as *Alu* and SVAs. In fact, L1 reverse transcriptase can also mobilize other mRNAs and small non-coding RNAs in the host (Ohms *et al.*, 2014).

The LTRs of retrotransposons provide a source of repeated sequences for homologous recombination which can reshape the chromosome, depending on crossovers events (Fig. 1.7) (Mieczkowski *et al.*, 2006). There are a few factors governing the chance of chromosome recombination including the number of repeats, the location of the repeats, the length of the repeats and the degree of sequence divergence between repeats (Mieczkowski *et al.*, 2006). It is likely that the chance for recombination is proportional to the number of repeats (Wilson *et al.*, 1994). The geographical location of the repeated gene also greatly affects the rate of recombination. Two *leu2* heteroalleles located 20kb apart in the same chromosome

have a 10-fold higher rate of recombination than distant loci on the same chromosome or repeats located on non-homologous chromosomes (Lichten and Haber, 1989). In yeast, the rate of recombination showed a sharp decline when repeats were less than 250 bp and increased linearly above 250 bp (Jinks-Robertson *et al.*, 1993). Notably the LTR regions of the *Ty* and *Tf* families are larger than the minimum sequence length required for a homologous recombination. Therefore, retroelements are possibly one of the forces driving genome restructuring. In support of this, three genes *CYC1*, *OSM1* and *RAD7* that are flanked by two *Ty1* elements have a high chance to be removed through homologous recombination in *S. cerevisiae* (Liebman *et al.*, 1981). In addition, such recombination events are one of the events that create solo LTRs where the retroelements are excluded from the genome (Roeder and Fink, 1980). In *S. pombe,* a homologous recombination (HR) event was observed when replication forks were arrested in at the 5' end of an LTR. The replication fork barrier was bound with the DNA-binding protein Sap1 (Zaratiegui *et al.*, 2011; Jacobs *et al.*, 2015). This blockage to triggered DNA double strand breaks, driving HR.

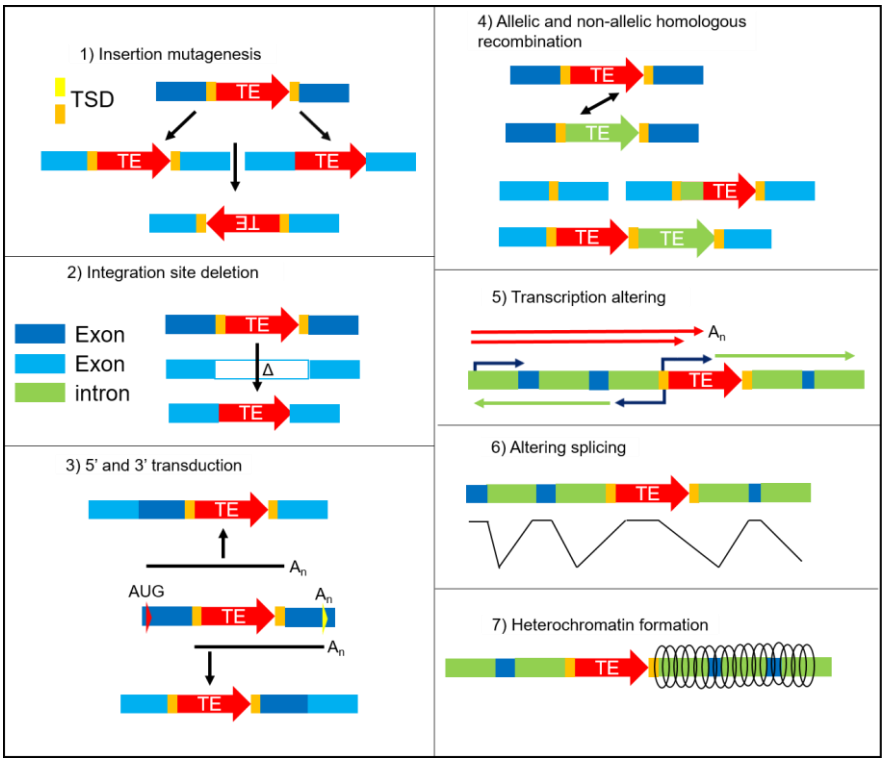


Figure 1.6 Transposable elements may affect the host genome in different ways.
(1) Insertion mutagenesis. (2) A random insertion may perform deletion at the new insertion site. (3) Either the adjacent sequence at 5' or 3' position of TE may be carried alone with during retrotransposition. (4) Recombination of two different TE could lead to TE removal or mispairing and crossing over. (5) Transcription may be terminated or truncated within TE sequence (red arrow). An antisense promoter may initiate production of transcript of opposite strand (green arrow). (6) Splicing of intron may be altered by TE, creating new exon. (7) TE may alter chromosomal architecture, changing nearby gene expression. Adapted from Kazazian, (2004)

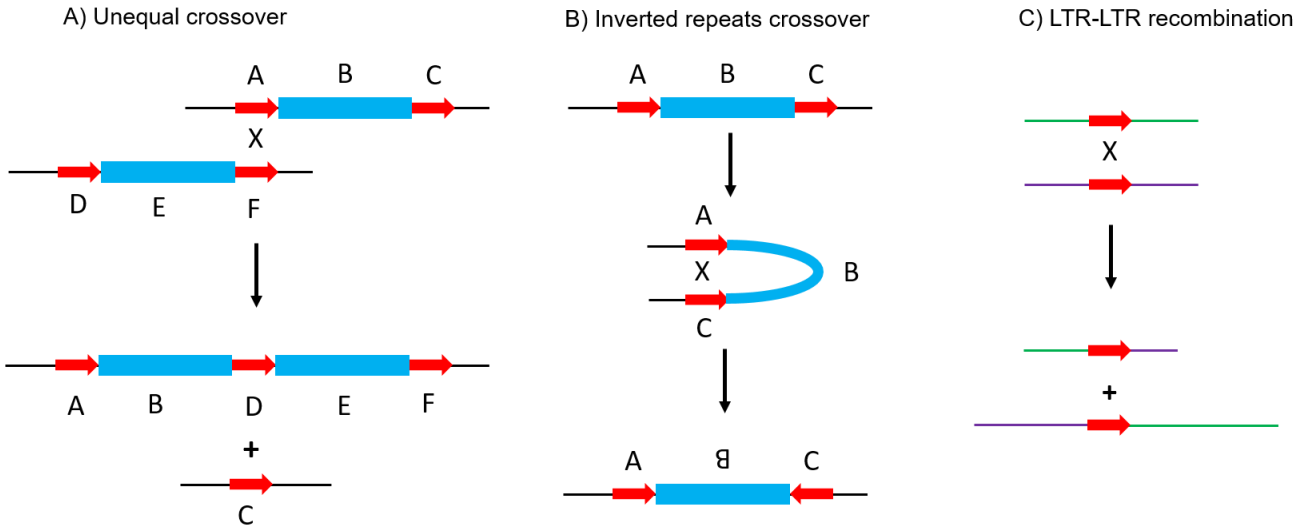


Figure 1.7 Chromosome rearrangements with repeat sequence recombination.

Red arrows represent repeated sequences and orientation. Blue boxes represent TEs. Different chromosome sequence are shown as green and purple. (A) Unequal crossover between repeated sequences. Exchange of genetic material between misaligned direct repeats results in either deletion or duplication of transposable elements in between. (B) Inverted repeat crossover. A crossover event is occurred by the inversion of repeated sequences flanking the TE. (C) Crossover between different repeated sequences on different chromosomes. Chimeric chromosome are generated by the translocated sequences. (Adapted from Mieczkowski et al., 2006)

1.11 Retroelements and disease

The rearrangements of chromosomes and gene mutations described above resulting from transposable elements as well as endogenous retroviruses may damage the fitness of the host and can result in the development of disease states. In humans, there are at least 96 single-gene diseases associated with retrotransposon mobilization and it is predicted that more will be discovered (Kazazian, 2004; Hancks and Kazazian, 2012). A study of Neurofibromatosis type I characterised 18 independent L1 retrotransposon insertions which cluster in three hotspot sites (Wimmer *et al.*, 2011). L1 site-specific mutation is also observed in leukaemia (Kaiser, 2003). The other potentially harmful retroelements in human are Human endogenous retroviruses (HERVs), which integrated themselves into the host germline cell genomes to perform vertical infection. Increased levels of HERV proteins has been observed under a variety of conditions such as diabetes, schizophrenia, leukaemia, multiple sclerosis and teratocarcinoma (Biemont and Vieira, 2006). However, there is no definitive proof that HERVs contribute to the pathology of these diseases. Nonetheless, HERVs which have lost their ability to complete cell-to-cell infection or transposition life cycles, have been linked to azoospermia, a disease caused by the deletion of one of the three regions in the human Y chromosome. This causes, spermatogenic failure and infertility because the AZF-1 gene is removed by a non-reciprocal recombination event involving HERV-1 (Kamp *et al.*, 2000; Sun *et al.*, 2000). Recombination between *Alu* elements in humans is also responsible for a whole range of human genetic disorders (Kim *et al.*, 2016)

Uncontrollable gene expression that leads to cancer could also potentially be brought about by transposons (Hughes and Coffin, 2005; Feinberg *et al.*, 2006). In mouse, the regulatory sequence of an ERV LTR can upregulate a high level of transcription in ageing tissue and cell lines that is associated cancer in mice (Maksakova *et al.*, 2011). Also, expression of L1 elements promotes double-strand chromosome breaks (DSB) and a greater number of L1-induced DSBs than the number of successful insertions is observed (Gasior *et al.*, 2006). Moreover, a high level of L1 expression is also linked to DNA damage, apoptosis signalling and senescence induction in some cell lines (Belgnaoui *et al.*, 2006; Wallace *et al.*, 2008; Rodriguez-Martin *et al.*, 2020).

1.12 Host benefits from retroelements

Although retroelements are potentially hazardous they have also assisted the restructuring and reorganisation of eukaryotic genomes and have contributed to evolution. This suggests that the host may have been received some benefits from these elements and therefore maintained their existence under neutral or positive selection. Theoretically, retroelements without infectious ability act only as parasites should be eliminated from the genome during evolution. However, that most organisms discovered have not excluded mobile elements from their genome, suggests that retrotransposons may also be of benefit to their host (Capy *et al.*, 2000). Detailed analysis has revealed that in some cases transposon DNA and proteins have been domesticated by their host (McClintock, 1984). This process has been termed "exaptation" (Volff, 2006). Indeed, retroelements have been suggested to play a role in assisting the host cell respond to external environmental signals (reviewed by Feschotte, 2008). Transposons also showed a significant impact to host genome in germ-line cells. A transcriptional derepression event occurred during specific stages of development of germline of animals and plants by a programmed TE reactivation mechanisms to desilencing precision development of TE (Maupetit-Mehouas and Vaury, 2020).

As discussed further below, the expression of transposons is commonly upregulated under stress conditions. For example the expression of the *Tnt1* element in tobacco *Nicotiana tabacum* is induced after various biotic and abiotic stresses (Grandbastien *et al.*, 1997). In *Drosophila*, both the *412* element and *mariner* element are induced in expression after a change of temperature (Giraud and Capy, 1996; Vieira and Biemont, 1996). SINE and LINE expression was reported to be upregulated by genotoxic poisons, radiation, heat shock, viral infection, and heavy metals (Farkash and Luning Prak, 2006). Later, studies have illustrated numerous ways that retroelements can directly influence the regulation of neighbouring gene expression (Feschotte, 2008). An example in human suggested that HERVs assist the expression of host genes by recruiting p53 with their p53 binding sites within the LTR and thereby influence the transcriptional activation of the closest adjacent gene (Wang *et al.*, 2007). In *S. pombe* the upregulation of some genes in response to hypoxia is controlled by binding of the Sre1 transcription factor to a nearby *Tf2* LTR (Sehgal *et al.*, 2007). Furthermore, the *Tf1* LTR element acts as transcriptional enhancer which is activated by heat shock

and oxidative stress and has been suggested to increase transcription of adjacent genes in some contexts (Feng *et al.*, 2013). Although the L1 retrotransposon creates double-strand breaks when integrating into the genome, they also provide DNA repair mechanisms after integration (Morrish *et al.*, 2002; Goodier and Kazazian, 2008; Stribinskis and Romos, 2010).

1.13 The regulation of transposons in host genome

Although transposons can benefit their host, uncontrolled retrotransposition can also be harmful. Therefore, host cells have evolved many diverse mechanisms and marshalled various forces to regulate retrotransposons activity (Fig. 1.8).

1.13.1 DNA methylation

Methylation of cytosine residues in DNA plays an important role in suppressing gene expression, especially retrotransposons in some eukaryotes. This DNA modification mechanism is employed in some eukaryotes including vertebrates, flowering plants and some fungi (Levin and Moran, 2011). In these organisms, retrotransposons are often directly methylated to repress transcription and the demethylation of the genome re-activates retroelement transcription. (Goll and Bestor, 2005; Maksakova *et al.*, 2008; Tsukahara *et al.*, 2009). In mice, the maintenance DNA methyltransferases Dnmt1, and *de novo* methyltransferases Dnmt3a and Dnmt3b form a defence by methylating the intracisternal A particle (IAP) retrotransposons in embryos and germ cells (Walsh *et al.*, 1998; Kato *et al.*, 2007). The loss of Dnmt3L, a Dnmt3a/b homologue without a catalytic domain, results in to the loss of *de novo* cytosine methylation across all types of retrotransposons in mice and their transcription level is induced in spermatocytes and spermatogonia (Bourc'his and Bestor, 2004). In plants, 24 nt RNAs target particular sequences with homology to retroelements for cytosine methylation (Levin and Moran, 2011). In human, hypomethylation has been linked to activation of L1 in cancer cells (Daskalos *et al.*, 2009). Despite the difference in methylation status between different tumours, hypomethylation was showed to be associated with both cancer progression and development (Wilson *et al.*, 2007). In addition, DNA methylation and the downstream RNAi machinery has been proposed to play a

germline-specific role in regulating transposons in mice and human (Aravin *et al.*, 2008; Kuramochi-Miyagawa *et al.*, 2008; Mugat *et al.*, 2020).

1.13.2 RNAi

RNA silencing mechanisms are commonly employed to regulate endogenous gene expression (Du and Zamore, 2005) and contribute to viral defence in *Drosophila* (Galiana-Arnoux *et al.*, 2006; Wang *et al.*, 2006), insects (Kolliopoulou *et al.*, 2019), plants (Zhu *et al.*, 2019) and humans (Schuster *et al.*, 2019). This RNA mediated silencing has also been shown to regulate repetitive DNA and retroelements. RNA interference (RNAi) uses short RNAs to bind target homologous RNA sequences and direct their degradation or translation suppression. There are a few RNAi pathways including micro-RNAs (miRNA), small-interfering RNAs (siRNA), repeat-associated small interfering RNAs (rasiRNAs), piwi-interacting RNAs (piRNAs) and endogenous siRNA (endo-siRNA) (Vasselon *et al.*, 2013).

miRNAs gene are transcribed by RNA Pol II to produce long pre-miRNAs and is maturated by cleavage to form double-stranded hairpin-loop pre-miRNA. This pre-miRNA is then translocated to cytoplasm for further processing by the ribonuclease Dicer in the cytoplasm to form mature miRNAs (Church *et al.*, 2017). These miRNA are bound by Argonaute proteins in a large multiprotein RNA induced silencing complex (RISC) complex. RISC is then targeted to cellular RNA sequences that are homologous to the miRNAs (Du and Zamore, 2005). Degradation of cellular RNA is directed by Argonaute-miRNA activity cluster mRNA processing bodies (P-bodies) and the disruption of P-bodies is linked to a reduction in RNAi mediated degradation.(Sen and Blau, 2005).

siRNA can be of endogenous or exogenous origin and commonly functions in the cellular defence against molecular parasites such as viruses and retroelements. After infection by, viral dsRNA is processed as a part of viral defence system. siRNAs can also be employed by cells to modulate gene expression in a tissue- or development-stage specific manner. In some organisms such as fission yeast siRNA can also play a role in maintaining genome structure and organisation by establishing and maintaining histone marks (Martienssen *et al.*, 2005; Okazaki *et al.*, 2018).

siRNAs from repeat sequences have been termed rasiRNAs. In eukaryotes, the development of 24-27 nt long sense and antisense rasiRNA was suggested to be one of the major class of RNAi mechanisms that plays a role in repressing both LTR and non-LTR retrotransposons from yeast to plant and mamml (Aravin and Tuschl, 2005; Vagin *et al.*, 2006; Ruiz-Ferrer *et al.*, 2018). In plant, rasiRNA participates as the first line of defence against transposons through a RNA-directed DNA methylation (RdDM) that directs the precise methylation of retroelements (Xie and Yu, 2015). To perform target gene silencing, RNA polymerase IV (Pol IV) is recruited to the silenced transposon by identification of the transcription repressing H3 lysine 9 methylation mark. The transposon transcript is converted into double-stranded RNA and then processed by Dicer cleavage into 24 nt rasiRNA. Associated with Argonaute 4 and 6 protein, the rasiRNA-protein complex then directs chromatin modification (Ruiz-Ferrer *et al.*, 2018).

piRNAs are a unique type of RNA that are expressed in the germline (Wang *et al.*, 2019). This special form of RNAi is expressed by genomic loci (piRNA clusters) that encode precursor RNAs of transposons and are processed into 24-32 nt length single-stranded RNA without the requirement of Dicer. This precursor RNA is then bound to an Argonaute protein subfamily called PIWI and undergoes a primary processing pathway and then is specifically amplified through a 'ping-pong' cycle which amplifies the complementary sequence that targets the active transposon (Siomi *et al.*, 2011). Studies in *Drosophila* revealed that a large proportion of the piRNAs are generated a derived from transposon sequences and target transposon mRNA (Vagin *et al.*, 2006; Huang *et al.*, 2017; Czech *et al.*, 2018).

1.13.3 Chromatin

A range of chromosomal modification and remodelling events can suppress the transcription of transposons. It has been reported that histone deacetylation is essential for transcriptional suppression of human LINE-1 elements in embryonic carcinoma cells (Garcia-Perez *et al.*, 2010). Furthermore, chromatin immunoprecipitation (ChIP) experiments in mice (Martens *et al.*, 2005; Mikkelsen *et al.*, 2007; Karimi *et al.*, 2011) have revealed that a range of elements, including, class I (e.g. MLV), class II (e.g. IAP and ETn/MusD) and endogenous retroviruses (ERVs) are enriched with methylation of lysine 9 on histone H3 (H3K9me) which is a classic

mark of heterochromatin. This modification promotes the recruitment of binding proteins such as HP1 homologues that mediate silencing of gene expression (Vermaak and Malik, 2009; Kwon and Workman, 2011b; Kwon and Workman, 2011a). Consistent with this, upregulation of transposon transcription was observed following mutation of the H3K9 methyltransferase *suv39* in mouse ES cells (Martens *et al.*, 2005). Histone H3 lysine 27 methyltransferases and the polycomb repressive complex have been also been identified as being required for retroviral LTR silencing in mammalian somatic tissues (Matsui *et al.*, 2010; Macfarlan *et al.*, 2011; Rowe and Trono, 2011).

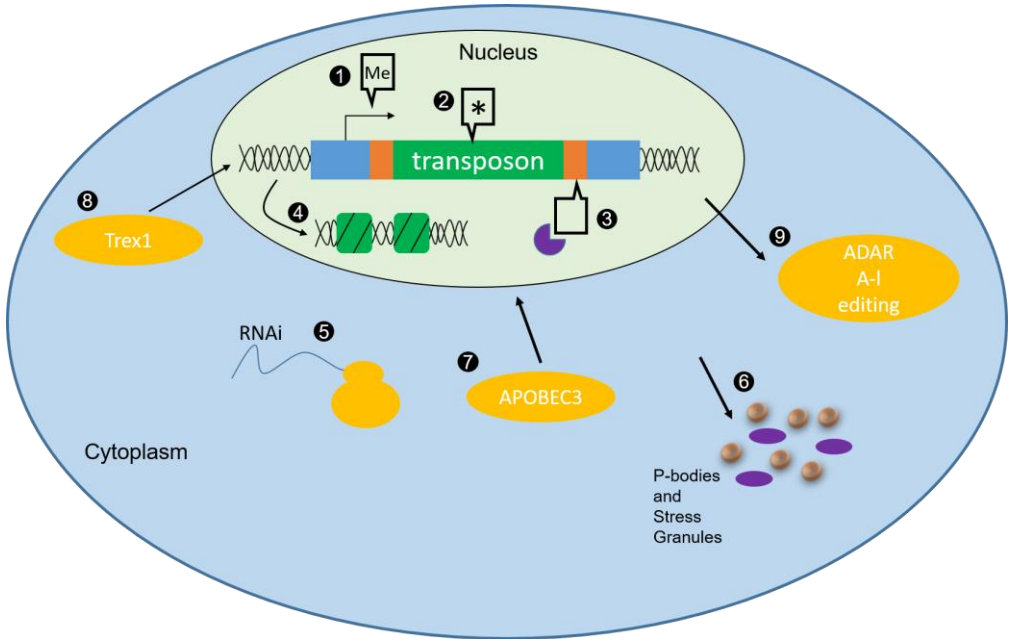


Figure 1.8 Host defences against retroelements.

1) DNA methylation represses transcription. 2) Mutation and rearrangement of coding region occur over time and inactivate or 'kill' the element. 3) Transcription stalling or premature termination results in in-complete, truncated or inactive transcripts. 4) Forming heterochromatin or non-classical repressive chromatin structures allows epigenetic silencing, preventing transcription and possibly recombination be tween elements. 5) RNAi mediated transcription silencing or RNA/cDNA degradation. 6) Transcripts can be sequestered in P -bodies and stress granules and 7) form VLPs containing APOBEC3 cytidine deaminase for repression by unclear mechanisms. 8) Trex1 nucleases degrades single -stranded DNA to prevent accumulation of cDNA for reverse transcription. 9) Adenosine to Inosine editing by ADARS editing. (Adapted from Goodier and Kazazian 2008).

1.14 Regulation of *Ty* element in *S. cerevisiae*

Expression of *Ty1* mRNA accounts for ~0.1-0.8% of the total polyadenylated RNA in *S. cerevisiae*, but despite this, the transposition frequency is relatively low (~1 event in every 10^7-10^8 cells) and VLPs and *Ty1* cDNA can hardly be detected (Curcio *et al.*, 1988). Sequence analysis reveals that majority of the *Ty1* elements are intact and with no mutations that should render them inactive (Curcio and Garfinkel, 1994) indicating that these elements are regulated at, a post-transcriptional level (Conte *et al.*, 1998; Lee *et al.*, 1998). At least five genetic screens have been carried out in *S. cerevisiae*, employing different approaches and identifying up to 130 factors that affect the mobilization of the *Ty1* or *Ty3* retrotransposons (Scholes *et al.*, 2001; Griffith *et al.*, 2003; Aye *et al.*, 2004; Irwin *et al.*, 2005; Risler *et al.*, 2012).

A *Ty1* mobilization assay with a mTn3 *lacZ/LEU2* bacterial transposon mutation library was used to identify genes that restrict *Ty1* retrotransposition (Scholes *et al.*, 2001). Twenty genes involved in DNA damage response, telomere maintenance and components of the MRX genome maintenance pathways were identified with mutants showing at least a 5-fold increase in *Ty1* mobilization (Scholes *et al.*, 2001). In addition, all these genes identified were suggested to involve post-transcriptional regulation of *Ty1* element since only a minimal impact on mRNA and cDNA levels was observed in these mutants (Scholes *et al.*, 2001).

Another screen was carried out by using the *S. cerevisiae* gene deletion library to search for *Ty1* regulators (Griffith *et al.*, 2003). In contrast to the previous screen using a natural genomic loci, a donor plasmid harbouring a *Ty1* element under the control of the *GAL1* promoter was introduced into deletion strains for the retrotransposition assay. In this screen, 101 mutants were identified that had altered levels of retrotransposition. Furthermore, among the 101 mutants identified, only 2 induced TE transposition while the remaining 99 repressed *Ty1* mobility (Griffith *et al.*, 2003). It is worth noting that the *Ty1* element expressed by the *GAL1* promoter overrides a post-translational repression of *Ty1* mobility (called copy number control) and results in ~1000 fold increase in retrotransposition. This explains why, mainly mutants that reduce retrotransposition, (which correspond to genes encoding activation factors of *Ty1* mobilization) were identified. This screen revealed that chromatin mutants had no effect on *Ty1* targeting but rather they are altering a different retrotransposition

process. (Griffith *et al.*, 2003). Half of the mutants showed no alteration in cDNA level while the other half showed an decreased *Ty1* cDNA (Griffith *et al.*, 2003).

A third screen employed a different approach to search through the haploid gene deletion collection. Instead of using *GAL/Ty1*, a *S. cerevisiae* strain either harbouring a *rtt101Δ* or *med1Δ* allele which confer a hypertransposition phenotype were employed. (Risler *et al.*, 2012). *RTT101* encodes a cullin-component of an E3 ligase and promotes replication fork progression through DNA damage, while *MED1* encodes a non-essential subunit of the RNA Pol II mediator complex involved in transcriptional regulation. The removal of either gene stimulates cDNA production and some subsequent step (or steps) in the *Ty1* life cycle. (Risler *et al.*, 2012). 275 retrotransposition host factors (RHF) were identified, where 45 factors were previously identified from other screens. 181/275 factors were involved in post cDNA synthesis steps while 43/275 RHF genes influenced *Ty1* cDNA levels. These included genes encoding specific ribosomal and ribosome biogenesis proteins and also RNA degradation, modification and transport proteins. Specially, four ribosome biogenesis mutants *bud21Δ, hcr1Δ, loc1Δ,* and *puf6Δ* showed reduced levels of *Ty1* Gag but not RNA (Risler *et al.*, 2012).

Little overlap was identified between the different *Ty1* screens. One possible reason is that the objective of the first two screens is quite different. One focused on the identification of restriction (repressive) factors (Scholes *et al.*, 2001) while another looked for activating factors that are required for the element to complete its life cycle (Griffith *et al.*, 2003).

A screen identifying *S. cerevisiae Ty3* regulators was also undertaken using a*GAL1/Ty3* plasmid based mobilization assay. This screening was conducted by using mTn3 insertion mutagenesis (Aye *et al.*, 2004). 25 genes were identified as encoding *Ty3* regulatory factors but surprisingly, they showed almost no overlap with the previously identified regulators of *Ty1*. This suggests that retroelement specific regulation may be in operation, but more likely demonstrates that each screen favours the identification of a special area or particular group of regulators.

Another screen on *Ty3* was carried by using a *HIS3* marked *Ty3* element and the *S. cerevisiae* haploid ORF deletion library (Irwin *et al.*, 2005). Factors involved in vesicular trafficking, RNA processing, DNA maintenance and nuclear transport were

identified, suggesting that retrotransposition was not limited by the level of *Ty3* proteins. The nuclear pore was identified as one of the key regulators of *Ty3* mobility and this is possibly related to the control of nucleus access by cDNA (Irwin *et al.*, 2005).

Further analysis was carried out to identify genes involved in the regulation of *Ty1* and *Ty3* retrotransposons. A*Ty1-his3AI* cassette with a natural *Ty1* promoter on plasmid and the *S. cerevisiae* haploid ORF deletion library was employed (Nyswaner *et al.*, 2008). It was suggested that genes that involved in RNA expression and processing, protein translation and folding, and trafficking were all required for *Ty1* retrotransposition while genes involved in chromatin structure, and the DNA damage response restrict the mobility of both *Ty1* and *Ty3* elements. The study suggested that some aspects of regulation are conserved between different elements and their hosts (Nyswaner *et al.*, 2008)

1.14.1 Post-transcriptional regulation

Although *S. cerevisiae* lacks RNAi machinery, one of the regulatory mechanisms of *Ty1* elements was shown to involve RNA. Antisense *Ty1* transcripts reduce IN and RT protein level post-transcriptionally to inhibit *Ty1* transposition and the copy number of the element (Matsuda and Garfinkel, 2009).

Human APOBEC3G (hA_3G) protein was showed to regulate the *Ty1* element life cycle when expressed in *S. cerevisiae*. Interaction between hA_3G and the *Ty1* Gag protein was followed by incorporation into the *Ty1* VLP in a similar manner to HIV-1 (Dutko *et al.*, 2005; Schumacher *et al.*, 2008). In mammalian cells, the interaction between A_3G and translation repression proteins and mRNA facilitates co-localization with P-bodies and stress granules (Gallois-Montbrun *et al.*, 2007). Previous studies have shown that P-bodies are associated with VLP assembly in both *Ty1* and *Ty3* retroelements. The *Ty* mRNA, and Gag proteins localise to P-bodies (Beliakova-Bethell *et al.*, 2006; Chiu and Greene, 2008). In addition, the proteins that associate with P-bodies were shown to be required for efficient *Ty3* retrotransposition (Irwin *et al.*, 2005; Beliakova-Bethell *et al.*, 2006), suggesting that the assembly of VLPs occurs within P-bodies.

A restriction mechanism called copy number control (CNC) was suggested for *Ty1* elements (Garfinkel *et al.*, 2016). It was observed that the retrotransposition rate decreases as element copy number increases (Garfinkel *et al.*, 2003). CNC

mechanisms which limit the number of elements in a genome have also been identified in other organisms such as *Drosophila* (Chaboissier et al., 1998) and *E. coli* (Johnson and Reznikoff, 1984). Studies that mutated different regions of the *Ty1* sequence revealed that the 5' region of *Ty1* especially the *GAG* gene is essential to establish CNC in *Ty1* (Garfinkel *et al.*, 2003; Saha *et al.*, 2015). Two novel proteins are encoded from the C-terminal half of *GAG*, p22 (22 kDa protein) and p18 (18 kDa protein) both of which inhibit retrotransposition. These proteins are initiated from two AUG codons halfway into *GAG*, and were shown to repress *Ty1* mobility ~35,000 fold (Saha *et al.*, 2015). The p22/p18 proteins alter other *Ty1* proteins such as IN and RT, they affect VLP assembly, decrease VLP yield, destabilize Pol and its processing, promote proteolysis of Gag, disrupt reverse transcriptase activity and prevent IN from maturation (Garfinkel *et al.*, 2016). Interestingly, both *Ty1* Gag and p22 share a nucleic acid chaperone domain while playing opposing roles in retrotransposition. Moreover, p18 competes with Gag for the same binding site of *Ty1* RNA (Pachulska-Wieczorek *et al.*, 2016).

1.14.2 Stress induced activation of *Ty* elements

There is increasing evidence indicating that *Ty* retroelements are activated under particular environmental stress conditions (Wessler, 1996; Capy *et al.*, 2000; Todeschini *et al.*, 2005; Nyswaner *et al.*, 2008). A response of retrotransposons to stress has been reported in plants and human (Beauregard *et al.*, 2008; Hunter *et al.*, 2013; Grandbastien, 2015; Negi *et al.*, 2016). In addition, activation of mammalian ERVs has been observed in response to infection and injury (Cho *et al.*, 2008). Therefore, it is not surprising that *Ty1* mobilization is influenced by exposure to environmental stresses such as ionising radiation, DNA damage, mating pheromone and nitrogen starvation (McClanahan and McEntee, 1984; Bradshaw and McEntee, 1989; Todeschini *et al.*, 2005; Mieczkowski *et al.*, 2006). The activation of *Ty1* expression and mobility could also upregulate nearby gene transcription by allowing differing transcription initiation from cryptic sites in the LTR (Todeschini *et al.*, 2005), reflecting that importance of transcription regulation from retrotransposons. In *Ty5*, integration site preference is changed upon the environmental stress. *Ty5* targets coding regions instead of silenced heterochromatin regions within the host genome

under conditions of amino acid, nitrogen or fermentable carbon starvation (Dai *et al.*, 2007).

1.15 Regulation of *S. pombe Tf* elements

Recently, a genome-wide screen was carried out to locate factors that contribute to *Tf1* transposition (Rai *et al.*, 2017). Using a *Tf1* element expressed from a multi-copy plasmid, 61 genes that promote integration were identified which were involved in nuclear transport, transcription, mRNA processing, vesicle transport, chromatin structure and DNA repair (Rai *et al.*, 2017). Moreover, a number of the proteins and pathways identified were found previously for *S. cerevisiae Ty1* and *Ty3* retrotransposons to promote integration, suggesting that the host factors that contribute to integration are common in distantly related organisms (Rai *et al.*, 2017). It worth noting that in this case the element is overexpressed from the *nmt* promoter which would circumvent native transcriptional controls. As such the screen described above is restricted to the identification of factors that contribute to the *Tf1* life cycle at a posttranscriptional level.

1.15.1 Transcriptional silencing of *Tf2* elements

A common strategy to restrict retrotransposon is to embed them in repressive chromatin in order to restrict their expression and limit their spread. In *S. pombe,* a number of proteins have been implicated in the transcriptional silencing of *Tf2* elements. Although *S. pombe* contains an RNAi machinery which is important for heteorochromatin silencing (Martienssen and Moazed, 2015), it only plays a very minor role in restricting *Tf2* expression (Hansen *et al.*, 2005). Furthermore, both H3K9 methylation and the heterochromatin protein 1 (HP1) homologue Swi6, (typical of RNAi-dependent heterochromatin regions), were not found to be associated with *Tf2* elements in genome-wide chromatin immunoprecipitation experiments (Cam *et al.*, 2005). Similarly, the mutation of RNAi factors and the H3K9 methyltransferase Clr4 have only a very mild effects on *Tf2* expression (Hansen *et al.*, 2005) and do not effect mobilization (Murton, 2012). Interestingly, in another fission yeast *Schizosaccharomyces japonicus,* a close relative of *S. pombe*, LTR retrotransposons do elicit a robust RNAi response and a large portion of LTR retrotransposon mRNAs are processed into siRNA for transposon silencing (Rhind *et al.*, 2011). Although RNAi

and heterochromatin factors do not play major role in restricting *Tf2* mobility in wild type cells, in the absence of the exosome a strong RNAi response is observed,*Tf2* mRNA is processed into siRNA and *Tf2* sequences become associated with H3K9 methylation.

The current evidence suggest that *Tf2* elements are subjected to a distinct form of transcriptional silencing that is mediated by specific histone chaperones, histone modifying enzymes and homologues of human centromere binding protein B (CENP-B) (Esnault and Levin, 2015).

CENP-B is thought to have evolved from the ancient Pogo/Tigger Class II transposon and is highly conserved from yeast to human (Volff, 2006). In human, CENP-B proteins localize to the pericentromeric alpha satellite repeats and bind to 17 bp CENP-B sequences for centromere formation (Masumoto *et al.*, 2004). Three CENP-B homologues have been identified in *S. pombe*: autonomously replicating sequence binding protein 1 (Abp1), CENP-B homologue 1(Cbh1) and Cbh2 (Murakami *et al.*, 1996; Lee *et al.*, 1997; Irelan *et al.*, 2001). They are enriched at the centromere region and localization of Abp1 and Cbh1 to *Tf2* elements has also been shown from genome-wide analysis (Cam *et al.*, 2005; Lorenz *et al.*, 2012). Deletion of Abp1 and Cbh1 induces the expression of both *Tf1* and *Tf2* and in turn, contributes to increased *Tf1* transposition (Cam *et al.*, 2008).

CENP-B proteins contribute to*Tf2* silencing by preventing RNA pol II binding to *Tf2* LTRs (Cam *et al.*, 2008; Lorenz *et al.*, 2012; Daulny *et al.*, 2016). Full-length Abp1 binds to two 10bp-AT-rich domains, which are found at the 3' end of the *Tf2* LTR and recruits the class I histone deacetylase (HDAC) Clr6 and the class II HDAC Clr3 (Cam *et al.*, 2008; Lorenz *et al.*, 2012). All three domains of Abp1 together, provide functions of high-order organisation, DNA sequence recognition and binding, protein stabilization and act as a platform for the association of other proteins, such as the H3K4 (Histone H3 at lysine 9) methyltransferase Set1 (Lorenz *et al.*, 2012). Together, Abp1 and Set1 repress the expression of both sense and antisense strands of *Tf2.*

In addition to mediating transcriptional repression, CENP-B proteins are also required for the physical clustering of *Tf2* elements into subnuclear structures called *Tf* bodies (Cam *et al.*, 2008). The formation of *Tf* bodies requires the recruitment of the Ku heterodimer complex of Ku70 and Ku80 and the condensin complex (Tanaka *et al.*,

2012). While *Tf* body formation is not required for transcriptional silencing, it has been proposed to restrict mobilization of *Tf* elements (Murton *et al.*, 2016).

The repression of *Tf2* elements also requires the HIRA histone chaperone complex, a conserved chromatin assembly factor, composed of Hip1, Slm9, Hip3 and Hip4 (Kanoh and Russell, 2000; Blackwell *et al.*, 2004; Anderson *et al.*, 2009; Anderson *et al.*, 2010). The HIRA complex also interacts with another H3-H4 histone chaperone Asf1 to mediate chromatin *Tf2* silencing. Indeed, loss of any of these proteins results in a dramatic increase in *Tf2* RNA (Greenall *et al.*, 2006; Anderson *et al.*, 2010; Yamane *et al.*, 2011). However, investigation of *Tf2* mRNA expression and mobilization frequency showed that while the expression of *Tf2* retrotransposons is increased dramatically only a very modest increase of mobilization was observed in HIRA mutants (Murton *et al.*, 2016). It is proposed that this is because *Tf* bodies are not disrupted by loss of HIRA (Murton *et al.*, 2016).

Interestingly, previous studies have also demonstrated that both HIRA and Asf1 in human are required for HIV latency by associating with other chromatin assembly factors that represses HIV transcription (Gallastegui *et al.*, 2011).

In addition, ATP-dependent chromatin remodellers regulate the accessibility of DNA by altering the nucleosome positioning and composition and are therefore involved in all DNA-dependent processes. In fission yeast, a family of Fun30 chromatin remodelers called Fft1, Fft2 and Fft3 have been identified (Stralfors *et al.*, 2011; Steglich *et al.*, 2015). Fun30 family members in other organisms have proved to be involved in transcription repression (Byeon *et al.*, 2013). Interestingly, the removal of Fft2 and Fft3 (*fft2Δfft3Δ*) or disruption of their catalytic subunits enhances expression of *Tf2*. This results from impaired positioning of nucleosomes over the *Tf2* transcription start site (Persson *et al.*, 2016). In addition, Fft2 and Fft3 regulate gene expression adjacent to solo LTRs by recruiting nucleosomes in stress conditions (Persson *et al.*, 2016).

Post transcriptional regulation of *Tf2* mRNA has also been identified as both sense and antisense *Tf2* transcripts are tightly controlled (Mallet *et al.*, 2017). The exoribonuclease subunit Rrp6 and the nuclear poly(A)-binding protein Pab2 regulate *Tf2* expression by preventing *Tf2* RNA accumulation via an RNAi-dependent pathway (Mallet *et al.*, 2017).

1.15.2 Regulation of *Tf* elements in response to stress

In *S. pombe*, transcription of *Tf2* elements is known to respond to stress. Microarray experiments indicated that *Tf2* mRNA levels increase in response to oxidative and heat stress (Chen *et al.*, 2003). Furthermore, transcription of *Tf2* elements is induced in response to hypoxic conditions (Sehgal *et al.*, 2007). This is mediated by a homologue of human sterol regulatory element binding protein (SREBP) called Sre1 (Espenshade and Hughes, 2007). Sre1 in *S. pombe* is required for growth under anaerobic conditions and regulates hypoxia responding genes (Todd *et al.*, 2006). Under conditions of oxygen sufficiency, Sre1 is membrane associated, but low oxygen results in cleavage of the N-terminal region which is localized to the nucleus and binds to the sterol response element (SRE) in target promoter regions which include *Tf2* LTRs. Anaerobic conditions therefore markedly increase *Tf2* transcription and mobilization and also the transcription of some genes that are adjacent to solo LTRs (Sehgal *et al.*, 2007).

Previous analysis has also linked *Tf1* elements to stress responses. While *Tf1* expression is stimulated by heat and oxidative stress, most of the neighbouring genes are not activated except for those which also induced by the same stress, suggesting a synergized enhancing response to a particular stress (Feng *et al.*, 2013). Furthermore, *Tf1* targets stress response promoters for integration which, potentially improves survival chance of cells (Esnault and Levin, 2015). During the course of this study *S. pombe Tf1* mobility was shown to be increased when cells are exposed to heavy metals, caffeine and the plasticizer phthalate (Esnault *et al.*, 2019). Furthermore, 255 genes adjacent to the *Tf1* insertions were analysed and the results were separated into five classes. Importantly, three of the five groups were significantly enriched for genes that are associated with the target of rapamycin (TOR) pathways (Esnault *et al.*, 2019). In addition, a recent study revealed that the homologues of the human tumour suppressor *TSC* genes that encode GTPase RHEB activating proteins regulate the expression of *Tf2* retrotransposons in *S. pombe* (Nakase and Matsumoto, 2018). This is interesting as TSC proteins regulate TOR signalling pathways. The loss of TSC1 or TSC2 in mammalian cells leads to constitutive activation of RHEB which then targets mTOR for continuous activation (Matsumoto *et al.*, 2002; Nakase *et al.*, 2006). Loss of either *tsc1*$^{+}$ or *tsc2*$^{+}$ triggers *Tf2* induction upon nitrogen starvation. In

addition, *Tf2* proteins were found to be degraded via autophagy, which is controlled by the Tor2 kinase, (Nakase and Matsumoto, 2018).

1.16 Rapamycin and Target of rapamycin (TOR)

Rapamycin, (also known as Sirolimus) is a natural antibiotic isolated from *Streptomyces hygroscopicus* NRRL 5491 from Easter Island (Rapa Nui) in 1972. It was reported to have antifungal activity (Sehgal *et al.*, 1975; Vezina *et al.*, 1975) and was later identified as an immunosuppressive drug (Mukherjee and Mukherjee, 2009). The activity of rapamycin is brought by binding to a family of intracellular receptors, termed FK506 binding proteins (FKBPs) by its effector domain, Rapamycin forms a surface with FKBP that interacts with, and inhibits, target of rapamycin (TOR) kinases (Koltin *et al.*, 1991; Van Duyne *et al.*, 1991).

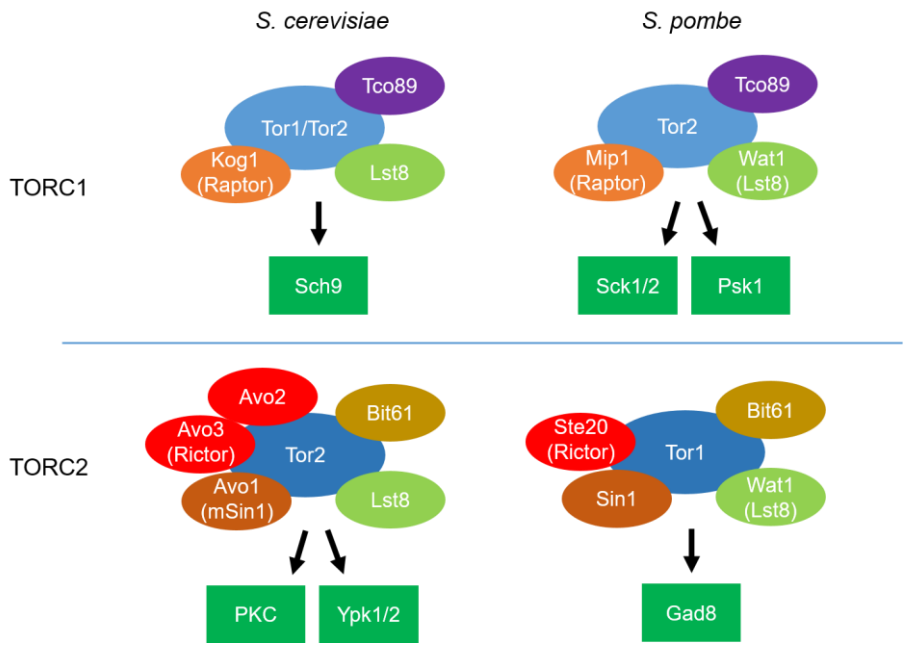


Figure 1.9 TORC1 and TORC2 subunits and downstream ACG kinases in *Saccharomyces cerevisiae* and *Schizosaccharomyces pombe* cells.

TORC1 and TORC2 complexes have shared and unique components. Homologues are indicated by colour. The target kinases of TORC1 and TORC2 are represented as green rectangles. Adapted from (Weisman, 2016).

1.17 The architecture of TOR and TOR complexes

TOR kinases are highly conserved in eukaryotes. However, unlike higher eukaryotes (nematodes, flies, mice, and human) where only one TOR gene is present, early studies in yeast revealed two genes, (*TOR1* and *TOR2*) and studies of these yeast genes provided the framework to the understanding of the more complex mammalian TOR (mTOR) system (Helliwell *et al.*, 1994). TOR kinases are members of the phosphatidylinositol 3-kinase-related kinase family. (Keith and Schreiber, 1995; Schmelzle and Hall, 2000). TOR kinase possess a number of characteristic sequence motifs, N-terminal HEAT repeats (derived from **h**untingtin, **e**longation factor, the **A** subunit of PP2A, and **T**OR1), a FAT (**F**ocal **A**dhesion **T**arget) domain, a FPB (**F**KBP-**R**apamycin **B**inding) domain, a Serine/Threonine kinase domain and a FACT (**FAT** domain in **C**-terminal) domain (Loewith and Hall, 2011). Sequence alignment suggested that *S. cerevisiae* TOR1 (*Sc*Tor1) and TOR2 (*Sc*Tor2) are highly conserved in sequence and the structural alignment of their HEAT repeats while in *S. pombe*, a divergence of HEAT repeats arrangements was observed in TOR1 (*Sp*Tor1) and TOR2 (*Sp*Tor2), suggesting that *Sp*TOR1 and *Sp*TOR2 do not shared the same structure and may have distinct functions (Weisman *et al.*, 2007; Shertz *et al.*, 2010).

Besides their known protein kinase function, they also function as evolutionary conserved scaffolds with several protein-protein interaction domains that mediate multi-protein complex formation (Alvarez-Ponce *et al.*, 2009). The major differences between Tor kinases of different species are the N-terminal HEAT repeats that are present in various number and function with the C-terminal conserved FATC domain as a scaffolding structure for protein-protein interactions (Kobe *et al.*, 1999). The FRP domain, is a highly conserved 100 amino acid region of Tor and residue S1975 is essential for the binding of the FKBP-rapamycin complex while residues W2041 and F2048 are critical for rapamycin interaction (Lorenz and Heitman, 1995; Shertz *et al.*, 2010). Meanwhile, mutation of L2031, F2309, and Y2105 of mTOR abolish the binding of phosphatidic acid, (Shertz *et al.*, 2010).

TOR kinases are found in two distinct evolutionarily conserved complexes termed TOR complex 1 (TORC1) and TOR complex 2 (TORC2) (Fig. 1.9). In both cases a TOR acts as the central catalytic subunit and their function is accompanied by TORC1- and TORC2-specific subunits as well as protein subunits that are shared between the

two complexes. The *Sc*TORC1 complex consists of either *Sc*Tor1 or *Sc*Tor2, essential protein Lst8, essential protein Kog1 and non-essential protein Tco89 while *Sc*TORC2 consists of scaffolding protein Avo1 and Avo3, Tor2, Lst8 and non-essential Avo2 and Bit61 (Loewith *et al.*, 2002; Wullschleger *et al.*, 2005). *Sp*TORC1 is composed of Lst8 homologue Wat1, *Sp*Tor2, Tco89 and the Kog1 homologue, Mip1 while *Sp*TORC2 consists of the Rictor homologue, Ste20, Sin1, *Sp*Tor1, Bit61 and Wat1 (Weisman, 2016).

1.18 The molecular mechanism of rapamycin

The drug-protein interaction between rapamycin and TOR depends on interaction with the peptidyl-prolyl cis-trans isomerase FKBP12 (FK506 binding protein 12) which was isolated as binding to the immunosuppressive drug FK506 (Kino *et al.*, 1987; Siekierka *et al.*, 1989). FKBP12 is a 12-kDa cytosolic protein (FKBP12) with an ability to catalyse *cis-trans* isomerization of peptidyl-prolyl bonds in peptides and proteins and the catalytic activity is inhibited by the binding of drug and drug-protein complex (Siekierka *et al.*, 1989). The crystal structure of human FKBP12-rapamycin complex showed that the pipecolinyl ring of rapamycin interacts with the hydrophobic cavity between the α-helix and β-sheet of FKBP12 (Abraham and Wiederrecht, 1996). FKBP12 homologues were isolated in *S. cerevisiae* and *S. pombe*, which are called Fpr1 and Fkh1, respectively. The disruption of these proteins confers rapamycin resistance (Koltin *et al.*, 1991; Weisman *et al.*, 2001). The Fpr1-rapamycin complex (rapamycin hereafter) targets a 196 amino acid fragment (amino acids 1886-2081) of *Sc*TOR2 with the critical Ser^{1975} (Stan *et al.*, 1994). Likewise, rapamycin targets a similar serine-containing domain in *Sc*TOR1 and any mutations of the serine residue confers rapamycin resistance (Zheng *et al.*, 1995). Surprisingly, despite the fact that Tor2 is able to bind rapamycin, rapamycin does not inhibit TORC2 in *S. cerevisiae* and only TORC1 is rapamycin-sensitive (Loewith *et al.*, 2002). The rapamycin insensitivity was shown to be the Fpr1-rapamycin binding site in TORC2 which is masked by the subunit Avo3 (Rictor) and prevents binding of the complex, meanwhile, no evidence suggested any role of Fpr1 in TOR signalling (Gaubitz *et al.*, 2015).

One remarkable feature of *S. pombe* is that rapamycin does not inhibit cell growth but some physiological process such as sexual development and amino acid uptake are inhibited (Weisman *et al.*, 1997; Weisman and Choder, 2001; Weisman *et al.*, 2005).

Further analysis showed that TORC1 activity is rapamycin sensitive and is inhibited by Fkh1-rapamycin complex in the FBP region of Tor2 and mutation of Ser^{1837} increases rapamycin resistance (Takahara and Maeda, 2012). The failure of rapamycin to inhibit cell growth has been attributed to incomplete inhibition of TORC1 activity. Also, unlike *Sc*TORC2, only part of the *Sp*TORC2 related function is rapamycin sensitive and the sensitivity is suppressed by either deletion of $fkh1^{+}$ or introduction of a rapamycin-binding defective *tor1* allele (Weisman *et al.*, 2005) but in general, the novel cellular roles of TORC2 are rapamycin insensitive (Schonbrun *et al.*, 2009).

1.19 The signalling cascade of TOR

The two distinct TOR complexes respond to different cellular conditions. In short, TORC1 is the master regulator of growth and starvation responses while non-essential TORC2 plays a role in cellular metabolism, growth and survival. Compared with the relatively well understood specific signals and mechanisms that operate downstream of TOR, the upstream stimuli are poorly understood (Weisman, 2016).

1.19.1 The downstream signalling of TORC1

The sensitivity of TORC1 to rapamycin provides an excellent tool for in dissecting the cellular functions of this complex. Inhibition of *Sc*TORC1 results in a phenotype that is similar to starved cells, including arrest at the G1 phase of the cell cycle, G0-like cellular morphology and physiology, repression of ribosomal gene expression, rapid drop in protein synthesis, induction of stress- and nutrient-starvation genes, and stimulation of autophagy (Barbet *et al.*, 1996; Noda and Ohsumi, 1998; Beck and Hall, 1999; Cardenas *et al.*, 1999). Similarly, *Sp*TORC1 disruption leads to phenotypes that specifically resemble nitrogen-starved cells including growth arrest at the G1, a small and round cell morphology, induction of nitrogen-starvation-induced genes, and activation of the sexual development pathway (Alvarez and Moreno, 2006; Uritani *et al.*, 2006; Hayashi *et al.*, 2007; Matsuo *et al.*, 2007; Weisman *et al.*, 2007).

A key substrate of TORC1 in *S. cerevisiae* is the AGC kinase Sch9, a homologue of mammalian S6 kinase 1 (S6K1) (Powers, 2007; Urban *et al.*, 2007). Six phosphorylation sites in the C terminal of Sch9 were identified to be directly phosphorylated by TORC1 resulting in increased stability and a change into an active

conformation (Powers, 2007; Loewith and Hall, 2011). Upon nitrogen, carbon, or phosphorylation starvation, high salt, high temperature, aberrant redox conditions, amino acid starvation or low nitrogen source quality, Sch9 becomes dephosphorylated and induces specific responses (Urban *et al.*, 2007; Stracka *et al.*, 2014). Other downstream targets of *Sc*TOR1 are the type 2A (Pph21, Pph22 and Pph3) and 2A-related phosphatases (Sit3, Sit4, Ppg1), which regulate the activity of a family of GATA transcription factors (Loewith and Hall, 2011; Weisman, 2016). Exposure to reduced quality or quantity of nitrogen source, causes the inactivation of *Sc*TORC1, and dissociation of the regulatory subunit Tap42 from the PP2A and PP2A-like phosphatases. This activates the phosphatases which in turn dephosphorylate GATA-transcription factors Gln3 and Gat1 causing translocation into the nucleus (Di Como and Arndt, 1996; Beck and Hall, 1999; Cardenas *et al.*, 1999; Jiang and Broach, 1999). Gln3 and Gat1 are the transcription factors required for transcription activation of genes that are normally repressed in the present of a high quantity/quality nitrogen source. Also they are critical regulators of nitrogen catabolite repression, where high-quality nitrogen sources are imported and assimilated in preference to poor-quality nitrogen sources (Weisman, 2016).

Other non-AGC kinase and PP2A kinase targets of TORC1 were also identified in budding yeast including the autophagy regulating serine/threonine kinase Atg1 (Kamada *et al.*, 2000; Kawamata *et al.*, 2008; Kabeya *et al.*, 2009). Upon nitrogen deprivation or rapamycin treatment, Atg13, the essential regulatory unit of the Atg1 kinase complex, rapidly losses its highly phosphorylated status and promotes the formation of the Atg1 complex and results in autophagy induction (Kamada *et al.*, 2000). In fission yeast, a similar autophagy pathway was identified. The key protein Atg13 is also highly phosphorylated under nutrient-rich conditions and loses its phosphorylation state after nitrogen starvation, after the inactivation of TORC1 (Kohda *et al.*, 2007).

A total of 78 proteins encoded from 137 genes make up the yeast ribosome and TORC1 was identified to coordinate the regulation of these genes via several mechanisms (Lempiainen and Shore, 2009; Loewith and Hall, 2011). Transcription of ribosomal protein genes was thought to be regulated by the controlled association of the activator protein Ifh1 and its constitutively promoter-bound partner protein Fhl1, a forkhead-like transcriptional factor (Martin *et al.*, 2004; Schawalder *et al.*, 2004; Wade

et al., 2004; Rudra *et al.*, 2005). The forkhead DNA binding domain of Fhl1, the DNA binding protein Rap1 and the high mobility group protein Hmo1 together promote constitutive association of Fhl1 to ribosomal protein (RP) gene promoters (Hall *et al.*, 2006; Berger *et al.*, 2007). TORC1 regulates RP gene transcription by controlling the interaction of Fhl1 with two FHB-containing phosphoproteins, the co-activator Ifh1 or the co-repressor Crf1 (Loewith and Hall, 2011). When TORC1 is active, Ifh1 is phosphorylated and binds to Fhl1 for RP transcription activation. In contrast, the inactivation of TORC1 triggers the displacement of Ifh1 with Crf1 to repress RP transcription (Loewith and Hall, 2011). The regulation of Ifh1 binding and phosphorylation is mediated though the AGC kinase Sch9 and an unknown protein because Sch9 does not directly interact with Ifh1 (Fig. 1.10) (Cai *et al.*, 2013; Albert *et al.*, 2016).

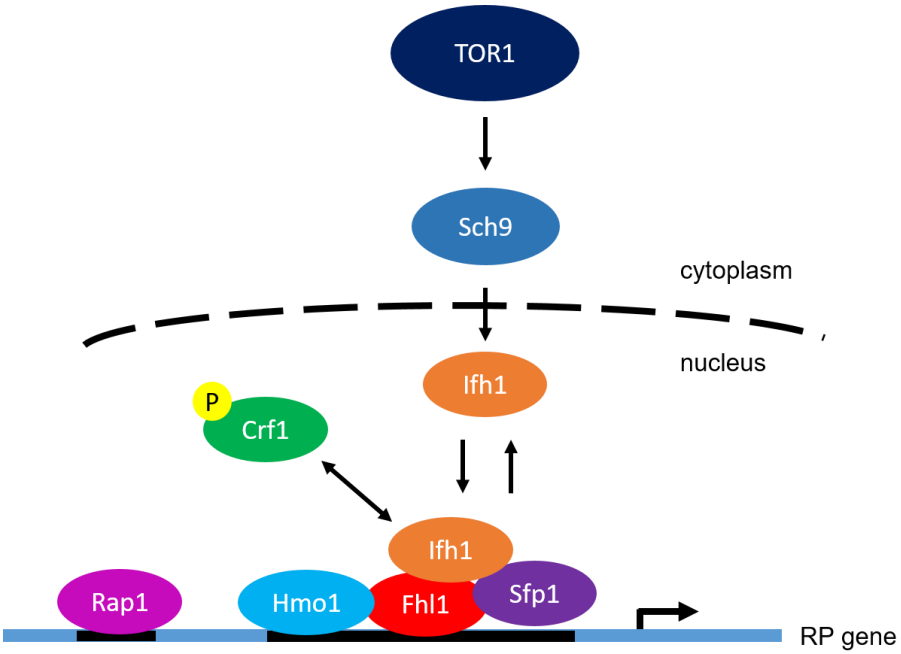


Figure 1.10 Schematic representation of the mechanism of RP gene expression that is regulated by TORC1 in *Saccharomyces cerevisiae*.

During exponential growth, the Sch9 kinase stimulates the activity of the coactivator Ifh1 to promote its binding on RP gene promoters to stimulate transcription. Upon nitrogen stress or rapamycin treatment TORC1 is inactive and phosphorylated Crf1 competes with Ifh1 for Fhl1 binding and represses transcription. Adapted from (Xiao and Grove, 2009; Albert *et al.*, 2016)

A Fhl1/Ifh1/Crf1 independent but TORC1-dependent regulation of RP gene transcription is also present in budding yeast and employs split zinc finger protein Sfp1 (Fingerman *et al.*, 2003; Marion *et al.*, 2004; Lempiainen *et al.*, 2009; Singh and Tyers, 2009). Sfp1 binds with TORC1 and its phosphorylation promotes binding to a subset of RP gene promoters regardless of osmotic or nutritional stress, suggesting that TORC1 regulates RP gene transcription by two different mechanisms (Lempiainen *et al.*, 2009).

An orthologue of *Sc*Fhl1 has been identified in *S. pombe* which is called Fhl1. Fhl1 also contains forkhead domains with forkhead associated domains (Szilagyi *et al.*, 2005). Genetic evidence suggests that *Sp*Fhl1 functions downstream of TORC1 and regulates al number of ribosomal, meiotic nitrogen-starvation and stress-response genes (Pataki *et al.*, 2017) However, the molecular mechanisms of Fhl1 function and regulation have not been investigated.

1.19.2 The upstream signalling of TORC1

In yeast cells, the activity of TORC1 is sensitive to a wide variety of stresses (high salt, redox stress, high temperature) and nutritional starvation, and mTORC1 is also sensitive to energy levels (ATP) and growth factors (Chantranupong *et al.*, 2015). To date, no direct signals or mechanism for TORC1 activation have been identified, however, evidence suggests that nitrogen sources and/or amino acids, which can also serve as a nitrogen source are the main stimuli for TORC1 activation (Weisman, 2016). The observation that the presence of rapamycin, causes yeast phenotypes that resemble nutrient starvation (a dramatic decrease in protein synthesis, autophagy induction and exit from the cell cycle and entrance into the quiescent G0 state) suggests that TORC1 is downstream of nutrient signal (Barbet *et al.*, 1996; Loewith and Hall, 2011). Transcription profiling further supports the evidence that TORC1 is responsive to the quality as well as the quantity of the nitrogen sources, where the activity of *Sc*TORC1 and *Sp*TORC1 are decreased in response to low levels or poor quality of the nitrogen source (Nakashima *et al.*, 2010; Laor *et al.*, 2014; Stracka *et al.*, 2014).

Nitrogen plays an essential role in the synthesis of amino acids, nucleotides, and other cellular components. Any high-quality nitrogen source (ammonium or glutamine) is able to promote rapid growth and repress nitrogen catabolite repression genes

(Cooper, 2002; Petersen and Russell, 2016). In addition, *Sc*TORC1 and *Sp*TORC1 also responds to specific amino acids such as leucine, glutamine, asparagine, arginine, aspartate, methionine, and cysteine (Bonfils *et al.*, 2012; Valbuena *et al.*, 2012; Stracka *et al.*, 2014; Peli-Gulli *et al.*, 2015), raising the possibility that the true nitrogen stressing component for TORC1 activation may be amino acids. Although amino acids were identified to be one of the stimuli of mTOR signalling (Hara *et al.*, 1998; Wang *et al.*, 1998; Jewell *et al.*, 2015), amino acids act through a different mechanism to the quantity/quality of the nitrogen sources (Stracka *et al.*, 2014; Davie *et al.*, 2015). Moreover, different amino acids seem to activate TORC1 with their unique pathways (*Sc*TORC1) (Bonfils *et al.*, 2012; Duran *et al.*, 2012).

TORC1 and some of its conserved upstream regulators were located at the membrane of the vacuole or the lysosome in higher eukaryotes (Eltschinger and Loewith, 2016). Residing either at the vacuole or lysosomal membrane, TORC1 is activated by two distinct guanosine triphosphate GTPases, Gtr (homologue of Rag in higher eukaryotes) and Rhb1 (Rheb in higher eukaryotes). The signalling cascade of Rag/Gtr-TORC1 is conserved across mammal, *S. cerevisiae* and *S. pombe* while the Rhb1/Rheb-TORC1 axis is only conserved in mammals and *S. pombe* but absent in *S. cerevisiae* (Fig. 1.11).

In *S. cerevisiae*, the Gtr complex is formed by Gtr1 (Rag A/B in mammal) and Gtr2 (Rag C/D in mammal). Activation of TORC1 by the Gtr complex can only be achieved by binding GTP to the Gtr1 or Rag A/B and GDP to the Gtr2 or Rag C/D heterodimer (Kim *et al.*, 2008; Sancak *et al.*, 2008; Binda *et al.*, 2009). The Gtr1-Gtr2 GTPase complex associates with the Ego (**e**xit from rapamycin-induced **gr**o**w**th arrest) complex, composed of Ego1, Ego2, and Ego3 which is anchored to the vacuolar membrane and tethers *Sc*TORC1 (Binda *et al.*, 2009; Kira *et al.*, 2016). The Ego complex is the homologue of the LAMTOR complex in higher eukaryotes that also resides on the lysosomal membrane and tethers mTORC1. A separate guanine exchange factor Vam6 was isolated in both *S. cerevisiae* and *S. pombe* that activates *Sc*TORC1 or *Sp*TORC1 in response to amino acids (Binda *et al.*, 2009; Valbuena *et al.*, 2012). However, the localization of both TOR complexes to the vacuolar membrane is nutrient independent (Binda *et al.*, 2009; Valbuena *et al.*, 2012).

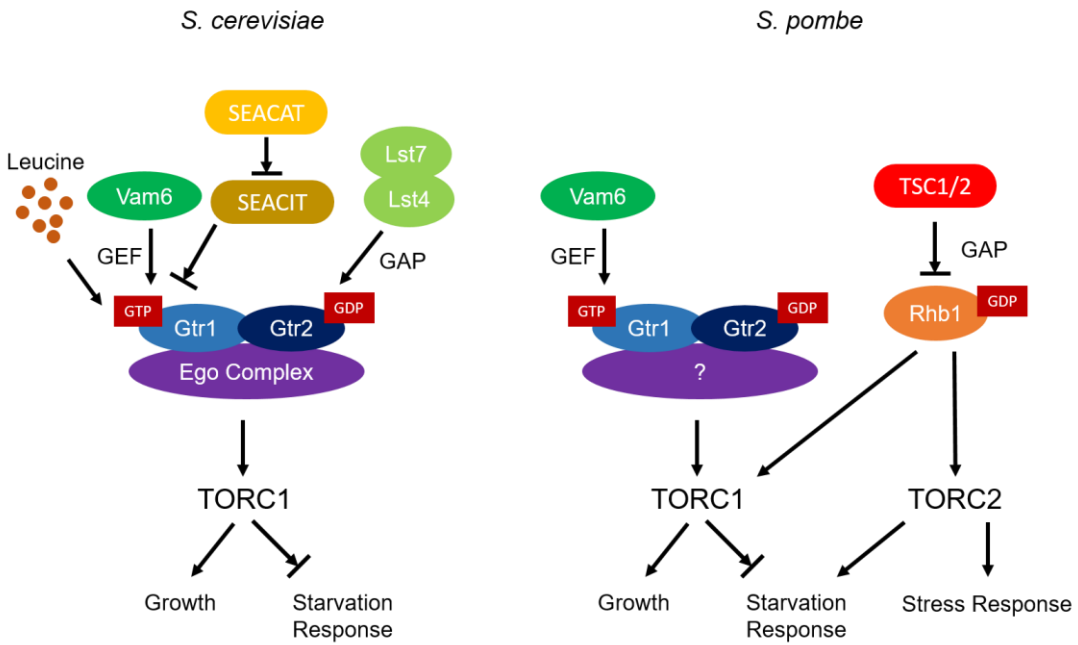


Figure 1.11 Upstream regulators of TOR in *S. cerevisiae* and *S. pombe*

*Sc*TORC1 and *Sp*TORC1 are activated by the GTPase complex Gtr1-Gtr2. The complex is activated when Gtr1 is bound with GTP and Gtr2 is bound with GDP. Vam6 is a guanine exchange factor (GEF) for Gtr1 that is conserved between both yeast. In *S. cerevisiae*, the Gtr1-Gtr2 complex is associated with the Ego (**e**xit from rapamycin-induced **gro**wth arrest) complex and is controlled by the SEACIT (**Se**h1-**a**ssociated sub**c**omplex **i**nhibiting **T**ORC1 signalling), SEACAT, leucine, and Lst4-Lst7 complexes. In *S. pombe*, either Ego, Lst4-Lst7 or SEACIT, SEACAT complexes equivalents are yet identified. *Sp*TORC1 is also regulated by the Rhb1 GTPase (mammalian Rheb) and the TSC (tuberous sclerosis complex, a tumour suppressor complex in mammals) that acts as a GAP towards Rhb1. The Tsc-Rhb1 signalling cascade also activates *Sp*TORC2 by direct binding. Adapted from (Eltschinger and Loewith, 2016; Weisman, 2016)

Leucine deficiency in *S. cerevisiae* promotes destabilization between Gtr1 and TORC1 and in turn, reduced Sch9 phosphorylation (Binda *et al.*, 2009). Similarly, the loss of Gtr1 reduces Sch9 phosphorylation with a slow growth and sick phenotype (Binda *et al.*, 2009). However, the disruption of *grt1/grt2* genes is not lethal in both *S. cerevisiae* and *S. pombe*, suggesting that they only play a limited role in TORC1 activation (Valbuena *et al.*, 2012). In *S. pombe*, the loss of *grt1/grt2/vam6* results in decreased TORC1 activity with a hyper-mating phenotype, but TORC1 is still responsive towards changes in the quantity or quality of the nitrogen sources (Valbuena *et al.*, 2012).

In *S. cerevisiae*, the Gtr complex is negatively regulated by the conserved GAP (GTPase activating protein) complex SEACIT (**Se**h1-**a**ssociatted sub**c**omplex **i**nhibiting **T**ORC1 signalling). This signalling regulation is conserved in higher eukaryotes with Rag complexes regulated by GAP GATOR1 (**GA**P activity **to**wards **R**ag**1**). Both of the complexes are negatively regulated by additional conserved complexes SEACAT (**Se**h1-**a**ssociatted sub**c**omplex **a**ctivating **T**ORC1 signalling) and GATOR2 (**GA**P activity **to**wards **R**ag**2**), respectively (Kira *et al.*, 2016). Another GAP Lst4-Lst7 complex was recently identified that acts through Gtr2 (Peli-Gulli *et al.*, 2015).

While in *S. pombe*, neither of the Lst4-Lst7 nor SEACIT-SEACAT GTPase-activating proteins was isolated, a homologue of human Rheb GTPase was identified (Mach *et al.*, 2000; Urano *et al.*, 2005; Uritani *et al.*, 2006). Disruption of Rhb1 showed a nitrogen starvation phenotype that is highly similar to the phenotype resulting from disruption of *Sp*TORC1 (Mach *et al.*, 2000; Yang *et al.*, 2001) Rhb1 is negatively regulated by the GAP TSC1-TSC2 (tuberous sclerosis complex) complex, which is conserved in humans (Urano *et al.*, 2005), suggesting that Tsc1/Tsc2 complex negatively regulates Rhb1. The deletion of the TSC complex caused a reduced transcription of nitrogen-starvation-induced amino acid permeases, prolonged phosphorylation of ribosomal S6 under nitrogen (Nakashima *et al.*, 2012), and reduced amino acid uptake (Matsumoto *et al.*, 2002; van Slegtenhorst *et al.*, 2004), leading to hyperactivation of *Sp*TORC1.

The signalling cascade of TSC-Rhb1 acts on several signalling pathways including AMPK, a serine/threonine kinase that coordinates cell growth and metabolism (Davie *et al.*, 2015). In fission yeast, nitrogen stress stimulates a decreased level of ATP, which in turn, increases AMPK activity to repress TORC1 activity though the TSC-Rhb1 signalling cascade (Davie *et al.*, 2015). A similar AMPK-dependent TSC-Rheb-

mTORC1 signalling pathway operates in mammalian cells (Inoki *et al.*, 2003; Jewell *et al.*, 2013).

1.19.3 The downstream signalling of TORC2

The participants in TORC2 pathways are less well understood compared with TORC1 due to the lack of a specific inhibitor and the finding that the phenotypes resulting from loss of TORC2 vary from organism to organism (Weisman *et al.*, 2014).

There is little overlap between the functions attributed to *Sc*TORC2 and *Sp*TORC2 *Sc*TORC2 is essential while *Sp*TORC2 is required only in under starvation and other stress conditions. The major substrates of *Sc*TORC2 are Ypk1 and Ypk2, an essential pair of homologous AGC kinases (Roelants *et al.*, 2004). In *S. pombe*, the AGC kinase substrate of TORC2 is Gad8 and overexpression of Gad8 can suppress a TORC2 defects (Matsuo *et al.*, 2003).

*Sp*TORC2 is required to perform two main responses to starvation, sexual development and the entry into stationary phase. As a result, cells that lack either TORC2 or Gad8 are highly infertile and die soon after exit from the logarithmic growth phase (Kawai M *et al.*, 2001; Weisman and Choder, 2001). In addition, the loss of TORC2-Gad8 causes sensitivity to a range of stresses such as low or high temperature, osmotic or oxidative stress, and DNA damage or replication stresses (Weisman and Choder, 2001; Ikeda *et al.*, 2008; Schonbrun *et al.*, 2009). During non-stress conditions, *Sp*TORC2-Gad8 is required for the G2/M transition and an elongated phenotype was observed in cells with disrupted *Sp*TORC2 due to a delay in entry into mitosis (Petersen and Nurse, 2007; Ikeda *et al.*, 2008; Ikai *et al.*, 2011). The loss of *Sp*TORC2-Gad8 also disables chromatin-mediated gene silencing and assembly of heterochromatic domains at subtelomeres, showing a decreased histone 3 lysine 9 dimethylation (H3K9me2) and histone 4 lysine 20 dimethylation (H4K20me2) but increased histone 3 lysine 4 trimethylation (H3K4me3) and histone 4 lysine 16 acetylation (H4K16Ac) (Cohen *et al.*, 2018). Similar observations were seen in the concerning the roles of *Sc*TORC2 and *Sp*TORC2 towards DNA damage sensitivity (Schonbrun *et al.*, 2009; Shimada *et al.*, 2013). In these situations, TORC2 induces survivability after DNA damage in a checkpoint independent manner. However, the mechanisms are different between budding and fission yeast in that TORC2 supresses

the level of DNA damage though actin cytoskeleton pathway in *S. cerevisiae* while no such a linkage was identified in *S. pombe* (Weisman et al., 2014).

1.19.4 The upstream signalling of TORC2

Compared with TORC1, the upstream regulation of TORC2 in yeast is poorly understood. The loss of *Sc*TORC2 does not confer a starvation-like phenotype, suggesting nutrient availability does not regulate *Sc*TORC2 (Loewith and Hall, 2011). However, the ribosome maturation factor Nip7, a homologue of mammalian mNip7 is required for TORC2 kinase activity (Zinzalla *et al.*, 2011). In mammalian cells, mNip7 is required for the direct activation of mTORC2 via ribosome maturation. This suggests that ribosomes ensure that TORC2 is only active in growing cells. *Sc*TORC2 localizes to the plasma membrane and is activated by plasma membrane stress which occurs from cell surface expansion or stress on the plasma membrane (Berchtold *et al.*, 2012). Therefore, a feedback loop mechanism is proposed that *Sc*TORC2 activity is triggered by membrane growth and in turn, regulates membrane biosynthesis (Eltschinger and Loewith, 2016). Visualisation of *Sp*TORC2 revealed that it has cytoplasmic and cortical localisation (Tatebe *et al.*, 2010). However, biochemical assays suggested that both Tor1 (the catalytic subunit of *Sp*TORC2) and Gad8 are found in the nucleus (Cohen *et al.*, 2016).

*Sp*TORC2 is activated by glucose stress but not nitrogen stress (Cohen *et al.*, 2014; Hatano *et al.*, 2015). An upstream GTPase Rhy1 is required to activate *Sp*TORC2-Gad8 in response to glucose (Hatano *et al.*, 2015). However, the regulation of *Sp*TORC2-Gad8 in response to glucose availability is rapid and requires no protein translation. *Sp*TORC2 activity is also regulated by the mitogen-activated protein kinase (MAPK) Sty1 (also known as Spc1, a homologue of Hog1 in *S. cerevisiae*) (Morigasaki *et al.*, 2019). The loss of Sty1 induces metabolites such as purine biosynthesis intermediates and nucleotide derivatives and showed a nuclear abnormal and viability phenotype which are rescued by rapamycin (Sajiki *et al.*, 2018). This indicates that a coordinated action between the SAPK and TORC2 pathways operates for fission yeast cells to survive environmental stress.

1.19.5 Cross-talk between TORC1 and TORC2

The differentiation response of fission yeast requires the presence of both mating types (h^- and h^+) and reduced nitrogen in the environment (Yamamoto, 1996). The reduced availability of nitrogen represses TORC1 activity which leads to increased mating by extension of the G1 phase and the induction of expression of the genes required for mating (Alvarez and Moreno, 2006; Uritani *et al.*, 2006). In contrast, TORC2 is essential during starvation and stress conditions. In the absence of TORC2-Gad8 cells are unable to arrest in G1, resulting in sterility (Kawai M *et al.*, 2001; Weisman and Choder, 2001). The expression of mating genes is regulated by transcription factor Fkh2, which is a substrate of the TORC2-Gad8 signalling cascade (Szilagyi *et al.*, 2005; Shimada *et al.*, 2008). The phosphorylation of Ser546 of Gad8 regulates differentiation responses and is countered by PP2A-B55^{Pab1}. As a result, active phosphorylated Gad8 is accumulates after the loss of PP2A-Pab1 repression, which is achieved by Ppk18-Igo signalling of the TORC1 pathway (Fig. 1.12) (Martin *et al.*, 2017; Martin and Lopez-Aviles, 2018). The loss of Ppk18 and Igo1 results in an inability to arrest in G1 in the absence of nitrogen and reduction in sporulation while the loss of PP2A-Pab1 leads to a hyperfertile phenotype (Perez-Hidalgo and Moreno, 2017). The connection between *Sp*TORC1 and *Sp*TORC2 with Ppk18-Igo1-PP2A-Pab1 explains the opposing roles of the two complexes (Weisman *et al.*, 2007) and suggests that the function of both TOR complexes is linked in a certain degree

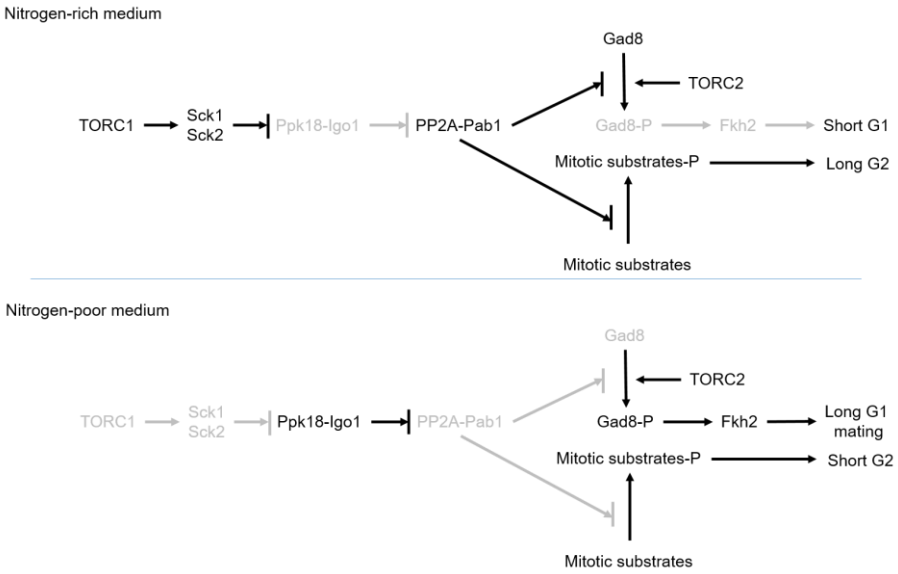


Figure 1.12 Current model of the cross-talk between TORC1 and TORC2 for differentiation in *S. pombe*

In nitrogen-poor conditions, PP2A-Pab1 is inactivated by the Ppk18-Igo1 pathway due to inactivation of TORC1-Sck1-Sck2. The inactivation of PP2A-Pab1s prevent the dephosphorylation and inactivation of Gad8, resulting in the extension of the G1 phase and sexual differentiation. Adapted from (Perez-Hidalgo and Moreno, 2017)

1.20 Aims

LTR retrotransposons share a similar life cycle to infectious retroviruses and present a threat to genomic integrity. Thus, host cells must tightly control of both expression and mobilization of retroelements. The molecular mechanisms that regulate these elements is only partially understood. Therefore, the aim of this study was to identify and characterise the host cell factors and the environmental stimuli that influence the activity of the *S. pombe Tf2* LTR retrotransposons. The initial aims were to *(i)* investigate the feasibility of a systematic screens to identify genes that regulate *Tf2* mobilization and *(ii)* to determine whether a copy number control mechanism operates in *S. pombe.* During these studies it was discovered that the composition of the growth medium impacts upon activity of *Tf2* retrotransposons and this finding this prompted an examination of the role of TOR signalling in the control of these elements.

Chapter 2 Materials and Methods

2.1 Strains and media

2.1.1 *Escherichia coli*

E. coli SURE cells (e14$^-$ *[mcrA$^-$]Δ [mcrB-hsdSMR-mrr] 171 endA1 supE44 thi-1 gyrA96 relA1 lac recB recJ sbcC umuC::Tn5[kan^r] ucrC[F'proAB lac^q Δmis Tn10 (Tet^{r}O)])* were grown in Luria-Bertani (LB) medium (2% [w/v] Bacto-tyrtone, 1% [w/v] Bacto-yeast extract. 1% [w/v] NaCl [pH 7.2]. Bacto-agar (2% [w/v]) was added if required for solid media. Ampicillin (Sigma) at a concentration of 0.1 mg/ml was added when required.

2.1.2 *S. pombe* strains and growth median

The *S. pombe* strains used in this study are listed in Table 2.1 and plasmids are listed in Table 2.2. *S. pombe* culture was performed in standard rich (YE5S) medium (0.5% [w/v] yeast extract, 3% [w/v] glucose and 225 mg/L adenine, histidine, leucine, uracil and lysine hydrochloride). 500 µg/ml Geneticin (G418) / 75 µg/ml ClonNAT / 200 µg/ml hygromycin B was added to sterilised media when required. When plasmid selection was required, cells were cultured in Edinburgh Minimal Medium (EMM) (5 g/L NH_4Cl, 3 g/L potassium hydrogen phthalate, 2.2 g/L Na_2HPO_4, 2% [w/v] glucose, 20 m/L salts [52.5 g/L $MgCl_2$•$6H_2O$, 50 g/L KCl, 2 g/L $NaSO_4$, 0.735 g/L $CaCl_2$.$2H_2O$], 1 m/L vitamins [10 g/L inositol, 10 g/L nicotinic acid, 1 g/L panthothenic acid, 10 mg/L biotin], 0.1 ml/L minerals [10 g/L citric acid, 5 g/L boric acid, 4 g/L $ZnSO_4$•$7H_2O$, 4 g/L $MnSO_4$, 2 g/L $FeCl_2$•$6H_2O$, 1 g/L KI, 0.4 g/L $CuSO_4$•$5H_2O$, 0.4 g/L molybdic acid] with the required amino acid supplements. Media was solidified by the addition of 2% [w/v] bacto-agar. For long-term cyrostorage, cells were inoculated into YE5S with 30% glycerol and stored at -80°C. Cells were incubated at the required temperature on the relevant solid agar plates for ~3 days. Liquid cultures were prepared by inoculating a single colony or loop of cells from a fresh agar plate in 5 ml of the appropriate media overnight with shaking at the required temperature. Cultures were then diluted to an OD_{595} 0.1-0.2 in the appropriate volume. Cell number was estimated by measuring of OD_{595} where OD_{595} 0.1 ≈ 2 x10^6 cells/ml.

Table 2.1 *S. pombe* strains

Strain Number	Other Name	Genotype	Source
SW5	*wt*	*h⁻ ade6-M216 leu1-32 ura4-D18*	Lab stock
SW4	*wt*	*h+ ade6-M216 leu1-32 ura4-D18*	Lab stock
HM19	*Tf2-12natAI*	*h⁻ ade6-M210 leu1-32 ura4-D18 his3D Tf2-natAI*	Lab stock
SW820	*Tf2-11natAI*	*h⁻ ade6M216 leu1-32 ura4-D18 Tf2-11-natAI*	Lab stock
HM284	*rhp51Δ Tf2-12natAI*	*h⁺ ade6-M210 leu1-32 ura4-D18 rhp51::ura4⁺ his3D Tf2-12natAI*	Lab stock
HM136	*Tf2-6 lacZ*	*h⁻ ade6-M216 leu1-32 ura4-D18 Tf2-6::lacZ(ura4⁺)*	Lab stock
HM137	*Tf2-11 lacZ*	*h⁻ ade6-M216 leu1-32 ura4-D18 Tf2-11::lacZ(ura4⁺)*	Lab stock
HM294	*sre1-N Tf2-6 lacZ*	*h⁻ ade6* leu1-32 ura4-D18 his* sre1-N(1-440aa)::kan Tf2-6::lacZ(ura4⁺)*	Lab stock
HM246	*sre1-N Tf2-12natAI*	*h⁻ ade6-M210 leu1-32 ura4-D18 Tf2-12natAI sre1N(1-440aa)::kan*	Lab stock
SW822	*Prototrophic wt*	*h⁺*	Lab stock
SW968	*Tf2-12natAI ura4⁺*	*h⁻ ade6-M210 leu1-32 ura4-D18 his3D Tf2-natAI (ura4⁺)*	This study
SW971	*sre1-N Tf2-12natAI ura4⁺*	*h⁻ ade6-M210 leu1-32 ura4-D18 Tf2-12natAI sre1N(1-440aa)::kan (ura4⁺)*	This study
CC1	*sre1-N Tf2-12natAI ura4⁺ hph*	*h⁻ ade6-M210 leu1-32 ura4-D18 Tf2-12natAI sre1N(1-440aa)::hph (ura4⁺)*	This study
SW904	*set1Δ Tf2-12natAI*	*h? ade6-M210 leu1-32 ura4-D18 his3D? set1::kan Tf2-12natAI*	Lab stock
SW1005	*Tf2-12natAI*	*h⁺ Tf2-12natAI*	This study
SW1006	*Tf2-6 lacZ*	*h⁺ Tf2-6 lacZ*	This study
CC2	*sre1-N Tf2-6 lacZ hph*	*h? ade6-M216 leu1-32 ura4-D18 Tf2-6::lacZ(hph⁺)*	This study
CC3	*hht2Δ*	*h⁺ hht2::kanMX6*	BIONEER v2.0
CC4	*red1Δ*	*h⁺ red1::kanMX6*	BIONEER v2.0
CC5	*cay1Δ*	*h⁺ cay1::kanMX6*	BIONEER v2.0
CC6	*nts1Δ*	*h⁺ nts1::kanMX6*	BIONEER v2.0
CC7	*fft3Δ*	*h⁺ fft3::kanMX6*	BIONEER v2.0
CC8	*xap5Δ*	*h⁺ xap5::kanMX6*	BIONEER v2.0
CC9	*pab2Δ*	*h⁺ pab2::kanMX6*	BIONEER v2.0
CC10	*set1Δ*	*h⁺ set1::kanMX6*	BIONEER v2.0
CC11	*pku80Δ*	*h⁺ pku80::kanMX6*	BIONEER v2.0
CC12	*rhp51Δ*	*h⁺ rhp51::kanMX6*	BIONEER v2.0
CC13	*nup124Δ*	*h⁺ nup124::kanMX6*	BIONEER v2.0
CC14	*clr4Δ*	*h⁺ clr4::kanMX6*	BIONEER v2.0
CC15	*rdp1Δ*	*h⁺ rdp1::kanMX6*	BIONEER v2.0
CC16	*rrl1Δ*	*h⁺ rrl1::kanMX6*	BIONEER v2.0
CC17	*set3Δ*	*h⁺ set2::kanMX6*	BIONEER v2.0
CC18	*exo1Δ*	*h⁺ exo1::kanMX6*	BIONEER v2.0
CC19	*hht2Δ*	*h⁺ hht2::kanMX6 Tf2-12natAI*	This study
CC20	*red1Δ*	*h⁺ red1::kanMX6 Tf2-12natAI*	This study
CC21	*cay1Δ*	*h⁺ cay1::kanMX6 Tf2-12natAI*	This study
CC22	*nts1Δ*	*h⁺ nts1::kanMX6 Tf2-12natAI*	This study
CC23	*fft3Δ*	*h⁺ fft3::kanMX6 Tf2-12natAI*	This study
CC24	*xap5Δ*	*h⁺ xap5::kanMX6 Tf2-12natAI*	This study

CC25	*pab2Δ*	*h⁺ pab2::kanMX6 Tf2-12natAI*	This study
CC26	*set1Δ*	*h⁺ set1::kanMX6 Tf2-12natAI*	This study
CC27	*pku80Δ*	*h⁺ pku80::kanMX6 Tf2-12natAI*	This study
CC28	*rhp51Δ*	*h⁺ rhp51::kanMX6 Tf2-12natAI*	This study
CC29	*nup124Δ*	*h⁺ nup124::kanMX6 Tf2-12natAI*	This study
CC30	*clr4Δ*	*h⁺ clr4::kanMX6 Tf2-12natAI*	This study
CC31	*rdp1Δ*	*h⁺ rdp1::kanMX6 Tf2-12natAI*	This study
CC32	*rrl1Δ*	*h⁺ rrl1::kanMX6 Tf2-12natAI*	This study
CC33	*set3Δ*	*h⁺ set2::kanMX6 Tf2-12natAI*	This study
CC34	*exo1Δ*	*h⁺ exo1::kanMX6 Tf2-12natAI*	This study
HM31	*Tf2 LTR*	*h- ade6-M216 leu1-32 ura4-D18 LTR-lacZ (ura4+):: chrm II*	Lab stock
HM60	*Tf1-lacZ*	*h- ade6-M216 leu1-32 ura4-D18 Tf1-lacZ (ura4+):: chrm II*	Lab stock
HM137	*Tf2-11 -lacZ*	*h- ade6 M216 leu1-32 ura4-D18 Tf2-11::lacZ(ura4+)*	Lab stock
HM369	*hip1∆ Tf2-12natAI*	*h- ade6 M? leu1-32 ura4-D18 hip1::??? Tf2-12natAI*	Lab stock
CC35	*tor2-51*	*h⁺ tor2-51:ura4+ ura4-d18 leu1-32 (BA120)*	Alvarez and Moreno, 2006
CC36	*tor1Δ Tf2-12natAI*	*h⁺ tor1::kanMX6 Tf2-12natAI*	This study
CC37	*tor1Δ lacZ*	*h⁺ tor1::kanMX6 Tf2-6::lacZ(ura4⁺)*	This study
CC38	*ste1Δ lacZ*	*h⁺ ste20::kanMX6 Tf2-6::lacZ(ura4⁺)*	This study
CC39	*ste1Δ Tf2-12natAI*	*h⁺ ste20::kanMX6 Tf2-12natAI*	This study
CC40	*fkh1Δ lacZ*	*h⁺ fkh1::kanMX6 Tf2-6::lacZ(ura4⁺)*	This study
CC41	*fkh1Δ Tf2-12natAI*	*h⁺ fkh1::kanMX6 Tf2-12natAI*	This study
CC42	*nfx1Δ lacZ*	*h⁺ nfx1::kanMX6 Tf2-6::lacZ(ura4⁺)*	This study
CC43	*nfx1Δ Tf2-12natAI*	*h⁺ nfx1::kanMX6 Tf2-12natAI*	This study
CC44	*gaf1Δ lacZ*	*h⁺ gaf1::kanMX6 Tf2-6::lacZ(ura4⁺)*	This study
CC45	*gaf1Δ Tf2-12natAI*	*h⁺ gaf1::kanMX6 Tf2-12natAI*	This study
CC46	*fhl1Δ lacZ*	*h⁺ fhl1::kanMX6 Tf2-6::lacZ(ura4⁺)*	This study
CC47	*fhl1Δ Tf2-12natAI*	*h⁺ fhl1::kanMX6 Tf2-12natAI*	This study
CC48	*crf1Δ lacZ*	*h⁺ crf1::kanMX6 Tf2-6::lacZ(ura4⁺)*	This study
CC49	*crf1Δ Tf2-12natAI*	*h⁺ crf1::kanMX6 Tf2-12natAI*	This study
CC50	*rap1Δ lacZ*	*h⁺ rap1::kanMX6 Tf2-6::lacZ(ura4⁺)*	This study
CC51	*rap1Δ Tf2-12natAI*	*h⁺ rap1::kanMX6 Tf2-12natAI*	This study
CC52	*sfp1Δ lacZ*	*h⁺ sfp1::kanMX6 Tf2-6::lacZ(ura4⁺)*	This study
CC53	*sfp1Δ Tf2-12natAI*	*h⁺ sfp1::kanMX6 Tf2-12natAI*	This study
CC54	*hmo1Δ lacZ*	*h⁺ hmo1::kanMX6 Tf2-6::lacZ(ura4⁺)*	This study
CC55	*hmo1Δ Tf2-12natAI*	*h⁺ hmo1::kanMX6 Tf2-12natAI*	This study
CC56	*pka1Δ lacZ*	*h⁺ pka1::kanMX6 Tf2-6::lacZ(ura4⁺)*	This study
CC57	*pka1Δ Tf2-12natAI*	*h⁺ pka1::kanMX6 Tf2-12natAI*	This study
CC58	*sty1Δ lacZ*	*h⁺ sty1::kanMX6 Tf2-6::lacZ(ura4⁺)*	This study
CC59	*sty1Δ Tf2-12natAI*	*h⁺ sty1::kanMX6 Tf2-12natAI*	This study

h$^{?}$ = mating type not determined. *ade6** = *ade6-M210* or *ade6-M216. his** = histidine marker not determined.

2.2 Genetic crosses and Random Spore Analysis

S. pombe strains of opposite mating types, h^+ and h^-, were streaked onto a rich YE5S agar plate and incubated overnight at the required temperature. A loop of each strain was mixed on an - EMM1/2G agar plate [as with EMM but replacing NH_4Cl with 0.5 g/L sodium glutamate] and incubated for 2-3 days at 25°C to allow conjugation, meiosis and the formation of tetrad asci. Sporulation was checked with light microscopy. Asci produced after mating were incubated in 1 ml of 0.5% [v/v] glusylase (Sigma) and incubated at 25°C overnight. Approximately 5 µl of this solution was diluted in 1 ml H_2O, mixed and 100 µl was taken and spread over YE5S plate and incubated at 30°C until colonies appeared. Genotypes of colonies were determined by selection on appropriately supplemented YE5S, or EMM agar.

2.3 DNA transformation

2.3.1 Preparation of competent *E. coli* and transformation with plasmid constructs

To a flask containing 200 ml of 2XLB medium, 2 ml of overnight *E. coli* SURE competent cells (Stratagene) were inoculated and shaken at 30°C for 2 hours. At OD_{595} = 0.2, sterile $MgCl_2$ was added to a final concentration of 20 mM and left to grow at 30°C until OD_{595} = 0.5. The culture was then incubated for 2 hours in an ice water bath. The cells were then pelleted by centrifugation at 4°C for 5 minutes at 900 x *g*. The cell pellet was resuspended in 100 ml of ice cold calcium/manganese medium (100 mM $CaCl_2$, 70 mM $MnCl_2$, 40 mM $C_2H_3O_2Na$, [pH 5.5]) and incubated in the cold room overnight. The cells were pelleted next day by centrifugation at 4°C for 5 minutes at 900 x g. The supernatant was discarded and the cell pellet was resuspended in 20 ml of ice cold calcium/manganese medium with glycerol (15% v/v). 200 µl aliquots of competent cells were transferred to pre-cooled eppendorf tubes, snap frozen in liquid nitrogen and stored at -80°C. Competent SURE cells were transformed with plasmid DNA by calcium chloride method (Sambrook, 1989). Transformed cells were plated on solid LB media (2% [w/v] bacto-tryptone, 1% [w/v] bacto yeast extract, 1% [w/v] NaCl pH 7.2) with 0.1 mg/mL ampicillin (Sigma-Aldrich) and incubated at 37°C overnight.

2.3.2 Transformation of *S. pombe*

S. pombe were transformed using the lithium acetate method described (Moreno *et al.*, 1991). Cells were cultured in YE5S at 30°C until they had reached exponential phase (OD_{595} 0.3-0.5) and then harvested by centrifuged at 3000 rpm (Mistral 2000, MSE) for 3 minutes. The cell pellet was washed with nH_2O and then washed with 1 ml of 1 x LiAc/TE (0.1M lithium acetate pH 7.5, 10 mM Tris-HCl pH 7.5, 1 mM EDTA pH 8.0) and resuspended in 1 ml 1 x LiAc/TE. Linearized DNA (~1 µg) or plasmid DNA (~0.5 µg) and 2 µl sonicated salmon sperm DNA (10 mg/ml) were added to 100 µl of competent cells and incubated at room temperature for 10 minutes. 260 µl PEG/LiAc/TE (0.1M lithium acetate pH 7.5, 10 mM Tris-HCl pH 7.5, 1 mM EDTA pH 8.0, 40% PEG-4000 [v/v]) was added to the solution and incubated for 60 minutes in 30°C. 43 µl 100% DMSO were added to these cells and the solution was subject to 42°C heat shock for 5 minutes. The mixture was then plated onto the appropriate EMM agar plate. For antibiotic selection, those cells were washed with 1 ml of H_2O and incubated in rich YE5S medium overnight before plating on YE5S plate supplemented with the appropriate antibiotic.

2.4 DNA isolation

2.4.1 Plasmid isolation from *E. coli*

A single colony of *E. coli* was incubated in 5 ml LB media with ampicillin (100 µM) and incubated overnight at 37°C. Isolation of plasmid DNA was achieved using GelElute Plasmid Miniprep (Sigma) kit in accordance with the manufacturer's instructions. Saturated overnight *E. coli* cultures were pelleted and lysed. Plasmid DNA was collected with membrane column and washed with ethanol-containing buffer. Plasmid DNA was then eluted with 100 µl nH_2O.

2.4.2 *S. pombe* genomic DNA isolation

S. pombe genomic DNA was extracted as described previously (Moreno *et al.*, 1991). 1 ml of cells from an overnight YE5S were pelleted at 9000 rpm in a microcentrifuge. The pellet was washed in 1 ml nH_2O and resuspended in 200 µl STET DNA breakage buffer (100 mM NaCl, 10 mM Tris-HCl pH 8.0, 1 mM EDTA pH 8.0, 2% Triton-X100 [w/v], 1% SDS [w/v]), 200 µl glass beads (0.5 mm, Biospec Products) and 200 µl

phenol:chloroform:isoamyl-alcohol (25:24:1). Cells were lysed with a Biospec mini-bead beater for 20 seconds at full power. 500 µl STET DNA breakage buffer was added to the lysate before centrifugation for 5 minutes at 13000 rpm. The supernatant was transferred to a fresh tube with 0.1 x volume 3 M sodium acetate [pH 5.2], and 2 x volumes of 100% ethanol. The Eppendorf tube was incubated at -20°C for 60 minutes, the DNA was then pelleted by centrifugation at 13000 rpm for 15 minutes. The pellet was then washed with 70% ethanol, allowed to air dry and then resuspended in 100 µl H_2O.

2.5 DNA Manipulation and analysis

2.5.1 Polymerase chain reaction (PCR)

PCR reaction was performed with Phusion PCR system (New England Biolabs). Oligonucleotide primers used in this study are listed in Table 2.3. Each 50 µl reaction contained 10 µl Phusion GC buffer, 1 µl DMSO, 20 µM dNTPs (including dATP, dCTP, dTTP, dGTP), 0.5 µM of each primers, ~50 ng genomic DNA (described in Section 2.4.2) or 1 µl of plasmid DNA (described in Section 2.4.1) and 1 unit of Phusion polymerase with nH_2O to make up to final volume. PCR reaction were performed in a T3 Thermocycler (Biometra).

2.5.2 DNA analysis by gel electrophoresis

DNA analysis was performed by agarose (1% [w/v]) gel electrophoresis in 1x TAE (40 mM Tris base, 20 mM acetic acid, 1 mM EDTA pH 8.0) with 2.5 µl ethidium bromide (Bio-rad, #161-0433) used to stain DNA. DNA ladders (Thermo Scientific, #SM0403) was used to illustrate the size of the DNA products. GeneJET Gel Extraction kit (Thermo Scientific) was used to extract DNA from agarose gel following the manufacturer's instructions.

2.6 RNA extraction, manipulation and analysis

2.6.1 RNA isolation and quantification

Cells were cultured in appropriate medium (OD_{595} ≈ 0.3) were harvested by centrifugation for 3 minutes at 3000 rpm in microfuge and the resulting pellet was snap frozen in liquid nitrogen and stored at -80°C until required. Pellets were resuspended

in 750 µl TES (10 mM Tris-HCl, 10 mM EDTA pH 8.0, 0.5% SDS [w/v]) and 750 µl acidic phenol:chloroform (25:24:1, pH 5.2), incubated for 1 hour at 65°C with rigorous vortexing for 10 seconds every 15 minutes. After incubation, samples were chilled on ice, vortexed for 20 seconds and then centrifuged at 4°C for 10 minutes at 13000 rpm in microfuge. The supernatant was transferred to a tube containing 750 µl acidic phenol:chloroform (25:24:1, pH 5.2), vortexed and centrifuged at 4°C for 10 minutes at 13000 rpm. The aqueous layer was collected to anther tube containing 750 µl phenol:chloroform:isoamyl alcohol (25:24:1, pH 6.4) and centrifuged at 4°C for 10 minutes at 13000 rpm in microfuge. The top aqueous layer (400 µl) was then transferred to an Eppendorf tube containing 40 µl 3 M sodium acetate pH 5.2 and 1 ml 100 % ethanol. RNA was precipitated at -20°C overnight. After centrifugation at 13000 rpm in microfuge for 15 minutes, the resulting pellet was washed once with 70% ethanol, air dried, and then finally resuspended in 50 µl nH_2O. The integrity of RNA extracted was analysed by running 5 µl aliquots on a 50 ml 1x TAE 1% agarose gel with 3 µl ethidium bromide and analysed using a UV transilluminator. The concentration of RNA was measured with the use of Nanodrop 1000 spectrophotometer (Thermo Scientific). Equal concentrations of RNA were achieved by diluting with nH_2O and absorbance was read at 260 nm and 280 nm with concentrations calculated in ng/µl.

2.6.2 DNase treatment of RNA

RNA extracted as described in Section 2.6.1 was treated with DNase with the Primerdesign Precision DNAse kit (DNAse-50, Primer design). DNA removal was performed using 45 µl of RNA, 5 µl precision DNase buffer with 1 µl of Precision DNase and incubated for 30 minutes at 30°C and the DNase was heat deactivated by incubating at 55°C for 5 minutes.

2.6.3 Reverse transcription and quantitative PCR (RT-qPCR)

RNA extracted and prepared as described in Sections 2.6.1 and 2.6.2 was used for reverse transcription and quantitative PCR. Reverse transcription was performed by using Precision Reverse Transcription Premix (Primer design, RT-Premix2-48) in accordance to manufacturer's instructions with 1 ng of RNA, and then diluted 10 fold. Quantitative PCR was performed with SYBR Green and was detected by a Rotor Gene

6000 Real-Time PCR machine using the following settings: Step 1 (Denaturation) 95°C for 10 seconds; Step 2 (Data Collection) 60°C for 60 seconds, with both steps being repeated 50 times. After the final step, a melt curve was generated in order to assess primer specificity. All expression levels displayed was normalised to *act1.* Primers used in qRT-PCR reactions are listed in Table 2.4.

2.7 Quantitative β-galactosidase assays

S. pombe cultures were grown in 30 ml of the required media at the required temperature until the OD_{595} = 0.3 - 0.5. 25 ml of each culture was collected by centrifugation at 3000 rpm for 3 minutes and the resulting pellet was resuspended in 600 µl of Z-buffer (60 mM Na_2HPO_4, 40 mM NaH_2PO_4, 40 mM KCl, 1 mM $MgSO_4$ pH 7.5) and snap frozen in liquid nitrogen and stored at -80°C until required. 50 µl of chloroform, 50 µl of 0.1% SDS and 600 µl of Z-buffer were added to an Eppendorf tube which were then pre-warmed to 30°C in a water bath. 200 µl of thawed cell resuspension was added to each tube and cells were permeabilised by vortexing for 30 seconds. 160 µl of ortho-nitrophenyl-β-D-falactopyranoside (ONPG, Sigma-Aldrich) (4 mg/ml in Z-buffer) was added to initiate the reaction. Eppendorf tubes were incubated at 30°C until the reaction mixture developed a yellow appearance, at which point 400 µl of 1 M Na_2CO_3 was added to terminate the reaction. Completed reactions were centrifuged for 10 minutes at 13000 rpm alongside a control mixture without any cells. The OD_{420} of the supernatant was measured alongside the OD_{595} of 30 µl of thawed cell suspension diluted with 970 µl of water in order to calculate the β-galactosidase activity as below:

$$\text{β-galactosidase (lacZ) Unit} = \frac{100{,}000 \times OD_{420}}{\text{Incubation time (min)} \times OD_{595} \times \text{volume of the cell used (µl)}}$$

2.8 *Tf2-12natAI* quantitative Mobilisation assays

A 6 ml aliquot of the required media was inoculated with a small loop of fresh cells and the cultures were incubated at 30°C with shaking or rotation for 2 nights, or until cultures reached saturation. 30 µl of the culture was used in ten-fold serial dilution and 100 µl of 10^{-5} dilutions were spread onto duplicateYE5S agar plates and incubated at 30°C for 3 days until colonies could be counted. A 5 ml aliquot of the saturated culture

was centrifuged for 3 minutes at 3000 rpm and resuspended in 500 µl nH_2O before being spread between two YE5S plates containing 75 µg/ml ClonNAT (Werner BioAgents). Plates were incubated at 30°C for 3-4 days until colonies appeared. The frequency of marked element mobilization was determined as the number of colonies gaining resistance to ClonNAT as a proportion of the total number of viable cells and subjected to fluctuation analysis using the method of the median (Lea and Coulson, 1949). Median values were calculated from a set of five assays. Mean values were calculated from at least 3 median values.

Table 2.2 Plasmid used in this study

Plasmid	Description	Source
pRep42	Empty vector control. *nmt* promoter, *ura4*$^+$ marker	Lab stock
pHL449-1	Overexpression of *Tf1* from *nmt* promoter	Dang *et al.,* 1999
pHL473	overexpression of *Tf1* allele with integrase encoding sequence deleted	From Henry L. Levin
pHL1260	Tf1-*neoA1* with deletion of amino acids 215 to 224 (ΔA)	Teysset *et al.,* 2003
pHL1262	Tf1-*neoA1* with deletion of amino acids 2 to 11, (ΔA)	Teysset *et al.,* 2003
pHL1264	Tf1-*neoA1* with deletion of amino acids 64 to 73 (ΔB)	Teysset *et al.,* 2003
pHL1258	Tf1-*neoA1* with deletion of amino acids 165 to 174 (ΔC)	Teysset *et al.,* 2003
pHL490-80	Tf1-*neoAI* with frameshift in PR	Dang *et al.,* 1999
pHL476-3	Tf1-*neoAI* with frameshift in IN	Dang *et al.,* 1999
pHL919-3	Tf1-*neoAI* with frameshift in RT	Dang *et al.,* 1999
pAL0122	ura4 5′ flanking sequence–hphMX4–ura4 3′ flanking sequence	Lorenz. A, 2015

Table 2.3 PCR Oligonucleotide primers

Primer name	DNA sequence 5'-3'
ura4$^+$ 5'	AAA TAG AGC TAC TGC TGG ACC
ura4$^+$ 3'	GTA AGG GTA CTA TTG CGT TAG
ura4$^+$ ck#1	TTC ATA CTG TAG TGG TAC GAG
ura4$^+$ ck#2	CAA GAG ATA TAG AGA AGC TGG
AL1 fw	AGC TAC AAA TCC CAC TGG
AL1 bk	GTG ATA TTG ACG AAA CTT TTT G
MX4/6cassUP	GAC ATG GAG GCC CAG AAT AC
MX4/6cassDWN	TGG ATG GCG GCG TTA GTA TC
hph diagnosUP	CAA TAG GTC AGG CTC TCG CTG

hph diagnosDWN	CAG AAC CAA CTT GAA CGA CCG

Table 2.4 qRT-PCR Oligonucleotide primers

Primer name	DNA sequence 5'-3'
act1 5'	GAA GTA CCC CAT TGA GCA CGG
act1 3'	CAA TTT CAC GTT CGG CGG TAG
Tf2 global 5'	GAT GGA ATT CAA ACA TCA GAC
Tf2 global 3'	CAT TTG TGA GTT TTA CCA TAC
isp4 5'	CTA GTA TGT TGT GGC CTG TG
isp4 3'	CAA GTG ACC CAA GCA AAT A

Chapter 3 Towards systematic genetic screens for factor that regulate *Tf2* mobilization

3.1 Introduction

Previously an assay has been established in the laboratory which allows the mobilization frequency of an endogenous *Tf2* element (*Tf2-12*) to be monitored (Murton, 2012; Murton *et al.*, 2016). A nourseothricin (ClonNAT) resistance cassette, interrupted by an artificial intron (*natAI*), was inserted into the non-coding region of *Tf2* adjacent to the 3' LTR (Fig. 3.1). The intron is orientated in the same transcriptional direction as that of *Tf2-12* (and opposite to that of the *nat* cassette). Therefore a functional *nat* cassette is generated after successful intron splicing from the *Tf2-12natAI* transcript and integration of the processed *Tf2* cDNA back into the genome (Fig. 3.1). Advantages of the employment of ClonNAT are that it offers a tight selection for fission yeast, as the spontaneous resistance frequency is extremely low ($<1 \times 10^{-12}$ events/cell) and selection is maintained even when cells are plated at high density (1×10^8 cells/per plate) (Murton, 2012).

3.2 Systemic genetic screens for host factors that regulate *Tf2* activity

The assay described above revealed that *Tf2* elements mobilize with only a very low frequency in wild type cells grown in rich medium (YE5S) under standard conditions (~2 mobilization events per 10^8 cells). This suggests that specific host cell proteins restrict *Tf2* mobilization. That, the loss of the histone methyl transferase Set1 or CENP-B proteins results in significantly increased mobilization is consistent with this notion and indicates that chromatin plays an important role in suppressing *Tf2* activity (Murton *et al.*, 2016). In *S. cerevisiae*, global genetic screens have been instrumental in the identification of factors that control the activity of the *Ty1* and *Ty3* LTR retrotransposons and have provided significant insight into retrotransposon biology (Scholes *et al.*, 2001; Griffith *et al.*, 2003; Irwin *et al.*, 2005; Nyswaner *et al.*, 2008). Commercial genome-wide mutant library is available for *S. pombe* from Bioneer (Kim *et al.*, 2010). This comprises a collection of more than 3000 haploid gene deletion strains. The availability of this library opened up the possibility for a systematic identification of factors that restrict *Tf2* mobilization. Therefore, an objective of this

work was to introduce the *Tf2-12natAI* reporter into the haploid gene deletion collection and isolate mutants with increased *Tf2* mobilization frequency (Fig. 3.2A).

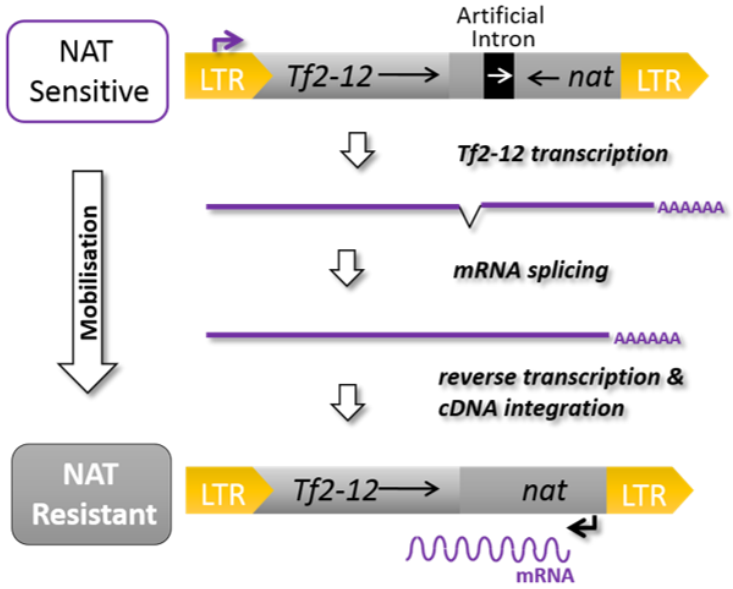


Figure 3.1 Schematic illustration of the Tf2-12natAI element.

The principle of the *Tf2-12natAI* reporter. The *nat* resistance cassette is disrupted by an artificial intron (AI) in the same orientation as the *Tf2-12* while in an opposite direction what *nat*. Therefore, the intron can be removed via splicing after mRNA transcription, originating from the *Tf2-12* LTR promoter. The production of cDNA followed by its integration into the genome results in the generation of a functional *nat* cassette and resistance to ClonNAT. Adapted from Murton (2012).

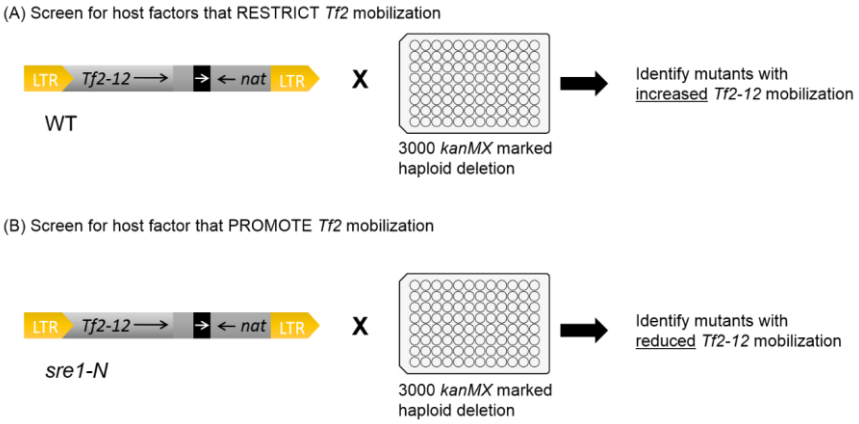


Figure 3.2 Schematic illustration of the proposed genetic screens.
Screen for factors that (A) restrict *Tf2* and (B) promote *Tf2*.

Genetic screens to identify cell factors that are required for *Tf2* mobilization are also of interest. The conservation between LTR retrotransposons and infectious retroviral life cycles means that the isolation of host cell factors that promote *Tf2* mobilization may reveal novel targets for antiretroviral drugs. However, the low frequency of *Tf2* mobilization when wild type cells are grown under standard condition (YE5S, 30°C) makes the identification of such mutants difficult.

A genetic background that provides a high starting level of *Tf2* activity would be beneficial to identify mutants that promote retrotransposon life cycle. Therefore, a *sre1-N* allele was employed which expresses a constitutively active form of the SREBP homologue, Sre1 which actives *Tf2* expression. Indeed, in the *sre1-N* background, *Tf2* mobilization is increased by ~45 fold (Hughes and Espenshade, 2008; Murton *et al.*, 2016).

3.3 Construction of *Tf2-12natAI* reporter strains compatible with high throughput genetic crosses.

In order for the *Tf2-12natAI* reporter to be introduced into the haploid gene deletion collection, it was first necessary to insert an *ura4*$^{+}$ selectable marker adjacent to the *Tf2-12natAI* reporter. A fragment containing the *ura4*$^{+}$ cassette flanked by a *Tf2* LTR and sequences immediately downstream of *Tf2-12* was amplified by PCR and introduced into *Tf2-12natAI* reporter strain. Correct integration of the *ura4*$^{+}$ marker was confirmed by PCR genotyping (Fig. 3.3). The resulting strains allows the cells that harbour the reporter to be selected for during automated genetic crosses using uracil prototrophy. In order to confirm that the *ura4*$^{+}$ marker does not affect the retrotransposition frequency of *Tf2-12natAI*, a mobilization assay was carried out. Comparison with the reference strain carrying the unmarked *Tf2-12natAI* reporter, showed no significant changes in retrotransposition frequency, indicating that *ura4*$^{+}$ marker does not interfere with the function of the *Tf2-12* element (Fig. 3.4).

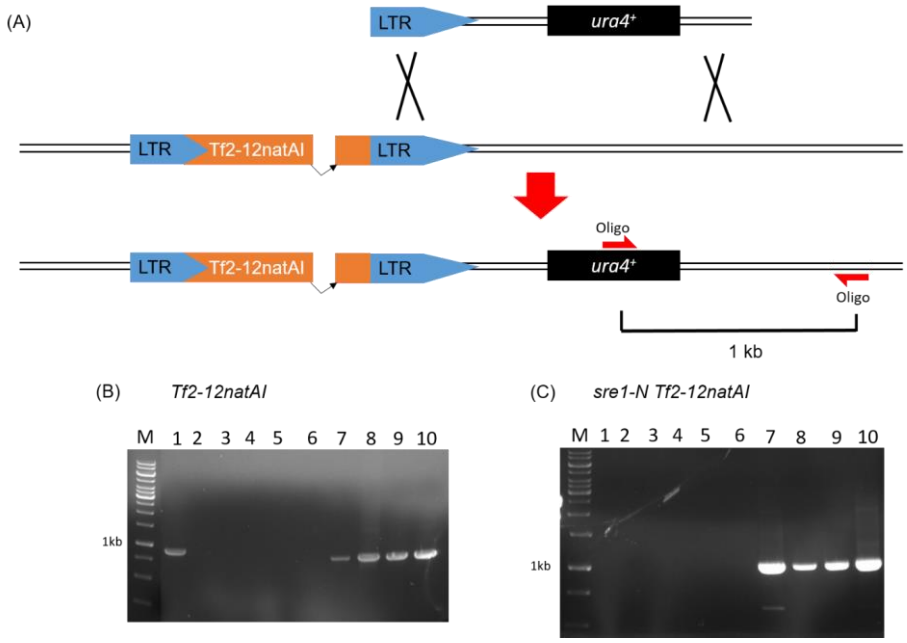


Figure 3.3 Construction of *ura4*⁺ marked *Tf2-12natAI* reporters.

Both wild type *Tf2-12natAI* and *sre1-N Tf2-12natAI* was introduced with *ura4*⁺ selection marker and the marker is confirmed with colonial PCR with a product size of 1 kb. (A) Ten transformed colonies of *Tf2-12natAI* strain were test against PCR reaction with diagnostic primer pairs for selection. Five of those (lane 1, 7, 8, 9 and 10) showed a positive result. (B) Ten transformed colonies of *sre1-N Tf2-12natAI* strain were test against PCR reaction with diagnostic primer pairs for selection. Four of those (lane 7, 8, 9 and 10) showed a positive result.

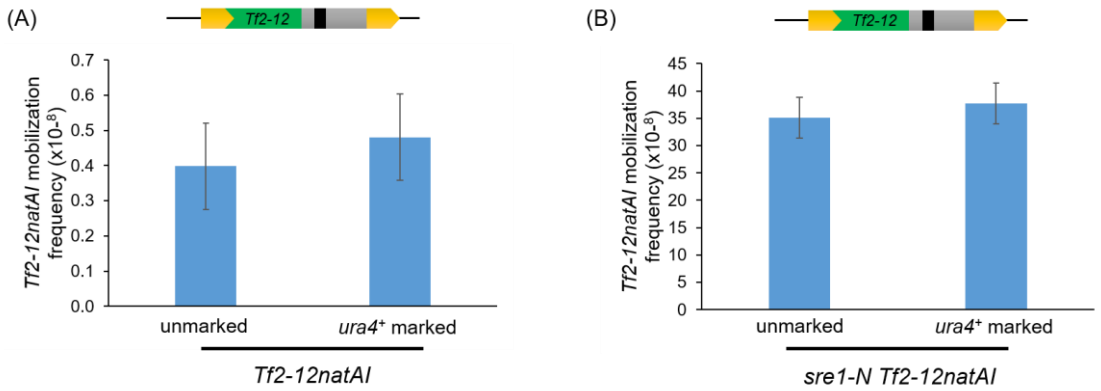


Figure 3.4 The *ura4*⁺ marker does not affect the frequency of *Tf2-12* mobilization.

Frequency of mobilisation of Tf2-12*natAI* in WT and *sre1-N* backgrounds before the addition of *ura4*⁺ (unmarked) and after tagged (*ura4*+ marked) in YE5S of (A) Wild type *Tf2-12natAI* and (B) *sre1-N Tf2-12natAI* were compared. Frequency of mobilization is defined as the proportion of ClonNAT resistance colonies from the total number of viable colonies using the mean of medium method. Average values determined by mean of medium analysis with a minimum of 30 repeats.

The *sre1-N* allele was constructed by introducing a marker cassette into one *sre1*$^{+}$ gene which results in the expression of the N-terminal region (bHLH) which continuously actives *Tf2* expression (Hughes and Espenshade, 2008). Unfortunately, the *sre1-N* allele is marked with the *kanMX* cassette (providing resistance to G418) which is the same marker employed for haploid gene deletion collection (Hughes and Espenshade, 2008). Therefore, in order to introduce the *sre1-N* allele into the Bioneer library, it was necessary to replace the *sre1-N(kanMX)* marker. An alternative marker, *hphMX* marker which allows selection against hygromycin B (Hentges *et al.*, 2005) was chosen for this purpose.

In order to exchange the *kanMX* marker, a 1.5 kb *hphMX* cassette was amplified by PCR from plasmid pLA0122 (Lorenz, 2015) and introduced into *sre1-N(kanMX)* cells. The *hphMX* cassette contains the same *TEF* promoter and terminator regions as the *kanMX* cassette allowing replacement of *kanMX* by homologous recombination (Fig. 3.5A). Cells in which the *kanMX* marker had been replaced with *hphMX* were identified by isolating hygromycin B resistant/ G418 sensitive colonies (Fig. 3.5B). Correct insertion of *hphMX* was confirmed by PCR genotyping (Fig. 3.5C).

To check that marker swapping of *sre1-N(kanMX)* for *sre1-N(hphMX)* did not affect *Tf2* activity, mobilization assays were carried out with wild type, *sre1-N(kanMX)* and *sre1-N(hphMX)* strains containing the *Tf2-12natAI* reporter. Surprisingly, *sre1-N(hphMX)* cells showed a significant decrease in *Tf2* mobilization (~10 fold) relative to *sre1-N(kanMX)* (Fig. 3.6A). To further investigate this, an independent *sre1-N(kanMX)* marker swap was performed in another strain harbouring a *Tf2-6 lacZ* reporter which allows *Tf2* expression be quantified using β-galactosidase assays. Comparison of *Tf2-6 lacZ* expression in both backgrounds confirmed that the *hphMX* cassette consistently had a deleterious impact upon the ability of *sre1-N* to active *Tf2* (Fig. 3.6B). The reason for this is not clear and because of the problems in constructing a *sre1-N* strain that is compatible with the gene deletion collection, this screen was not pursued further.

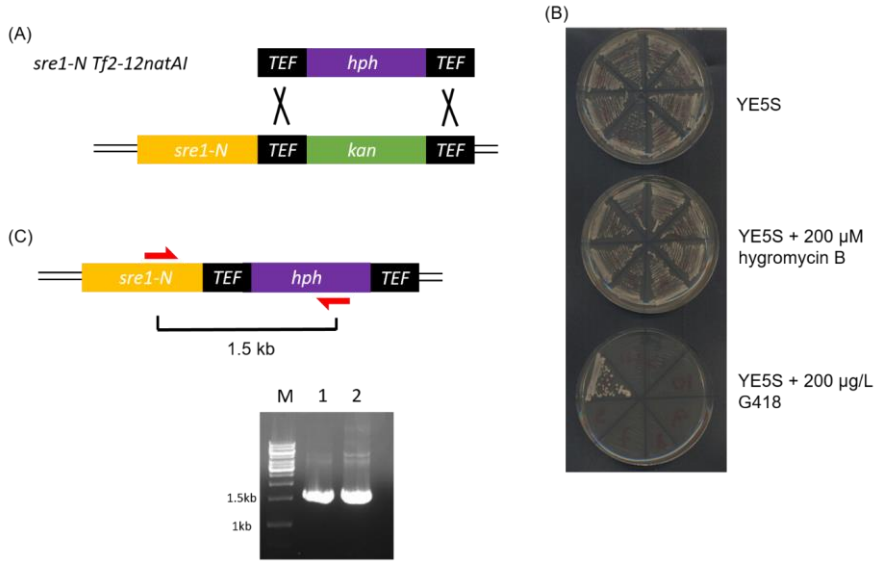


Figure 3.5 Marker switch of *sre1-N* from kanamycin to hygromycin.

(A) The selection of *sre1-N* for *sre1-N::kanMX* Tf2-12*natAI* strain is replaced with *hphMX* marker which provide hygromycin resistance, expressed with TEF promoter and TEF terminator. (B) Transformed cells were tested against hygromycin B and kanamycin for selection. (C) The genotyping of hygromycin B resistance colonies are confirmed with 1% agarose gel with EB and yield a 1.5 kb PCR fragment.

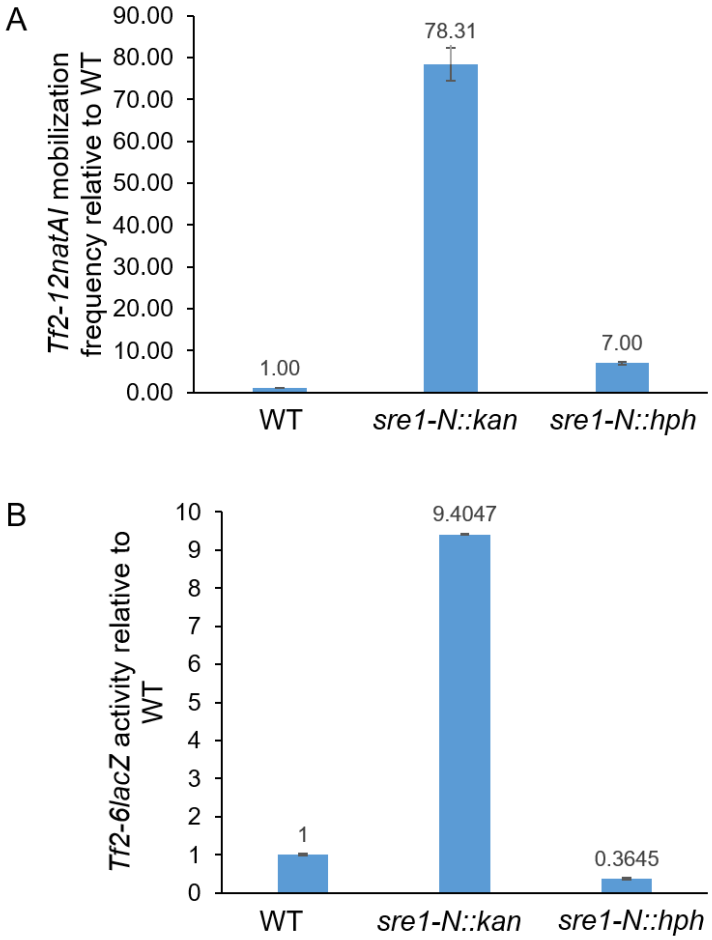


Figure 3.6 Escalated expression and mobilization of Tf element from *sre1-N* was impaired after switch from *kan* cassette to *hph* cassette.

(A) *Tf2-12natAI* mobilization frequency was determined as the proportion of ClonNAT resistance colonies from the total number of viable colonies using the mean of medium method. Average values determined by mean of medium analysis with a minimum of 30 repeats. (B) Strains were grown at 30°C in YE5S to mid-log phase before being collected for use in quantitative β-galactosidase assays. Values shown represent the average of 8 biological repeats assayed in duplicate and error bars indicate ±SEM.

3.4 Construction and analysis of a pilot library for systematic screen

In order to test the feasibility of using a high throughput screen to identify factors restricting *Tf2* mobilization, a pilot library was constructed by automated genetic crosses by Dr Peter Banks (High Throughput Faculty, Newcastle University). The pilot library was constructed by introducing the *Tf2-12natAI(ura4$^+$)* reporter into 16 different (*kanMX*) gene deletion strains. For each different deletion, four replicate strains were made. The gene deletion strains selected for the pilot library are detailed in Table 3.1. The library was constructed to contain alleles that have been shown to cause a robust increase in mobilization (*set1Δ* and *hht2Δ*), *rhp51Δ* which has reduced mobilization and *clr4Δ* and *rdp1Δ* which do not alter mobilization (Cam *et al.*, 2005; Murton, 2012; Murton *et al.*, 2016). Other alleles selected for the pilot library were chosen based upon their impact upon *Tf2* mRNA levels, *Tf* body formation, and impact upon *Tf1* retrotransposition or involvement in chromosome biology.

As a first test of the pilot library, 16 strains were picked at random and their ability to generate ClonNAT –resistant colonies was tested. Only 14 strains are viable and could produce ClonNAT resistant colonies indicating that the *Tf2-12natAI* reporter is co-segregating with the *ura4*$^+$ marker (data not shown).

In order to further confirm the validity of the pilot library, the mobilization frequency of the four independent replicate *set1::kanMX* strains from the pilot library was determined and compared to mobilization frequency of the laboratory *set1::kanMX Tf2-12natAI* reference strain. No significant change in retrotransposition frequency was observed between the pilot library *set1Δ* strains and the reference strain providing validation of the library (Fig. 3.7). The result also suggested that automated crosses are functioning as expected and that the strains generated were worthwhile.

Table 3.1 Deletion strain table for pilot library screen

Gene	Systematic ID	Function of encoded protein	Impact on Tf2 mobility	Comments	References
hht2	SPBC8D2.04	Histone H3.2	Increase	Reduction in H3 gene dosage results in a robust increase in *Tf2* mobilization	Hugh Cam personal communication
red1	SPAC1006.03c	RNA elimination factor	Predicted increase	*Tf2* mRNA levels increase in in *red1*Δ cells	Suzuki *et al.*, 2011
cay1	SPBC2F12.12c	cactin, spliceosome complex subunit	Predicted increase	Unprocessed *Tf2* mRNA increases in *cay1*	Lorenzi *et al.*, 2015
nts1	SPCC24B10.19c	Clr6 histone deacetylase complex subunit	Predicted increase	*Tf2* mRNA levels increased in *nts1Δ*	Zilio *et al.*, 2014
fft3	SPAC25A8.01c	ATP-dependent chromatin remodeller	Predicted increase	*Tf2* mRNA levels increased in *fft3Δ*	Steglich *et al.*, 2015
xap5	SPCC1020.12c	Yeast X-chromosome Associated Protein 5	Predicted increase	Tf2 mRNA levels increased in *xap5Δ*	Anver *et al.*, 2014
pab2	SPBC16E9.12c	Poly(A) binding protein	Predicted increase	Loss of Pab2 results in a modest increase in *Tf2* mRNA	Mallet *et al.*, 2017
set1	SPCC306.04c	Histone H3 lysine 4 methyltransferase	Increase	Loss of Se1 results in a #10 fold increase in *Tf2* mobilization	Murton *et al.*, 2016
pku80	SPBC543.03c	Ku80	Predicted modest increase	Loss of Ku70-80 causes loss of Tf bodies	Cam *et al.*, 2008
rhp51	SPAC664.14c	RecA protein required for homologous recombination	Reduced	Rhp51 is required for cDNA recombination/ Loss of rhp51 results in a 5 fold reduction in *Tf2* mobilization	Sehgal *et al.*, 2007; Murton *et al.*, 2016
nup124	SPAC30D11.04c	nucleoporin	Predicted decrease	Nup123 is required for the nuclear import of Tf1. Retrotransposition of *Tf1* is reduced in nup12Δ	Sistla *et al.*, 2007
clr4	SPBC428.08c	Histone H3 lysine 9 methyltransferase	No change	Loss of heterochromatin does result in increased *Tf2* activity under nutrient rich conditions	Murton, 2012
rdp1	SPAC6F12.09	RNA-directed RNA polymerase Rdp1	No change	Loss of RNAi does result in increased *Tf2* activity under nutrient rich conditions	Murton, 2012
nrl1	SPBC20F10.05	RNAi splicing factor binding protein	No change	Loss of RNAi factors does result in increased *Tf2* mRNA levels in nutrient rich conditions	Lee *et al.*, 2013
set2	SPAC29B12.02c	Histone H3 lysine 36 methyltransferase	Unknown	Impact of *set2*Δ on *Tf2* mRNA not reported	
exo1	SPBC29A10.05	exonuclease	Unknown	Impact of *exo1*Δ on *Tf2* mRNA not reported	

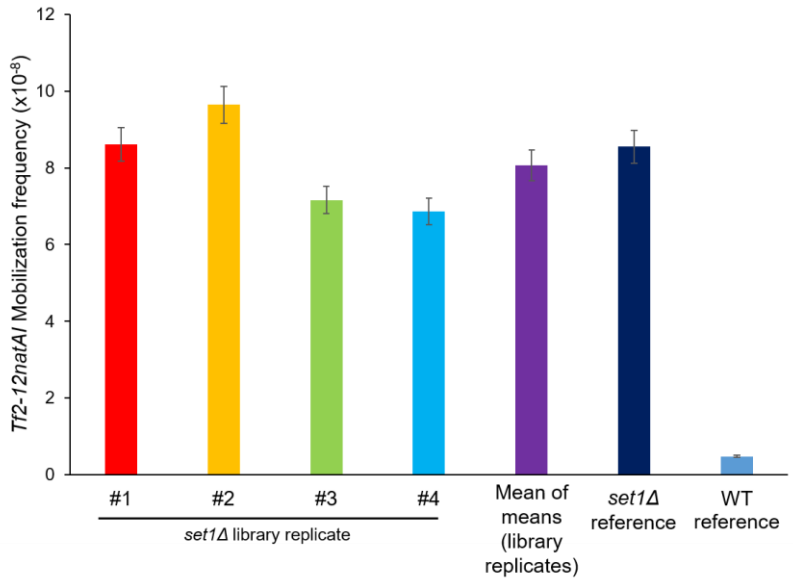


Figure 3.7 Analysis of *Tf2-12natAI* activity in pilot library *set1Δ* strain

Tf2-12*natAI* mobilization frequency was determined as the proportion of ClonNAT resistance colonies from the total number of viable colonies using the mean of medium method. Average values determined by mean of medium analysis with a minimum of 15 repeats. Error bars indicate ±SEM. The means of the four individual replicated from the pilot library was compared with lab reference strains in the lab.

The methodology for measuring mobilization frequency is labour intensive and is not compatible with a genome-wide screen as it requires determining the proportion of ClonNAT resistant colonies from the total number of colonies for at least 15 cultures (Fig. 3.7). Therefore, to establish a methodology compatible with a high throughput screen, a 'semi-quantitative' mobilization assay was tested. Instead of a standard mobilization assay determining the proportion of ClonNAT resistant colonies, the total number of ClonNAT resistant colonies from five - (5 ml) cultures was determined (Fig. 3.7). It was planned that two replicates from the library would be analysed for the primary screen and the mutants of interest would be further analysed by repeating this analysis with the remaining two replicates.

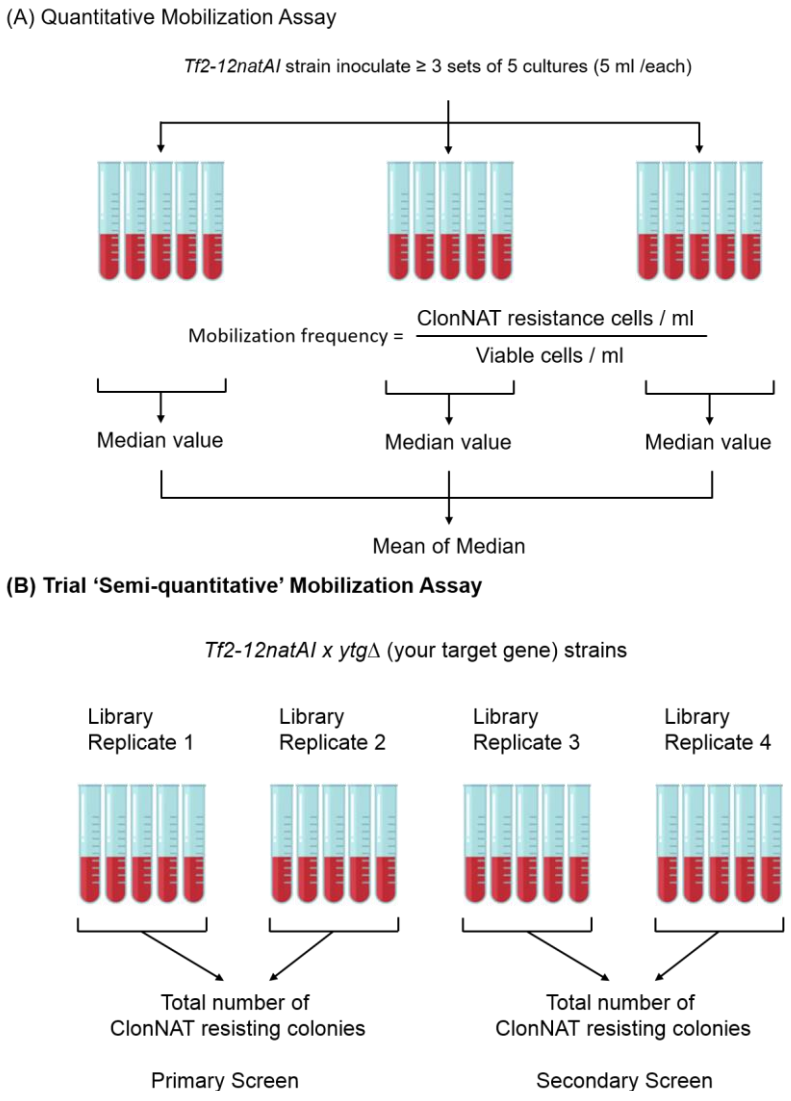


Figure 3.8 Illustration of quantitative and semi-quantitative mobilization assay

(A) Quantitative mobilization assay was carried out with inoculating a loop of cells in 5 ml of medium according to the experiment requirement. The culture is allowed to incubate at 30°C for 2 or more days until saturation. Yeast cells are collected and spread evenly into two YE5S plate containing 75 µg/ml nourseothricin ClonNAT. These plates are then incubated at 30°C for 4 or more days until colonies appeared. Each experiment was carried out in at least three groups of 5 tubes. Mean of each median value is determined for mobilization frequency. (B) Semi-quantitative mobilization assay was carried out by following the quantitative mobilization assay while only one median value was determined.

The strains from the pilot library were analysed in this semi-quantitative manner to determine feasibility of this approach (Fig. 3.9). Firstly, in many cases, the results were inconsistent between the primary and secondary screen (e.g. *set1Δ*, *set2Δ*, *pab2Δ*, *nts1Δ* and *xap5Δ*). Furthermore, using this approach, *set1Δ* would not have been identified as a mutant of interest (Fig. 3.9). It was therefore concluded that this approach would generate an unacceptable number of both false positives and false negatives. To further investigate this three mutants from the pilot library were analysed using quantitative mobilization assay. The *nup124* strain was analysed because it showed a high level of mobilization in both the primary and secondary screen. The *xap5* and *cay1* mutant were also analysed because of the level of mobilization in the primary and secondary screen (Fig. 3.10) (Sistla *et al.*, 2007; Anver *et al.*, 2014; Lorenzi *et al.*, 2015).

The result of *xap5Δ* indicated that the automatic crosses are not generating consistent replicates as *xap5Δ* might be ignored as false negative on the first screen. A similar false negative was also be seen in *cay1Δ* as only one of the two quantitative mobilization assay showed an increased mobility with a huge standard deviation value. *nup124Δ* would be taken as a false positive mutant of interest during the screen that a constant increase was present yet it is predicted as negative factor from previous experiments (Sistla *et al.*, 2007).

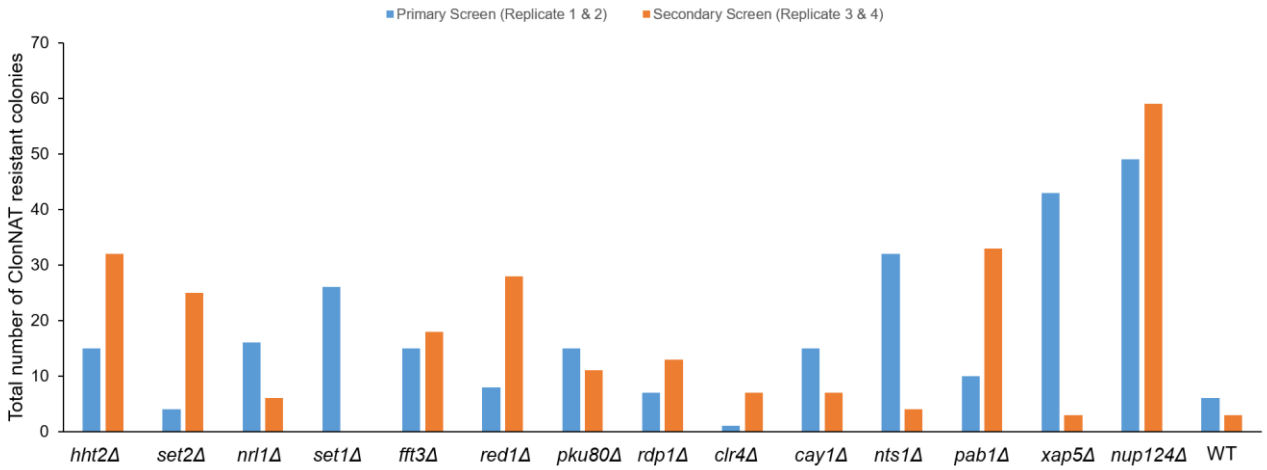


Figure 3.9 Quadruplicate of the mating strains were un-consist in the two semi-quantitative mobilization assay.
Semi-quantitative mobilization assay (Fig 3.8B) was carried out for screening in the pilot library. The total number of ClonNAT resistant colonies from replicate 1 and 2 were compared with replicate 3 and 4. A wild type *Tf2-12natAI* strain was included as a control and standard for comparison.

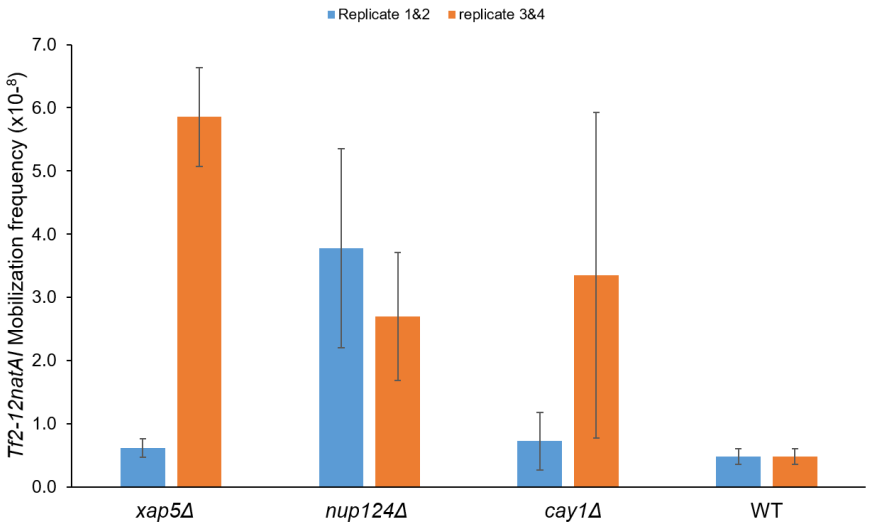


Figure 3.10 Quantitative mobilization assay of *xap5Δ*, *nup124* and *cay1*.

Tf2-12*natAI* mobilization frequency was determined as the proportion of ClonNAT resistance colonies from the total number of viable colonies using the mean of medium method. Average values determined by mean of medium analysis with a minimum of 15 repeats. Error bars indicate ±SEM value indicates significance between each means. The means of the four individual replicated from the pilot was compared with the reference strain in the lab stock.

3.5 Discussion

At the time of starting this work, genome-wide experiments identifying host factors affecting LTR retrotransposons mobility were limited to the budding yeast *Saccharomyces cerevisiae*. Several different reporter systems had been used to investigate the activity of the *Ty1* (Copia) and *Ty3* elements (Scholes *et al.*, 2001; Griffith *et al.*, 2003; Aye *et al.*, 2004; Irwin *et al.*, 2005; Nyswaner *et al.*, 2008). Since *S. pombe* is evolutionarily divergent to *S. cerevisiae*, it was thought that a screen for factors affecting *Tf2* activity would be valuable. Therefore, the feasibility of performing a systemic screen using the *Tf2-12natAI* reporter was investigated.

To date, only one large scale genetic screen, has employed an endogenous element as the reporter system. Scholes and co-workers used transposon mutagenesis to identify 21 genes that suppress *Ty1* mobility (Scholes *et al.*, 2001). The majority of experiments, and indeed all genome-wide systematic screens, of *S. cerevisiae* retrotransposon mobility have been carried out using plasmid-based elements most of which were under the control of heterologous promoter (e.g. *GAL*) (Griffith *et al.*, 2003; Irwin *et al.*, 2005; Nyswaner *et al.*, 2008; Sangesland *et al.*, 2016). This is beneficial since the basal transposition rate of endogenous elements is relative low. For example, for the *Ty1* element the frequency is estimated as 1 event in 10^7 cells (Nyswaner *et al.*, 2008; Esnault *et al.*, 2019). However, overexpressing transposable elements from plasmids could circumvent host cell expression controls, and possible evade defence /restriction mechanisms. Screens using plasmid heterologous promoters will not be able to identify factors that are involved in the transcriptional control of endogenous elements. Since this is a major interest in the laboratory, the use of plasmid based *Tf2* reporter under the control of the *nmt* promoter was discounted (Hoff *et al.*, 1998).

There are some advantages of employing the *Tf2-12natAI* reporter as the basis of a genome-wide screen. First, *Tf2-12natAI* uses an endogenous element with a native promoter, and thus presumably native chromatin and transcriptional control. Moreover, previous experiments have shown that the frequency of spontaneous resistance to ClonNAT is extremely low (< $1x10^{-12}$ events/cell) and offers an extremely tight selection for fission yeast. In addition, this selection is preserved when extremely high density cells are plated (1 x 10^8 cells/plate), allowing large numbers of cells to be assayed simultaneously with only a relatively small number of agar plates required

(Murton, 2012). The only other mobilization assay that utilises an endogenous *Tf2* element is based upon G418 selection (Rai *et al.*, 2017). The rate of spontaneous resistance to G418, means that a low density of cells is necessary and so a large number (~50) of plates is required for a measurement of mobilization. This makes studies of *Tf2* mobilization with multiple mutants or conditions impractical with this reporter.

However, there are disadvantages for the *Tf2-12natAI* system. The basal mobilization rate for *Tf2-12natAI* is very low (0.4-2 x 10^{-8} events/cell) when standard growth condition (YE5S medium at 30°C) are used. This is around an order of magnitude lower that the *Ty1* element in *S. cerevisiae* (Krastanova *et al.*, 2005). The low mobilization frequency for *Tf2-12natAI* creates major difficulties for high throughput analyses since it means that a large numbers of cells are required for a single experimental measurement. This is then not compatible with the small quantities or volumes processed in robotic/automated handling. Furthermore, it prevents the typical screening method for *Ty* elements, which involves replica plating patches of cells to selective agar to identify cells in which a mobilization event has occurred (Aye *et al.*, 2004). Indeed this method was found not to be practical with *Tf2-12natAI* due to the low mobilization rate, as too few colonies could be selected by replica plating. A number of attempts were used to reduce culture volume by using concentrated culture media (i.e. 5x YE5S and 10x YE5S), as they theoretically should have 5 to 10 times more cells relative to the same volume of standard medium. In practice, though the mobilization data collected from either of the concentrated cultures gave highly variable results (data not shown).

An alternative to screening the complete set of haploid gene collection is to select a subgroup of mutants for assay. Since a major of interest is the role that chromatin plays in the regulation of *Tf2* element mutants with GO-terms associated with chromatin could be screened. Such an approach has been employed to systematically map genetic interactions among 550 genes involved in chromosome function (Roguev *et al.*, 2008). However, even screening this many mutants with a quantitative assay would be extremely labour intensive. The major limiting factor is the requirement to perform numerous viable cell counts. Access to spiral plater (Gilchrist *et al.*, 1973) and automated colony reader would be required and unfortunately these are not available in the laboratory.

In addition, an alternative to *Tf2-12natAI* mobilization reporter could be carried out to screen for mutants which have altered *Tf2* expression. Integrated *Tf2 lacZ* reporters have been constructed and have been used extensively in the lab (Anderson *et al.*, 2009). β-galactosidase assays require relatively small numbers of cells and therefore could be automated. Indeed, high throughput assays for yeast have been described that are based upon Beta-Glo, a commercially-available luminescent β-galactosidase substrate (Hook, 2007; de Almeida *et al.*, 2008; Napper *et al.*, 2011). However, one possible drawback is that for some mutants, such as *hip1Δ*, increased *Tf2* expression does not result in increased mobilization (Murton *et al.*, 2016). This would limit the findings, only identifying factors that might only affect expression but not necessarily mobilization.

For selection of the *Tf2-12natAI* reporter, an *ura4*$^{+}$ marker was inserted immediately downstream of the 3' LTR. Initial experiments indicated that this *ura4*$^{+}$ marker is sufficient to allow reliable selection of the reporter in automated genetic crosses. More importantly, it does not affect either expression or mobilization rate of *Tf2-12natAI* (Fig. 3.4). For initial examination of the pilot library, the *set1Δ* strains were selected as positive control as this background is known to be associated with increased mobilization (Murton *et al.*, 2016). The analysis of *set1Δ* strains from the pilot library suggested that these automated strains behaved properly (Fig. 3.7). Since fully quantitative assays are not possible for >3000 strains, a 'semi quantitative' mobilization assay method was employed for the initial screening (Fig. 3.8). One of the significate drawback is that viable cell counts data could not be provided for the experiment. A simple alternative would be to estimate cell number in the cultures being tested by optical density and this was the initial plan going forward.

Unfortunately, from the results of semi-quantitative assay showed that the replicates of some strains behaved differently. For example, Clr6 histone deacetylase complex subunit *nts1* mutant strain would be identified as restricting factor from the primary screen but would not have been confirmed by the second screen. The only three strains that performed consistently were *fft3Δ*, *pku80Δ* and *nup124Δ*. Both *fft3Δ* and *pku80Δ* showed a modest increase in mobilization while a marked increase was observed in the *nup124Δ* strain. This increase was using a quantitative mobilization assay and was surprising because Nup124 has been shown to be necessary for the *Tf1* life cycle. Nup124 is a nuclear pore complex nucleoporin that is key to the nuclear

entry of retrotransposons, affecting their transposition ability (Balasundaram *et al.*, 1999; Dang and Levin, 2000; Kim *et al.*, 2005; Varadarajan *et al.*, 2005; Sistla *et al.*, 2007). The C terminus of Nup124 interacts with the N-terminal of nuclear localization signals of Gag Protein of *Tf1* to facilitate nuclear translocation of *Tf1-Gag.* A reduced *Tf1* transposition was observed in the *nup124* null mutant (Balasundaram *et al.*, 1999; Dang and Levin, 2000; Kim *et al.*, 2005; Varadarajan *et al.*, 2005). Also, the nuclear importation action is conserved between *Tf1* retrotransposon and retrovirus HIV-1 where a human nucleoporin homologue Nup153 is essential for HIV-1 infection (Varadarajan *et al.*, 2005). It is also suggested that the transposition event of *Tf1* requires two key domains, the FXFC-repeat domain and a conserved C-terminal peptide of Nup124 (Sistla *et al.*, 2007). This result suggested that the mode of importing *Tf2* cDNA may be different from *Tf1* as the transposition of *Tf2* is increased in Nup124 null mutant (Fig. 3.10). Quantitative mobilization assays were also carried for *xap5Δ* and *cay1Δ*. Previously, the *xap5* mutant was shown to have a modest upregulation of *Tf2* mRNA (Anver *et al.*, 2014) and *cay1* mutant was shown to repress *Tf2* retrotransposons (Lorenzi *et al.*, 2015). Of particular concern was the finding that the mobility of *Tf2-12natAI* differed between the replicates (*xap5Δ* ~10 fold and *cay1Δ* ~3 fold). These findings indicated that the strains generated by automated crosses may not be as reliable as suggested by the analysis of the *set1Δ* replicates.

A genetic background with a high level of *Tf2* mobilization would be advantageous to isolate mutants with reduced mobility and thereby identify factors required for the *Tf2* life-cycle. Therefore, a strain (*sre1-N*) that constitutively expresses an active form of Sre1 was employed (Hughes and Espenshade, 2008; Murton *et al.*, 2016). Unexpectedly, the replacement of *kanMX* cassette associated with the *sre1-N* allele with *hphMX*, resulted in a severe reduction in *Tf2* expression and mobilization (Fig. 3.6). An alternative *sre1-N* to stimulate *Tf2* elements would be carry out the experiments in hypoxic conditions which induces high levels of mobilization (Hughes and Espenshade, 2008; Murton *et al.*, 2016). This would have disadvantages though as access to a dedicated anaerobic cabinet would be required or a large number of anaerobe jars.

Ultimately, an alternative to the *sre1-N* allele was not pursued as during this research, the Levin lab published a systematic analysis of factors required for mobilization of a plasmid encoded *Tf1* element (Rai *et al.*, 2017). By utilizing screens and immunoblot

measurements of *Tf1* protein, 61 genes that promote integration was identified. The candidate factors participate in a range of non-chromatin process including nuclear transport, protein synthesis, mRNA processing, vesicle transport, ubiquitin-mediated proteolysis, signal transduction, metabolism, kinetochore, cytoskeleton and chromatin-associated factors, ranging chromatin structure, transcription, splicing and DNA repair (Rai *et al.*, 2017). It is noted that a significant portion of these factors identified overlap with functional homologue with host factors that promote transposition of *Ty1* and *Ty3* (Rai et al., 2017). It would be interesting to further analyse whether mutants identified by the Levin lab also affect *Tf2* activity, especially since *Tf1* relies on integrase-dependent insertion while *T2* predominantly integrates its cDNA into the host genome via homologues recombination (Esnault and Levin, 2015).

Finally, it is also important to note that although systematic screens are useful tool, they do have limitations. An analysis of the *S. cerevisiae Ty* screens revealed that there is only limited overlap in the factors identified (Rai *et al.*, 2017). Also, the Levin lab screen failed to identify the nucleoporin Nup124 in their screen, which mentioned before, is essential for *Tf1* integration (Rai *et al.*, 2017). This suggested that there are some inherent limitations on library screening and the factors identified are highly dependent on the method used to monitor retroelement activity (Sangesland *et al.*, 2016).

Chapter 4 Impact of *Tf1* copy number on the activity of *Tf2*

4.1 Introduction

Retrotransposons transpose via an RNA intermediate and the insertion of a new cDNA copy into the genome. This additive method of replication can potentially lead to large increases in element copy number which can threaten the host genome. Understanding the controls that prevent the over amplification of transposons is therefore important and has been studied in detail in the budding yeast *S. cerevisiae*. Most laboratory strains of *S. cerevisiae* contain between 30 and 35 *Ty1* elements and while natural isolates exhibit wider variation in copy number, no strains have more than 40 complete elements. Indeed, many natural isolates contain just a few copies of *Ty1* (Wilke and Adams, 1992; Wilke *et al.*, 1992), suggesting the existence of mechanisms which limit element spread. However, *S. cerevisiae* lacks RNAi machinery and APOBEC proteins which are used by other eukaryotic organisms to restrict retroelements (Drinnenberg *et al.*, 2011; Garfinkel *et al.*, 2016). Instead in *S. cerevisiae*, the frequency of mobilization of the *Ty1* LTR retrotransposon is subject to post-transcriptional co-suppression, which has also be call copy number control (CNC) (Saha *et al.*, 2015; Garfinkel *et al.*, 2016). This was discovered through the observation that increased *Ty1* element number results in a decrease in *Ty1* mobility (Garfinkel *et al.*, 2003). As the number of *Ty1* elements increases from 1 to 20 the transposition frequency of a marked *Ty1* element decreases over a 4800-fold range (Garfinkel *et al.*, 2003). This is because the *Ty1* retrotransposon is regulated by a small protein called p22 (22-kDa protein) which is produced from within the C-terminal half of the capsid gene *gag*. The p22 protein is encoded from a truncated *Ty1* transcript call *Ty1i* and inhibits several steps in the mobilization process before reverse transcription including the assembly of the virus like particle (Matsuda and Garfinkel, 2009; Nishida *et al.*, 2015; Saha *et al.*, 2015; Garfinkel *et al.*, 2016; Ahn *et al.*, 2017).

CNC-like mechanisms have been shown to regulate transposons in some other organisms such as *Drosophila* and *E. coli* (Simons and Kleckner, 1983; Chaboissier *et al.*, 1998), but currently, it is not known whether a CNC mechanism exists in *S. pombe*. It is known that in some mutant backgrounds (i.e. *hip1Δ*) the expression of *Tf2* elements is not directly proportional to mobilization frequency (Murton *et al.*, 2016). Therefore, the possibility that *Tf2* is also regulated by CNC was tested.

4.2 A high copy number of the *Tf1* element activates the mobilization of *Tf2*.

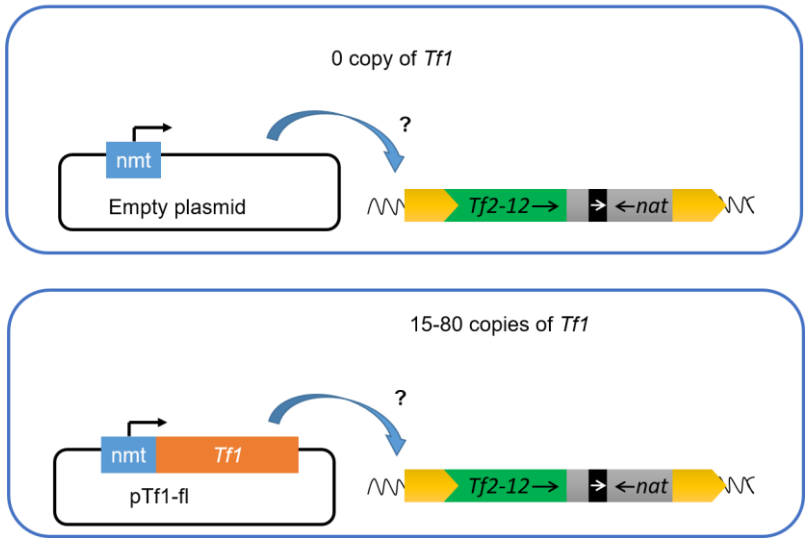
The study of the CNC mechanism in *S. cerevisiae* has been helped greatly by the availability of a range of laboratory strains with variations in *Ty1* copy number, including *Ty*-less strains which have no full length elements (Wilke *et al.*, 1992). Unfortunately, for *S. pombe* there are only few alternatives to the standard lab strain (972) and *Tf2*-less strains are not readily available. Therefore, as an initial test of whether a CNC inhibitor mechanism is present in fission yeast, a plasmid (pHL449-1) expressing the full-length *Tf1* element under the control of the *nmt1* promoter (Levin *et al.*, 1993) was transformed into the *Tf2-12natAI* reporter strain (Fig. 4.1A). The plasmid pHL449-1 is a multicopy vector that contains the *ars1* replication origin allowing between 15-80 copies per cell (Losson and Lacroute, 1983; Maundrell *et al.*, 1988). The standard lab strain contains no full length *Tf1* elements and as a result, this experimental system allows the impact of a massive increase in *Tf1* copy number upon *Tf2* activity to be determined. Importantly, the *Tf1* element is closely related to *Tf2* and in *S. cerevisiae* it has been demonstrated that a *Ty2* element can exert CNC upon the closely related *Ty1* element (Matsuda and Garfinkel, 2009).

The signature of CNC is that transposition rate is reduced after the retroelement is highly expressed (Matsuda and Garfinkel, 2009). Surprisingly, rather that inhibiting *Tf2* mobilization, high *Tf1* copy number dramatically increased *Tf2* mobilization frequency (~30 fold) compared to the control containing an empty plasmid (Fig. 4.1B), supported by the thiamine conditions used (data not shown). This indicates that a high copy number of one LTR retrotransposon can lead to the activation of a related element.

The expression of *Tf2* elements is subjected to a form of transcriptional silencing that is dependent upon specific histone deacetylases, CENB-P proteins, histone methyltransferases, histone chaperones and ATP-dependent chromatin remodelling complexes (Esnault and Levin, 2015). One possibility is that a multiple copies of the *Tf1* LTR element titrate away an important component of the silencing machinery which leads to de-repression of *Tf2* transcription. Therefore, the influence of high copy *Tf1* on *Tf2* expression levels was investigated by transforming empty vector and *Tf1* expressing plasmids into the *Tf2-6::lacZ* background of a distinct strain. Quantitative β-galactosidase assays showed that high copy *Tf1* resulted in only a very small increase in *Tf2* expression. The experiment of *Tf2* expression and mobilization was

carried out in different strains, this suggests high copy *Tf1* stimulates *Tf2* mobilization predominantly via a post transcriptional mechanism (Fig. 4.2). Unfortunately, due to the time constrain, experiment with the employment of Thiamine should be carried out to test whether the inhibition of plasmid expression could influence the result.

(A)



(B)

Plasmid	*Tf2-12natAI* mobilization	Fold increased
empty vector	$2.64 \times 10^{-7} \pm 6.26 \times 10^{-8}$	1
pTf1-fl	$7.98 \times 10^{-6} \pm 1.65 \times 10^{-6}$	30

Figure 4.1 The impact of high *Tf1* copy number on *Tf2* mobilization

(A) Diagram showing the experimental scheme. The *Tf2-12natAI* reporter strain was transformed with either pRep42 (empty vector) or pHL449-1 (pTf1-fl) which has a full length *Tf1* element under the control of *nmt* promoter.

(B) Frequency of mobilization is defined as the proportion of ClonNAT resistance colonies from the total number of viable colonies using the mean of medium method. Average values determined by mean of medium analysis (±SEM) with a minimum of 30 repeats.

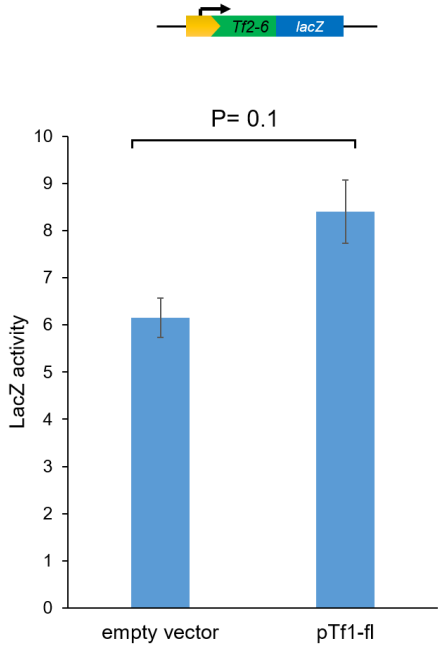


Figure 4.2 Increased *Tf1* copy number does not affect *Tf2* expression

The activity of a *Tf2-6 lacZ* reporter was determined in cells harbouring either empty vector or pTf1-fl. Strains were grown at 30°C in EMM to mid-log phase before being collected for use in quantitative β-galactosidase assays. Values shown represent the average of 8 biological repeats assayed in duplicate. Error bars indicate ±SEM and p value was calculated using a *t*-test.

4.3 Role of *Tf1* integrase in the stimulation of *Tf2* mobilization

Tf2 and *Tf1* have major differences when it comes to the mechanism of cDNA insertion back into the genome. *Tf1* cDNA is inserted upstream of Pol II transcribed genes in an integrase-dependent manner. In contrast, *Tf2* cDNA preferentially inserts into an existing *Tf2* element via homologous recombination (HR) (Levin *et al.*, 1990; Behrens *et al.*, 2000; Esnault and Levin, 2015). To investigate whether the function of *Tf1* integrase (IN) is required for the stimulation of *Tf2* mobilization, a plasmid harbouring a *Tf1* element with a frame shift mutation in the integrase gene (pTf1-IN ft) was employed (Levin, 1995). Compared to wild type *Tf1* expression of the *Tf1 IN* frame shift mutant resulted in a ~4 fold reduction in *Tf2* mobilization. This indicates that the function of *Tf1* IN is required for full stimulation of *Tf2* mobilization and suggested that *Tf1* IN is capable of mediating the integration of *Tf2* cDNA into genome (Fig. 4.3).

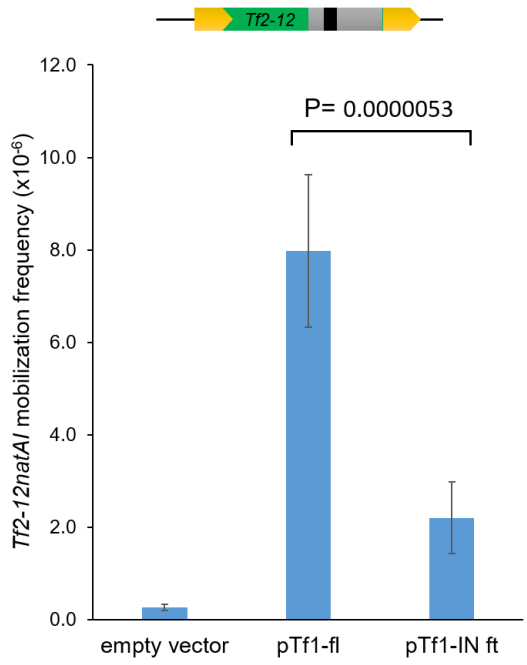


Figure 4.3 *Tf1* IN is required for full stimulation of *Tf2* mobilization

Tf12-12natAI mobilization frequency was determined in cells harbouring the indicated plasmids. Frequency of mobilization is defined as the proportion of ClonNAT resistance colonies from the total number of viable colonies using the mean of medium method. Average values determined by mean of medium analysis with a minimum of 30 repeats. Error bars indicate ±SEM value. p values were calculated using t test.

To further investigate this, an *rhp51Δ* deletion strain was employed which lacks homologous recombination (HR) ability. (Prudden *et al.*, 2003). In this strain, the ability of *Tf1* to stimulate *Tf2* mobilization should be completely dependent upon *Tf1* IN function because the alternative pathway of integration *Tf2* cDNA into an existing *Tf2* element via HR is not available. Mobilizations confirmed that in the *rhp51Δ* background the plasmid expressing *Tf1* IN ft had no ability to stimulate *Tf2* mobilization relative to the empty vector control (Fig. 4.4).

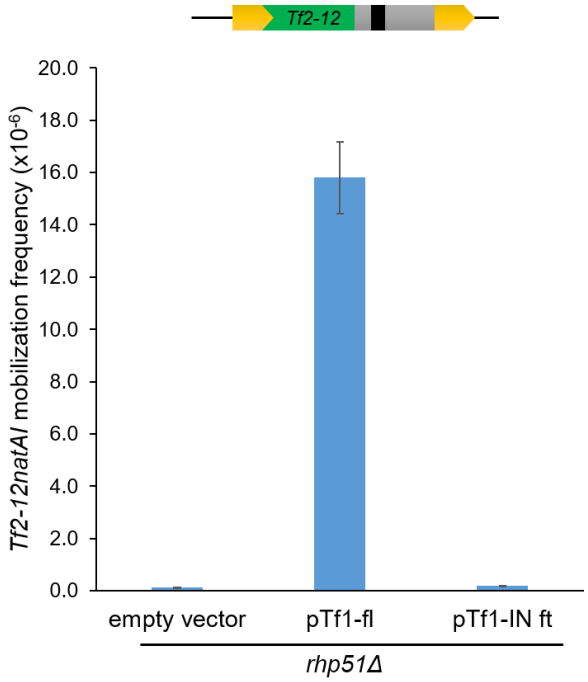


Figure 4.4 *Tf1* IN is required to stimulate *Tf2* mobilization in the absence of homologous recombination

Tf2-12natAI mobilization frequency was determined in *rhp51Δ* cells harbouring the indicated plasmids. Frequency of mobilization is defined as the proportion of ClonNAT resistance colonies from the total number of viable colonies using the mean of medium method. Average values determined by mean of medium analysis with a minimum of 30 repeats. Error bars indicate ±SEM value.

4.4 The *Tf1* protease (PR) and Gag proteins are required to stimulate *Tf2* mobilization

The previous results suggests that *Tf1* IN is mediating the integration of *Tf2* cDNA into the genome. To determine whether *Tf1* proteins are likely to be stimulating the production of *Tf2* cDNA as well as its integration, a plasmid containing a frame shift (ft) mutant in the *Tf1* reverse transcriptase gene (RT) and protease (PR) were employed (Dang *et al.*, 1999). Mobilization assay results indicate that the RT activity of *Tf1* is absolutely required for the increased mobility of *Tf2* (Fig. 4.5). Furthermore, a frameshift mutation in PR resulted in a reduction in mobilization relative to the empty vector control. This mutation will block the production of not only PR but also RT and IN and presumably leads to the production of a mutant (and unprocessed) poly-protein. The results indicate that this mutant polyprotein has an inhibitory impact on *Tf2* activity.

The Gag protein is essential for the life cycle of LTR retrotransposons. Analysis of *S. cerevisiae Ty1* has shown that the Gag protein is required for the formation of a virus like particle (VLP) and it is within this particle that LTR retrotransposon mRNA is converted to cDNA. Furthermore, some essential protein processing occurs in VLPs which is mediated by an element encoded protease (PR) (Leis *et al.*, 1988; Hansen *et al.*, 1992; You and McHenry, 1994). Teysset and co-workers have also demonstrated the essential nature of Gag for *Tf1* retrotransposition (Fig. 4.6A) (Teysset *et al.*, 2003). Experimental characterisation of the *Tf1* Gag protein has identified some functionally important domains (termed A, B, C and D) (Fig. 4.6A). The deletion of regions B (ΔB) and C (ΔC) which are in the centre of Gag destabilize the protein and result in no virus-like particle (Teysset *et al.*, 2003). As a result, the ΔB and ΔC mutations abolish the transposition of *Tf1*. To investigate the role of the *Tf1* Gag protein in stimulating *Tf2* mobilization, *Tf1* plasmids with the ΔB and ΔC partial deletions of the Gag gene were transformed in to the *Tf2-12natAI* reporter strain. Relative to the empty vector control the ΔB and ΔC expressing plasmids showed no ability to stimulate mobilization of *Tf2*, demonstrating that the *Tf1* Gag is necessary for this process (Fig. 4.6B).

Two separation of function Gag mutants, ΔA and ΔD were then examined. The ΔA mutation does not hinder the production of *Tf1* cDNA but is defective in *Tf1* transposition because nuclear transport of the cDNA is impaired (Teysset *et al.*, 2003). Consistent with this the ΔA *Tf1* Gag mutant had a reduced ability to stimulate *Tf2*

mobilization relative to wild type *Tf1* (Fig. 4.6C). Finally a mutant that is defective for *Tf1* RNA packaging (ΔD) was examined (Fig. 4.6D). This mutant impairs (but does not abolish) the production of *Tf1* cDNA and transposition. Surprisingly, a *Tf1* plasmid with the ΔD Gag mutation resulted in massive increase in *Tf2* mobility relative to the wild type *Tf1* (Fig. 4.6D). This suggests that this mutant form of *Tf1* Gag has increased ability to bring about either the processing of *Tf2* mRNA into cDNA and or its integration into the genome. Taken together, the results suggest that the mobilization of *Tf2* is limited at a post-transcriptional level and that *Tf2* mRNA can be packaged successfully into *Tf1* VLPs converted into cDNA and integrated into the genome using *Tf1* IN. As such *Tf2* can take advantage of the proteins from a related LTR retrotransposon to complete its life cycle.

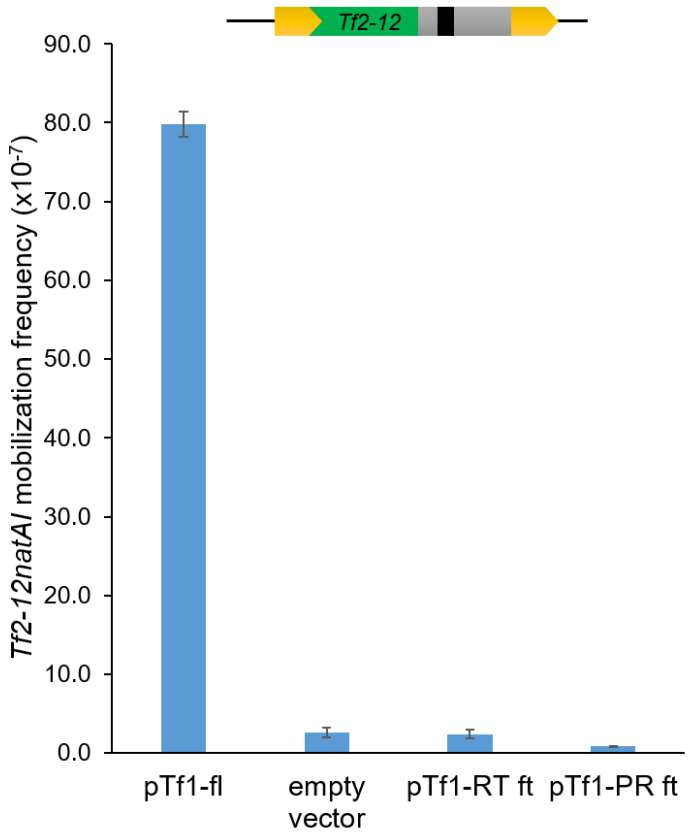
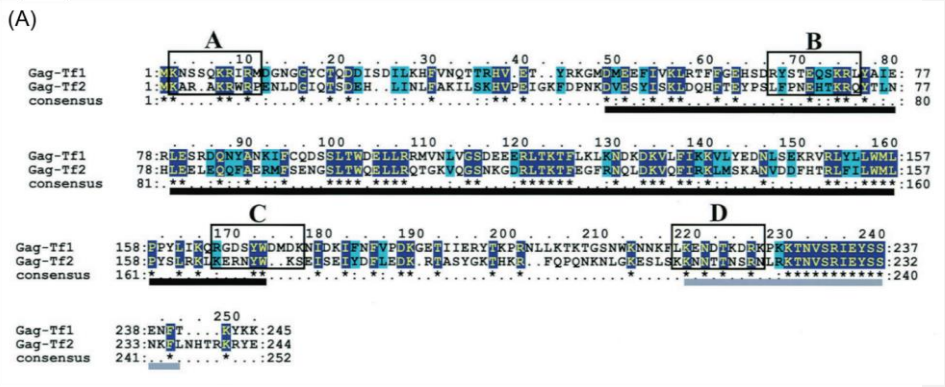


Figure 4.5 *Tf1* RT is required to stimulate *Tf2* mobilization

Tf2-12natAI mobilization frequency was determined in cells harbouring the indicated plasmids. Frequency of mobilization is defined as the proportion of ClonNAT resistance colonies from the total number of viable colonies using the mean of medium method. Average values determined by mean of medium analysis with a minimum of 30 repeats. Error bars indicate ±SEM value.

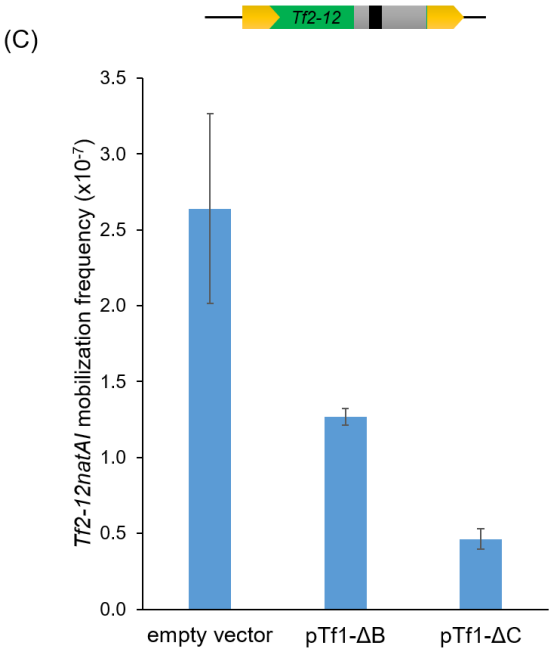
(A)



(B)

Tf1 element	Transposition	cDNA synthesis	Gag	RT	IN
ΔA	-	+++	+++	++	++
ΔB	-	-	-	++	++
ΔC	-	-	-	+++	+++
ΔD	+	+	+++	++	++
WT	++++	+++	+++	+++	+++

(C)



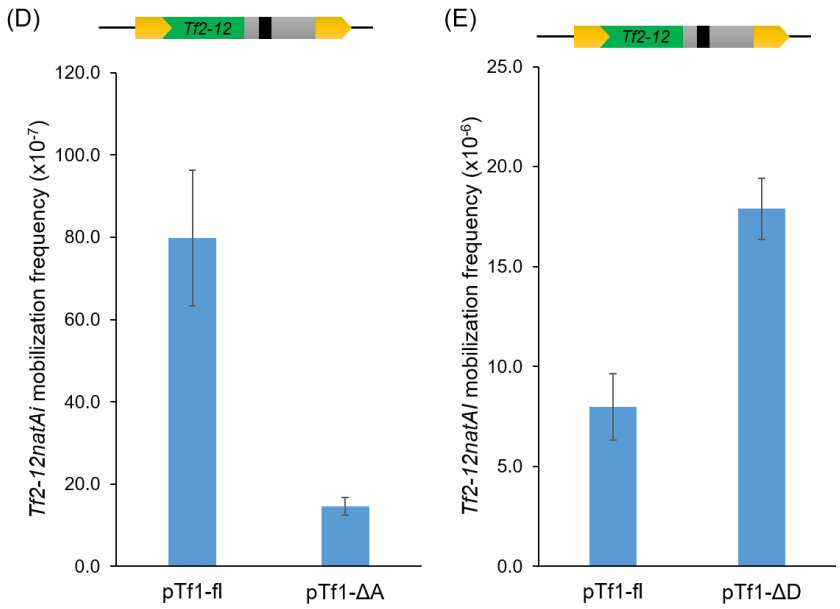


Figure 4.6 The *Tf1* Gag protein is required to stimulate *Tf2* mobilization

(A) Sequence alignment of the Gag proteins from Tf1 and Tf2. The dark blue boxes with yellow letters highlight identical amino acids, while the light blue boxes with black letters highlight similar amino acids. The four amino acid regions which are deleted in the ΔA, ΔB, ΔC and ΔD mutant respectively are indicated by boxes. Taken from (Teysset et al., 2003).

(B) Table summarising the properties of the mutant Gag proteins for Tf1 function (Teysset et al., 2003).

(C-E) Tf2-12natAI mobilization frequency was determined in cells harbouring the indicated plasmids. Mobilization frequency is the proportion of ClonNAT resistant colonies in the total number of viable colonies. Values were determined using the mean of the median with a minimum of 30 repeats. Error bars indicate ±SEM value.

4.5 Multiple copies of the *Schizosaccharomyces japonicas Tj1* LTR retrotransposon does not stimulate *Tf2* mobilization.

To see if a high copy number of other LTR retrotransposons could also stimulate *Tf2* mobility, the *Tj1* LTR retrotransposon from *S. japonicus* was investigated (Guo *et al.*, 2015). The *Tj1* element is related to both *S. pombe Tf1* and *Tf2* and belongs to the same Gypsy/Ty3 family. Furthermore, expression of *Tj1* from a plasmid from a *nmt* promoter has shown that this *S. japonicus* retroelement is capable of transposition within *S. pombe* (Guo et al., 2015). Therefore, a multicopy plasmid containing *Tj1* was transformed into the *Tf2-12natAI* reporter background. Mobilization assays indicated that a high copy number of this exogenous retroelement did not influence *Tf2* mobility relative to the empty vector control (Fig. 4.7B). This suggests that the ability to stimulate *Tf2* mobilization is limited to very closely related LTR retrotransposons.

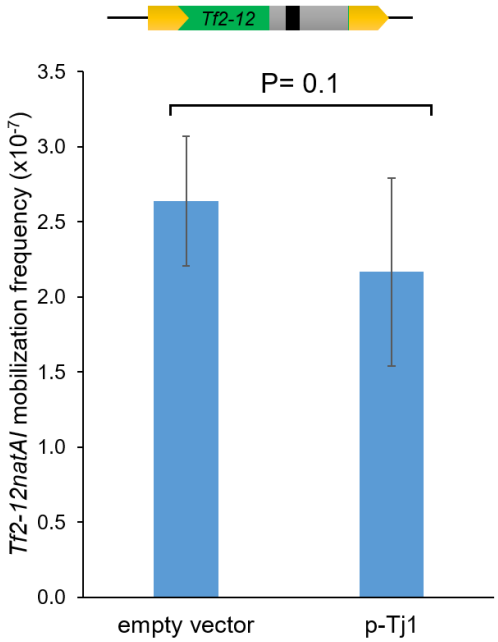


Figure 4.7 *Tj1* does not induces *Tf2* mobilization

Tf2-12natAI mobilization was determined in cells harbouring the indicated plasmids. Frequency of mobilization is defined as the proportion of ClonNAT resistance colonies from the total number of viable colonies using the mean of medium method. Average values determined by mean of medium analysis with a minimum of 30 repeats. Error bars indicate ±SEM value indicates significance between each means and p value is calculated by *t*-test.

4.6 Discussion

The evolution of host defence mechanisms for repressing retrotransposon activity is linked to the maintenance of genome integrity and fitness. One of the main reasons to limit the frequency at which an element can transpose is to avoid a significant increase in the copy number which can cause various mutations and gross chromosome rearrangements. Copy number control (CNC) has been discovered in *S. cerevisiae* to limit the spread of *Ty1* elements (Saha *et al.*, 2015; Garfinkel *et al.*, 2016). CNC-like mechanisms have also been shown to operate in other organisms suggesting that this self-restricting mechanism may be a widespread way for retroelements to limit their copies in the host cells (Johnson and Reznikoff, 1984; Lohe and Hartl, 1996; Chaboissier *et al.*, 1998). However, in *S. cerevisiae*, a CNC mechanism limits the copia family retroelement *Ty1*, but not the *Ty3* or *Ty5* elements (Saha *et al.*, 2015; Garfinkel *et al.*, 2016; Ahn *et al.*, 2017). Furthermore, to date, there is no evidence for the existence of CNC mechanisms that limit *S. pombe Tf1* and *Tf2* elements. This is possibly because unrestricted mobilization of these elements is controlled at other levels.

Ty1 of *S. cerevisiae* is highly expressed, and in fact, 30 copies of the *Ty1* can contribute as much as 10% of the total mRNA in yeast cells (Jiang, 2002). Therefore, *Ty1* elements are not subjected to transcriptional silencing which is a common host restriction mechanism (Jiang, 2002). CNC appears to be an alternative mechanism for preventing uncontrolled spread of *Ty1* retroelements. On the other hand, *S. pombe Tf2* elements are subjected to transcriptional repression which is proposed to limit mobility of the element (Murton *et al.*, 2016). Repression of *Tf2* expression requires the recruitment of histone deacetylases to *Tf2* loci by CENP-B proteins, which repress transcriptional at LTRs and *Tf2* internal cryptic promoters (Cam *et al.*, 2008; Lorenz *et al.*, 2012). The repression of *Tf2* expression is also dependent upon Set1 (histone H3 K4 methyltransferase), HIRA (histone chaperone) and Fun 30 ATP-dependent chromatin modellers (Lorenz *et al.*, 2012; Steglich *et al.*, 2015; Murton *et al.*, 2016). Furthermore, *Tf2* mRNA transcripts are promoted to undergo rapid degradation (Mallet *et al.*, 2017). *Tf* elements are physically clustered in the nucleus into structures called *Tf* bodies (Cam *et al.*, 2008; Lorenz *et al.*, 2012). While *Tf* bodies are not required for transcription silencing after cluster removal in Ku mutants, it has been suggested that

Tf bodies help to limit mobilization (Murton *et al.*, 2016). The control of *Tf2* activity at the transcriptional level may therefore make CNC mechanisms unnecessary.

The way that *Tf2* integrates into the genome may be another reason why CNC might not be required for these elements. The majority of the integration events for *Tf2* (~70%) occur via HR and are IN (integrase) independent (Hoff *et al.*, 1998). This HR strategy is thought to avoid disrupting the host genome coding sequence by recycling of integration sties (Hoff *et al.*, 1998). In contrast the *Tf1* mobilizes primarily via IN-dependent integration. Interestingly, this difference in transposition method is not determined by the 2% difference between the amino acid sequence of *Tf1* and *Tf2* IN proteins but may result from the sequence difference of *Tf1* and *Tf2* Gag (capsid) and PR (protease) proteins which, as discussed below, have an impact on proteolytic processing of precursor proteins (Esnault and Levin, 2015). The HR method of cDNA integration means that mobilization of the *T2* element does not increase copy number. As a result, the cell might not require a CNC to repress the mobility of *Tf2* as they will not threaten genome integrity. Also, it is worth noting that both *Tf1* and *Tf2* are member of the Ty3/gypsy transposon family and therefore, are more closely related to the *S. cerevisiae Ty3* retrotransposon rather than *Ty1* which is a member of the copia transposon family. Currently, no CNC mechanisms have been reported in *Ty3* regulation (Curcio *et al.*, 2015).

Multicopy expression of a marked *Ty1* element is known to swamp the CNC mechanism in *S. cerevisiae* (Saha *et al.*, 2015). Therefore, a multicopy expression system is not the best way to examine copy number control. Ultimately, proper investigation requires the construction of strains with alternative numbers of *Tf2* elements. There are 13 full-length *Tf2* elements present in the standard lab *S. pombe* strain with 249 solos LTRs (Bowen *et al.*, 2003; Esnault and Levin, 2015). Construction of a lab strain lacking *Tf2* elements would therefore be time consuming. However, it may be possible to identify natural isolates of *S. pombe* that lack full length *Tf2* elements.

It is possible that CNC does exists in *S. pombe* but high copy *Tf1* is not capable of exerting an effect on *Tf2* or vice versa. It is worth noting that for *S. cerevisiae Ty1* CNC is regulated by an alternative protein produced from the *gag* gene (Saha *et al.*, 2015; Garfinkel *et al.*, 2016). Interestingly, *Tf1* and *Tf2* sequences are almost identical for

RT and *IN* gene while a major difference is located in the *gag* region (Levin *et al.*, 1990; Weaver *et al.*, 1993). Therefore, a CNC protein produced from *Tf1 gag* may not function on *Tf2* or vice versa.

The data in this chapter instead suggest that *Tf2* mRNA can be packaged into *Tf1* virus-like particles and take advantage of *Tf1* proteins to complete its life cycle (Fig. 4.3). As the *Tf2* mRNA level does not increase in response to high *Tf1* copy number (Fig. 4.2), the large stimulation of *Tf2* mobilization is believed to operate at a post-transcriptional level. This suggests that despite the mechanisms that 'silence' Tf2 expression, the mRNA level of *Tf2* is not the limiting factor for mobilization bur instead the production or activity of the *Tf2* proteins. *Tf2* proteins are made first as a long polyprotein which is then processed into mature protein activities by PR. Previous studies suggests that *Tf2* polyprotein processing is less efficient than *Tf1* (Levin *et al.*, 1993; Hoff *et al.*, 1998). The polyprotein processing pathway is suggested to be different between *Tf1* and *Tf2* (Hoff *et al.*, 1998). PR of *Tf1* polyprotein is first cleaved and generates an RT-IN intermediate while IN of Tf2 is cleaved first and leaves behind a PR-RT intermediate (Atwood *et al.*, 1996; Hoff *et al.*, 1998). The significance of the difference between *Tf1* and *Tf2* polyprotein processing is that for *Tf2* there is an accumulation of PR-RT species while no detectable RT is observed (Hoff *et al.*, 1998).

It is interesting that *Tf1* can mediate integration of *Tf2* cDNA via an IN-dependent mechanism (Hoff *et al.*, 1998). The reason why *Tf2* and *Tf1* prefer different modes of integration is not really understood, particularly as the IN proteins from *Tf1* and *Tf2* are almost identical (Levin *et al.*, 1990; Hoff *et al.*, 1998). In fact, the *Tf1* and *Tf2* share a greater than 90% homology between RT, IN and the C-terminal regions of PR. The N-terminal regions of *Tf1* and *Tf2* PR share 80-90% homology but less than 50% homology is found between the two transposons in the *gag* region (Levin *et al.*, 1990; Lin and Levin, 1997). Differences in the *Tf1* and *Tf2* life cycles may be related to differences in protein processing, which in turn, may ultimately result from differences in the PR and Gag proteins of *Tf1* and *Tf2*.

From the results, the stimulation of *Tf2* activity by high copy *Tf1* is dependent upon the RT, Gag and IN proteins produced from *Tf1*. The importance of IN was confirmed by employing the HR-deficient mutant *rhp51Δ*. Increased transposition *Tf2* rates were abolished by removing functional *Tf1* RT and a *Tf1* PR frame shift mutant (PR ft),

results in a *Tf2* mobilization rate that is lower than the negative control (vector alone). The frameshift mutation of PR would also block RT and IN expression and the mutated polyprotein may have a dominant negative impact. Furthermore, activation of *Tf2* events requires *Tf1* Gag. In the mutants that cannot produce a functional Gag protein (pTf1-ΔB and pTf1-ΔC), the rate of *Tf2* mobilization is decreased compared to the wild type *Tf1* control. In the case of the pTf1-ΔD (ΔD) construct, the production of mutant Gag proteins stimulates the mobilization rate of *Tf2* relative to the wild type control. There may a competition between *Tf1* and *Tf2* mRNAs for incorporation into *Tf1* VLPs and in the ΔD mutant incorporation of *Tf2* mRNA may be favoured.

In contrast to *Tf1*, a *Tj1* retroelement from *S. japonicas*, could not stimulate the activity of *Tf2*, suggesting that only closely related elements can provide their proteins for co-activation. An interaction between different retroelements was observed in *S. cerevisiae Ty1* and *Ty2* for the co-suppression in CNC. Transposition of *Ty1* is suppressed by overexpressing the *Ty2* element but not the *Ty3* and *Ty5* elements (Garfinkel *et al.*, 2003). The sequences of *Ty1* and *Ty2* share ~70 % identity and the LTR regions are closely related (Jordan and McDonald, 1999a), suggesting that CNC could be triggered between closely related retroelements.

The results indicate that, instead of co-suppression as seen in *Ty1* and *Ty2*, a co-activation mechanism for *Tf1* and *Tf2* is present. A hypothesis could be drawn that the ability of *Tf1* to stimulate the mobilization of *Tf2* is reminiscent of the situation for transforming replication-defective retroviruses. Such retroviruses have had part of their native viral sequences replaced with an oncogene (*v-onc*). Many such viruses produces a Gag-v-onc fusion protein instead of the Gag-pol and Env proteins and so are not themselves replication competent. These viruses depend upon proteins produced from replication competent helper retroviruses to produce their cDNA and converse their life cycle (Coffin JM *et al.*, 1997). Furthermore, infection of mice with an exogenous retrovirus results in mobilization of an endogenous retrovirus, demonstrating that the activation of one retroelement can have a big impact on the spread of other retroelements (Evans *et al.*, 2009).

It was also argued that overexpression has influenced the ability for splicing to have occurred, which alter the expression and mobility of *Tf2* element. Further experiments could be carried out in testing the mRNA level could possible evaluate in this area.

In conclusion the results of this Chapter suggest that a CNC mechanism might not present for *Tf1* and *Tf2*. However, a relationship between the two closely related *Tf1* and *Tf2* was identified, where the activation of one element could stimulate the activation of the other element.

Chapter 5 Growth medium affects Tf2 activity

5.1 Introduction

Cells are exposed to a wide range of natural stresses that can lead to growth restriction or even death. In response to extracellular stress, cells not only active expression of networks of genes that together increase resistance but also mobilize retrotransposons (Esnault *et al.*, 2019). For example plant retrotransposons are largely quiescent during development but are activated in response to diverse stresses such as temperature, wounding and pathogen attack (Hou *et al.*, 2019). It has been proposed that this may be a survival strategy and or a generator of genomic diversity (Huang *et al.*, 2012). Nutrient deficiency is one of the stresses that induces retrotransposon activity and 'cross-talk' with other genes in budding yeast *S. cerevisiae* and also in plants (Dai *et al.*, 2007; Negi *et al.*, 2016). The *Ty1* retroelement was found to be activated by growth in low-nitrogen medium both at the level of transcription and retrotransposition (Morillon *et al.*, 2000). Furthermore, severe adenine deficiency is also known to activate *Ty1* (Varadarajan *et al.*, 2005). Dai and co-workers showed that the restriction of *Ty5* retrotransposon integration to heterochromatic domains was lifted under conditions of amino acid, nitrogen or fermentable carbon starvation (Dai *et al.*, 2007). In fission yeast, *Tf2* retrotransposons are known to be induced in response to hypoxic conditions (Sehgal *et al.*, 2007) but the impact of nutrient stress on retrotransposition is not well understood. Under low glucose conditions, *Tf2* elements become associated with elevated levels of the heterochromatic chromatin mark histone H3 lysine 9 dimethylation (Yamanaka *et al.*, 2013; Esnault and Levin, 2015), however no impact of low glucose on mobilization rate has been reported. Furthermore, the impact of the growth medium on *Tf2* activity has received little attention. Indeed, all previous experiments using an integrated *Tf2* mobilization reporters have utilised rich (YE5S) medium (Sehgal *et al.*, 2007; Murton, 2012). It was therefore surprising that the experiments reported in the previous chapter indicated that the growth medium can affect *Tf2* mobilization frequency. Cells transformed with an empty vector (pRep42) and grown in Edinburgh minimal medium (EMM), were found to have a *Tf2-12natAI* mobilization frequency of 2.46 x 10^{-7} (Fig. 4.3). In comparison wild type cells grown in rich YE5S medium at 30°C have a mobilization frequency of ~0.5-2.0 x 10^{-8}.

Therefore, the aim of this chapter was to characterise the impact of growth media and nutrient conditions on the activity of the *Tf2* retrotransposon.

5.2 Growth in minimal (EMM) medium induces *Tf2* expression and mobilization.

In order to investigate whether the high level of *Tf2* mobilization observed in Fig. 4.3 is due to the presence of the pRep42 plasmid or the EMM growth medium, *Tf2-12natAI* cells lacking pRep42 were inoculated into minimal EMM medium. This showed that when compared to YE5S, growth in EMM results in a ~100 fold increase in the mobility of the *Tf2* element. Furthermore, experiments using the *Tf2-6 lacZ* reporter revealed that growth in EMM results in ~6 fold increase in expression relative to YE5S, confirming that minimal growth medium induces *Tf2* activity (Fig. 5.1). The results suggest that either a component of YE5S medium is resulting in repression of the *Tf2* element or alternatively a component of EMM is activating *Tf2*.

In *S. pombe*, the LTR sequences of *Tf1* and *Tf2* differ significantly, especially the central portions of the LTRs (Fig. 5.2A) (Levin *et al.*, 1990). These differences mean that the transcriptional regulation of *Tf1* and *Tf2* is distinct. For example *Tf2* expression is induced in response to low oxygen via Sre1 but *Tf1* is not (Sehgal *et al.*, 2007; Murton *et al.*, 2016). Furthermore while 12 of the 13 *Tf2* elements exhibit very high levels of identity in their LTR regions, *Tf2-11* has a 'hybrid' 5' LTR that contains a region of homology to *Tf1* which is likely due to the recombination event between a pre-existing *Tf1* LTR (or LTR fragment) and the*Tf2-11* element (Bowen *et al.*, 2003). Previous experimental data has shown that *Tf2-11* has a different pattern of expression to the other *Tf2* elements (Sehgal *et al.*, 2007; Anderson *et al.*, 2009).

To investigate the LTR region responsible for high activity when cells are grown in EMM, reporter strains were used, where the *lacZ* gene is under the control of a endogenous 'typical' *Tf2* LTR, a *Tf1* LTR or the 'hybrid' *Tf2-11* LTR (Fig. 5.2B). β-galactosidase assays revealed that expression from neither *Tf1* nor *Tf2-11* was stimulated by growth EMM. Therefore, the sequences that allow high expression when cells are grown in minimal medium are absent from *Tf1* and *Tf2-11* LTRs.

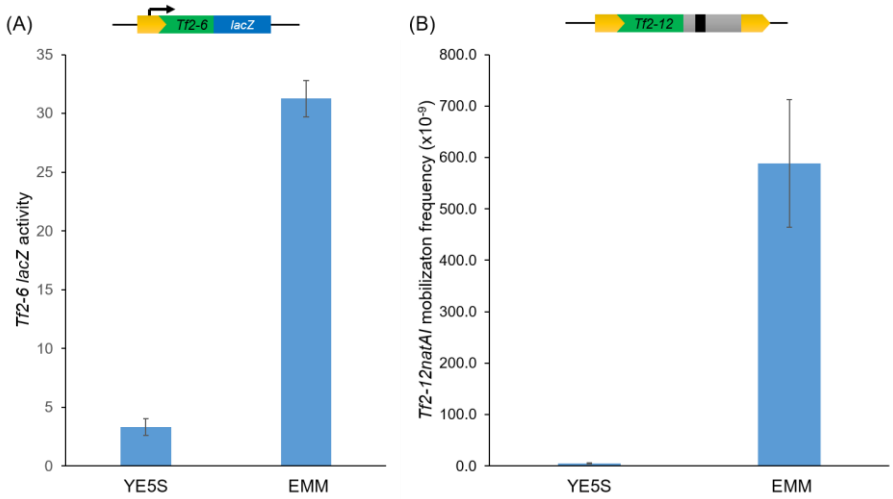


Figure 5.1 *Tf2* retrotransposon activity is induced in minimal (EMM) medium.

(A) *Tf2-6 lacZ* strains were grown at 30°C in YE5S or EMM to mid-log phase before being collected for use in quantitative β-galactosidase assays. Values shown represent the average of 8 biological repeats assayed in duplicate and error bars indicate ±SEM.

(B) A*Tf2-12natAI* reporter strain was cultured in either YE5S or EMM medium for 2 days at 30°C. Mobilization frequency was determined as the proportion of ClonNAT resistant colonies in the total number of viable colonies using the mean of medium method. Average values determined by mean of medium analysis with a minimum of 30 repeats are shown. Error bars indicate ±SEM.

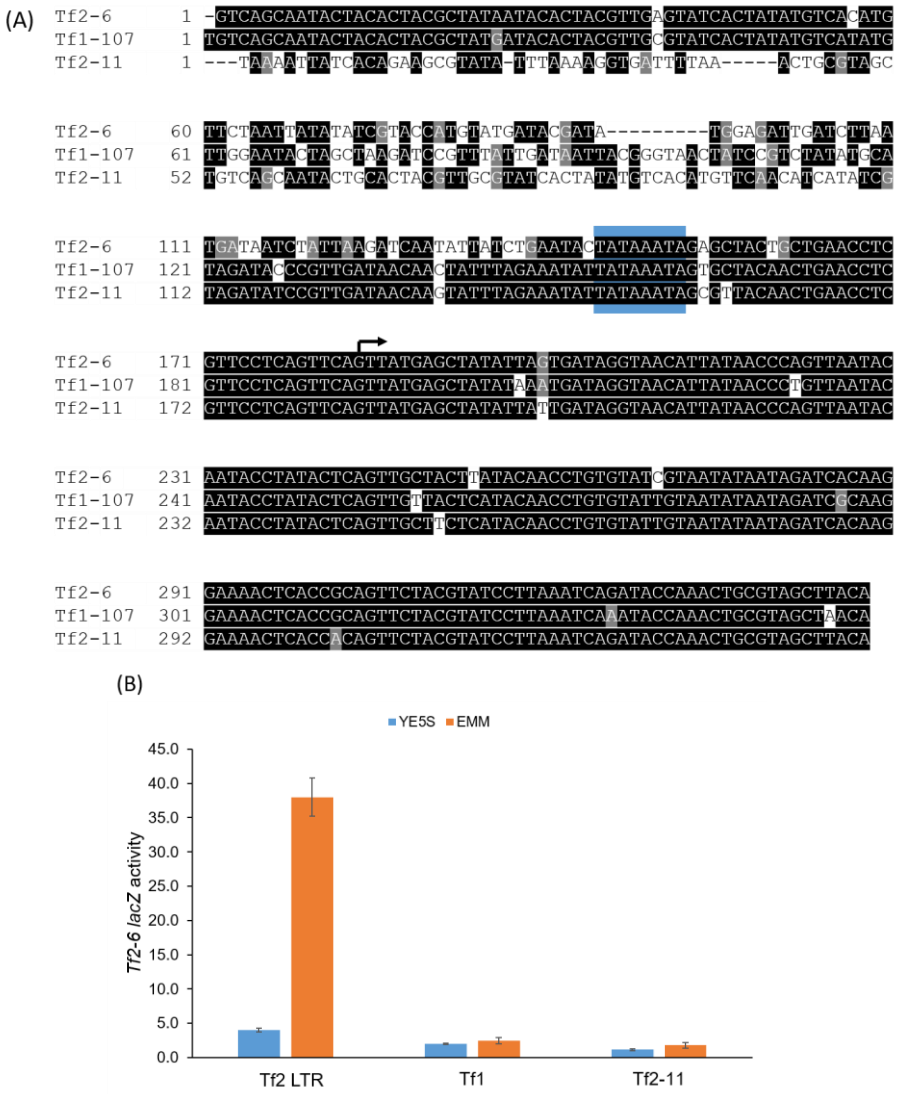


Figure 5.2 The expression of neither *Tf1* nor *Tf2-11* is induced by EMM

(A) The 5' LTR sequences of *Tf1-107*, *Tf2-6* and *Tf2-11* were aligned using ClustalW alignment (www.ch.ebnmet.org). Box shading with 1.0 fraction shading is shown. Note *Tf2-6* is included as an example of a 'typical' Tf2 LTR. Putative TATA box highlighted in blue and transcription start site indicated by arrow.

(B) *Tf2-LTR- lacZ, Tf1-lacZ*, *Tf2-11-lacZ* strains were grown at 30°C in either YE5S or EMM to mid-log phase before being collected for use in quantitative β-galactosidase assays. Values shown represent the average of 8 biological repeats assayed in duplicate and error bars indicate ±SEM.

Tf2 expression is also limited by the HIRA histone chaperone complex (Murton *et al.*, 2016). Previous data demonstrated that the loss of the HIRA complex subunits (*hip1Δ*) induces a very large increase in *Tf2-lacZ* expression when cells are grown in YE5S (Murton *et al.*, 2016). Therefore, *Tf2* expression was also measured in the *hip1Δ* background when cells were grown in EMM. In this background (*hip1*Δ), similar levels of expression were observed irrespective of whether cells were grown in EMM or YE5S. One explanation of these results would be that growth in EMM overcomes the repressive effects of HIRA on *Tf2* expression. Another explanation would be that in the *hip1*Δ background the expression of *Tf2* has reached its maximum level and cannot be further upregulated. Although, loss of HIRA leads to high level of *Tf2* expression, a corresponding increase in mobilization was not observed. Indeed, when cells are grown in EMM the deletion of *hip1*$^{+}$ led to a (~2.5 fold) decrease in the frequency of *Tf2* mobilization. It is not clear why this is the case but it is possible that while HIRA may repress *Tf2* transcription, it may promote later stages in the lifecycle, such as cDNA integration.

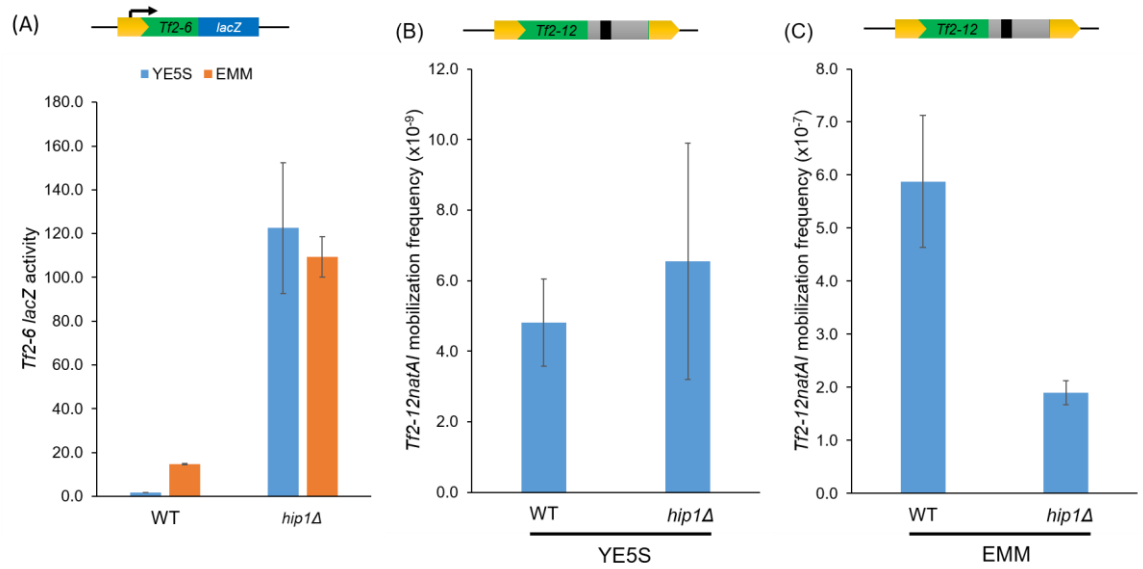


Figure 5.3 Loss of HIRA-mediated silencing in EMM does not result in uncontrolled *Tf2* mobilization

(A) *Tf2-6 lacZ* strains (*wt* and *hip1*Δ) were grown at 30°C in EMM or YE5S to mid-log phase before being collected for use in quantitative β-galactosidase assays. Values shown represent the average of 8 biological repeats assayed in duplicate and error bars indicate ±SEM. (B&C) Strains were grown in the indicated medium for 2 days at 30°C. *Tf2-12natAI* mobilization frequency was determined as the proportion of ClonNAT resistant colonies in the total number of viable colonies using the mean of medium method. Average values determined by mean of medium analysis with a minimum of 30 repeats. Error bars indicate ±SEM.

5.3 Impact of nitrogen source on *Tf2* activity

Edinburgh minimal media (EMM) was first developed by Murdoch Mitchison for his cell cycle analyses as a synthetic defined media (Mitchison, 1970) and changes to the nitrogen or carbon sources of the media are commonly used to manipulate growth and cell cycle controls and promote sexual differentiation (Petersen and Russell, 2016).

The data described above demonstrate that a switch of the growth medium is sufficient to induce *Tf2* mobilization and indicate that differences in one or more of the components of EMM and YE5S influence *Tf2* activity. A key candidate for one of these factors is the nitrogen source. YE5S is known as a rich medium, made up from yeast extract and glucose with amino acids (lysine, leucine and histidine) and nucleobase supplements (adenine and uracil) and supports a fast growth rate as a general use media (Petersen and Russell, 2016). The nitrogen source of YE5S is not precisely defined but yeast extract contains a complex mixture of peptides ranging from 6 to more 30 amino acids in length (Proust *et al.*, 2019). In contrast, EMM employs ammonium chloride, which is defined as a good nitrogen source for *S. pombe*. Therefore, the impact of changing nitrogen source from ammonium chloride to an alternative 'good' nitrogen source, glutamate and also a 'poor' nitrogen source in proline was determined.

Quantitative β-Galactosidase assay results indicated that changing the nitrogen source to glutamate or proline had only a small effect on *Tf2* expression. Also the actual mobility of *Tf2-12natAI* did not change when glutamate was swapped for ammonium (Fig. 5.4). Unfortunately, with proline being a poor nitrogen source, the growth of *Tf2-12natAI* strains was severely restricted and no mobility data was collected in for cells inoculated in proline medium. Nonetheless, the results suggest that the nitrogen source *per se* has only a minor impact on *Tf2* activity.

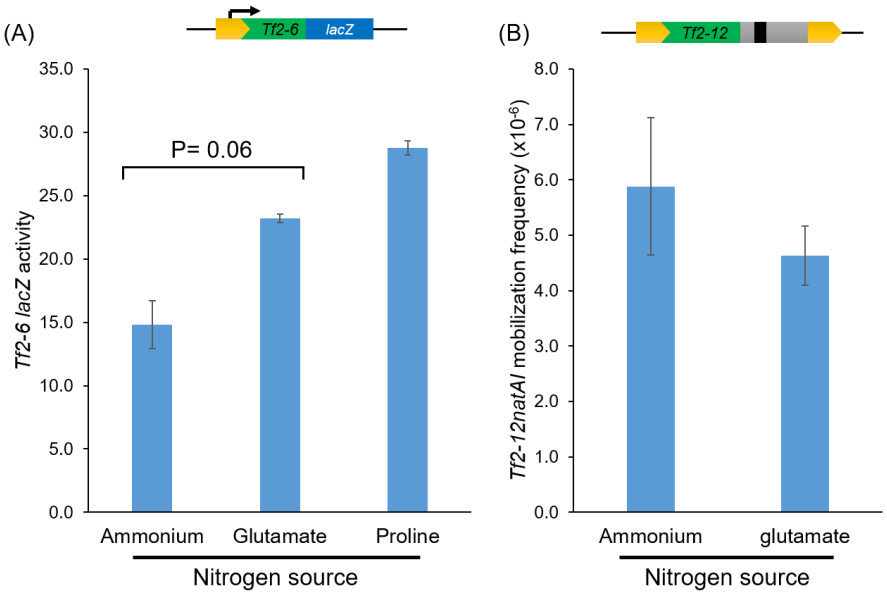


Figure 5.4 Quality of nitrogen source has only a modest effect on *Tf2* activity.
(A) *Tf2-6 lacZ* strains were grown at 30°C in EMM with either ammonium chloride, 3.75 g/L glutamate or 0.1% proline as nitrogen source before being collected for use in quantitative β-galactosidase assays. Values shown represent the average of 8 biological repeats assayed in duplicate and error bars indicate ±SEM. (B) *Tf2-12natAI* mobilization frequency was determined as the proportion of ClonNAT resistant colonies in the total number of viable colonies using the mean of medium method. Average values determined by mean of medium analysis with a minimum of 30 repeats. Error bars indicate ±SEM.

5.4 Impact of amino acid and nucleobase supplements on *Tf2* activity

One of the big differences between rich (YE5S) and minimal medium (EMM) is the abundance of amino acids and other important compounds such as nucleobases. It was hypothesized that the low activity of *Tf2* in YE5S medium may be due to the abundance of amino acids which may change cell physiology and cell signalling (Weisman *et al.*, 2005; Bonfils *et al.*, 2012; Han *et al.*, 2012; Kim *et al.*, 2013; Petersen and Russell, 2016). Many of the fission yeast cell strains used during the study are auxotrophic (e.g. *ade*$^-$ *leu*$^-$ *ura*$^-$ *his*$^-$). Therefore, to be able to manipulate amino acid and nucleobase levels properly, prototrophic strains harbouring the *Tf2-6 lacZ* and *Tf2-natAI* reporter were employed. Analysis revealed that prototrophic strains did not differ from the auxotrophic strains with respect to *Tf2* expression and *Tf2-12natAI* mobilization frequency in EMM (Fig. 5.5).

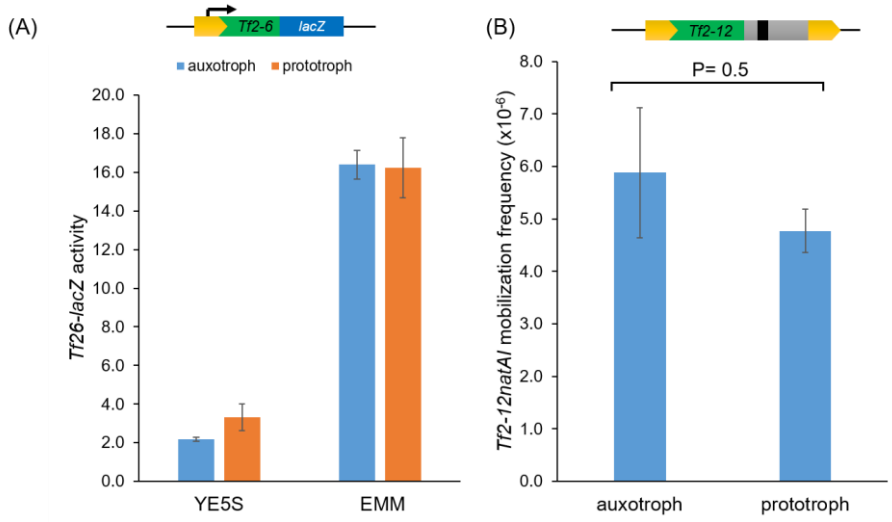


Figure 5.5 *Tf2* activity is not changed in prototrophic strain.

(A) *Tf2-6 lacZ* auxotroph and prototroph strains were grown at 30°C in the indicated medium to mid-log phase before being collected for use in quantitative β-galactosidase assays. Values shown represent the average of 8 biological repeats assayed in duplicate and error bars indicate ±SEM. (B) *Tf2-12natAI* mobilization frequency was determined as the proportion of ClonNAT resistant colonies in the total number of viable colonies using the mean of medium method. Average values determined by mean of medium analysis with a minimum of 30 repeats. Error bars indicate ±SEM. p value (*t*-test).

Excess levels of leucine and histidine amino acids and also the nucleobase adenine, were used in different combinations to determine their effect on the activity of *Tf2*. A two-fold decrease in *lacZ* expression was observed when leucine, histidine and adenine were added to the medium (Fig. 5.6A), suggesting that these supplements slightly repress*Tf2* expression. Further investigation into which of these plays a major role in this repression was approached by removing one from the medium each time. The removal of adenine or histidine has no significant effect on the activity of *Tf2-6 lacZ* while the removal of leucine increased the expression of *Tf2* (Fig. 5.6A), suggesting that leucine is important for the activity of *Tf2*. A fivefold increase in leucine concentration (to 500 mg/L) did not repress expression, indicating that repression requires also requires the addition of either adenine or histidine. Overall, although the addition of amino acids and nucleobases does not reduce *Tf2* expression to the level observed in rich YE5S medium.

The same experiment was repeated in the *Tf2-12natAI* strain to determine the effect on the mobility of *Tf2*. There was no significant difference of the mobility of *Tf2* between cells inoculated with and without (Ade, Leu and His) supplements (Fig. 5.6B). This suggests that high levels of amino acids and nucleobases is unlikely to be the basis for the low activity of *Tf2* retrotransposons when cells are grown in rich YE5S medium. Media that utilise yeast extract are also rich in B vitamins such as thiamine (Proust *et al.*, 2019). Therefore, the potential role of thiamine was also investigated. β-galactosidase and mobilization assays revealed the addition of thiamine to EMM medium did not influence on *Tf2* activity (Fig. 5.6AB).

Excess supplements do suppress *Tf2* expression, but do not lower the expression and mobilization to the YE5S level. Therefore, amino acid/nucleobase levels do not seem to be the source of the difference between YE5S and EMM.

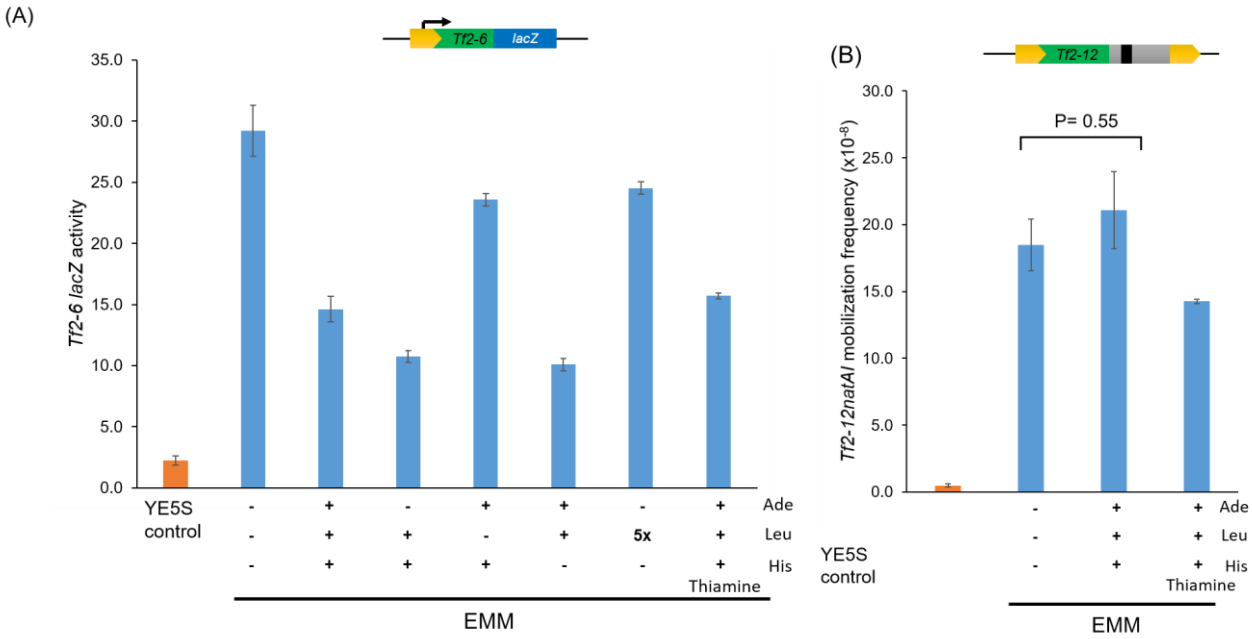


Figure 5.6 Tf2 activity is dependent on leucine with either adenine or histidine.

(A) *Tf2-6 lacZ* prototroph strains were grown at 30°C in YE5S or EMM with the indicated supplements to mid-log phase before being collected for use in quantitative β-galactosidase assays. Adenine was added at 100 mg/L, leucine at 100 mg/L and histidine 100 mg/L. Values shown represent the average of 8 biological repeats assayed in duplicate and error bars indicate ±SEM.

(B) Strains were grown for two days at 30°C in media as detailed in (A). *Tf2-12natAI* mobilization frequency was determined as the proportion of ClonNAT resistant colonies in the total number of viable colonies using the mean of medium method. Average values determined by mean of medium analysis with a minimum of 15 repeats. Error bars indicate ±SEM value. p value (*t*-test)

To further investigate the impact of the medium on expression, *Tf2-6 lacZ* cells were grown in YE5S with increasing amounts of EMM in the medium (Fig. 5.7). An increase in β-galactosidase activity was observed when EMM content increased from 10% to 90%. This suggests that rather that some compounds in YE5S repressing *Tf2* expression, EMM contains one or more ingredients that stimulates *Tf2*.

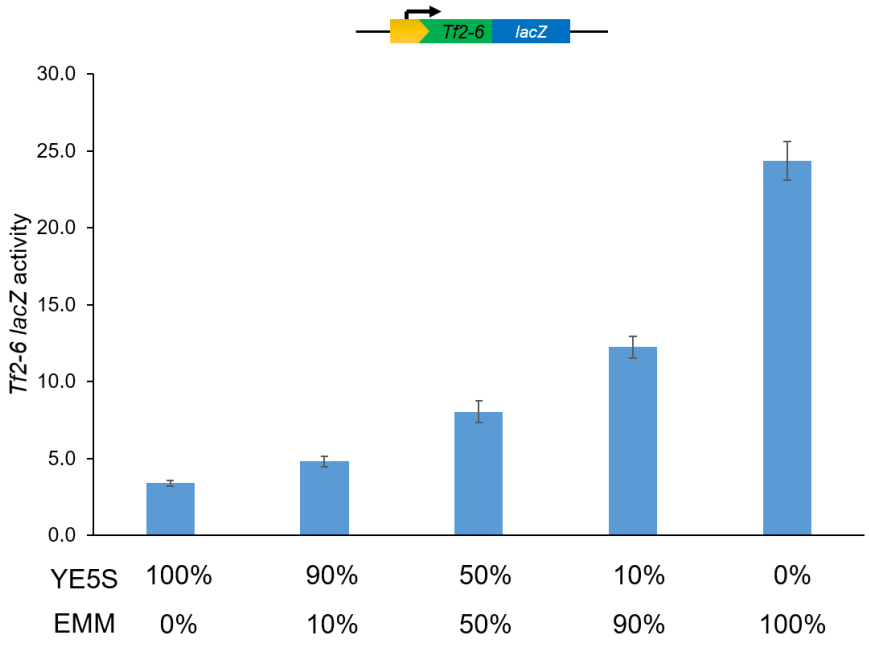


Figure 5.7 The addition of EMM to YE5S increases Tf2 expression.

Tf2-6 lacZ strains were grown at 30°C in YE5S and EMM amino acids supplement in different mixture to mid-log phase before being collected for use in quantitative β-galactosidase assays. Values shown represent the average of 8 biological repeats assayed in duplicate and error bars indicate ±SEM

5.5 Ammonium ions and phthalate are the key ingredient in EMM that activate *Tf2*

As previously discussed, EMM is a defined medium developed in the 1970s (Mitchison, 1970; Nurse, 1975). The composition of EMM is listed in Table 2.2. To determine what is responsible for the high activity of *Tf2* elements when cells are grown in this medium, individual components of EMM were added to YE5S medium and the expression of *Tf2* was monitored using a *lacZ* reporter. Unsurprisingly, majority of the ingredients (e.g. salts, vitamins, minerals) had no impact on *Tf2* expression while cells inoculated in YE5S with sodium phosphate (Na_2HPO_4) showed a modest increase in *Tf2* expression (Fig. 5.8A). There were two components, ammonium chloride (NH_4Cl) and potassium hydrogen phthalate ($C_8H_5KO_4$) from EMM that resulted in a significant increase in *Tf2* expression (~10 fold and ~8 fold, respectively) (Fig. 5.8A). Potassium hydrogen phthalate is a buffering agent and replaced sodium acetate in the original EMM formulation in order to grow *cdc5* mutant cells (Nurse, 1975). Therefore, the impact of sodium acetate on *Tf2* induction was also examined (Fig. 5.8A). The addition of sodium acetate resulted in only a modest increase (~2 fold) of *Tf2* expression, suggesting that the presence of a pH buffering agent *per se* does not account for the increase in*Tf2* expression but instead the chemical property of phthalate stimulates *Tf2* expression.

To determine if either ammonium ions or chloride ions were required for the activation of *Tf2*, ammonium sulphate (NH_4SO_4) and sodium chloride (NaCl) were added to the rich media. Unsurprisingly, the exchange from ammonium chloride (NH_4Cl) to sodium chloride (NaCl) abolished the induction of *Tf2* expression while ammonium sulphate (NH_4SO_4) activated *Tf2* expression to a similar level as ammonium chloride (NH_4Cl). This suggests that ammonium ions and phthalate are the two major ingredients in EMM that activate *Tf2* expression (Fig. 5.8B). The addition of both ammonium chloride and potassium hydrogen phthalate did not result in a further increase in expression suggesting that the two compounds activate *Tf2* expression via the same signalling pathway or there is an upper limit of *Tf2* expression (Fig. 5.8B).

The same experiment was repeated using the *Tf2-12natAI* reporter to analyse mobility in these conditions. Mobilization of the *Tf2-12natAI* cassette significantly increased following the addition of ammonium chloride or potassium hydrogen phthalate to YE5S

(Fig. 5.9). Again the addition of both did not result in a further increase. Therefore it is the presence of ammonium ions and potassium hydrogen phthalate that stimulates the activity of *Tf2* when cells are grown in EMM.

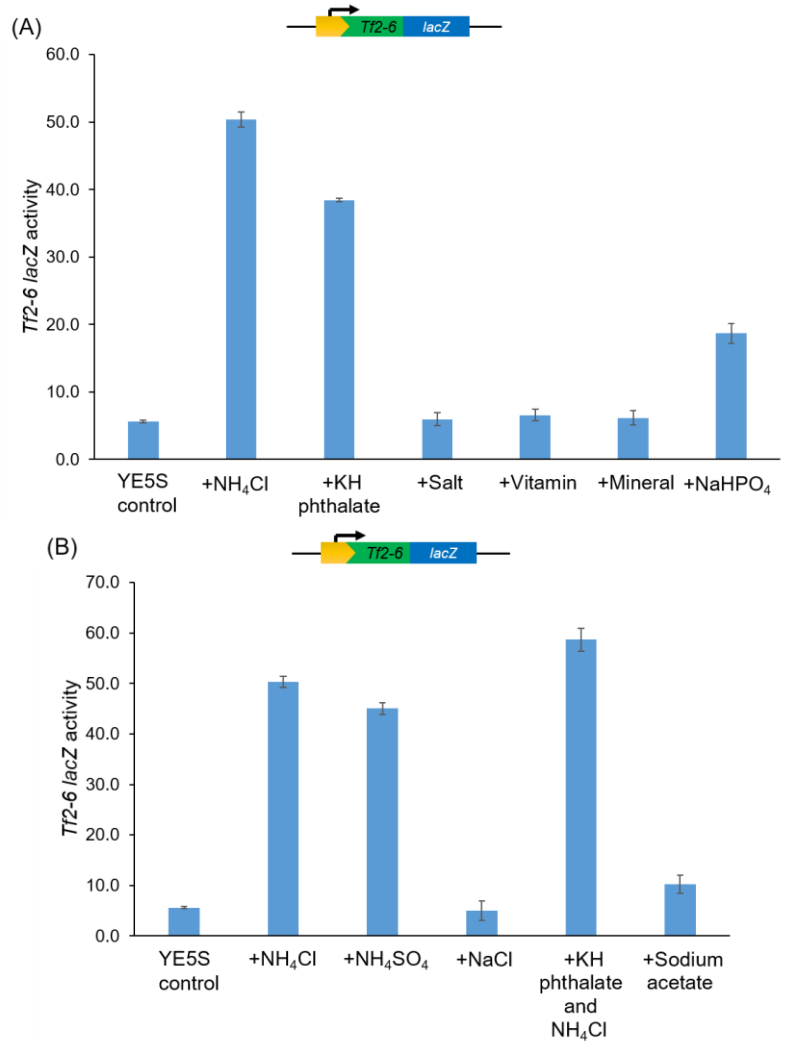


Figure 5.8 ***Tf2*** **activity is increased by ammonium and phthalate.**

Tf2-6 lacZ strains were grown at 30°C in YE5S medium with the addition of the indicated selected components to mid-log phase before being collected for use in quantitative β-galactosidase assays. When present ammonium chloride was added at 93.5 mM, ammonium sulphate at 61.7 mM, sodium chloride at 27.3 mM, potassium hydrogen phthalate at 14.7 mM and sodium acetate at 12.2 mM. Values shown represent the average of 8 biological repeats assayed in duplicate and error bars indicate ±SEM.

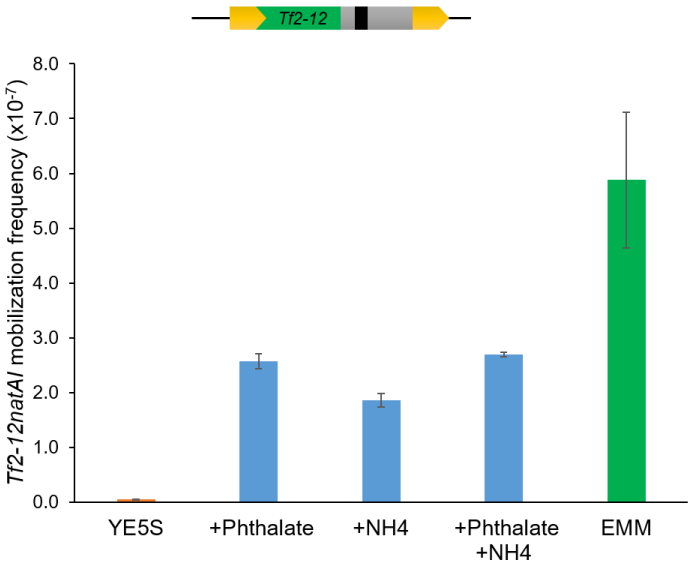


Figure 5.9 *Tf2* mobility is induced by ammonium and phthalate.

Cells were grown in YE5S medium with the indicated additions, ammonium chloride was added at 93.5 mM, and potassium hydrogen phthalate at 14.7 mM. *Tf2-12natAI* mobilization frequency was determined as the proportion of ClonNAT resistant colonies from the total number of viable colonies using the mean of medium method. Average values determined by mean of medium analysis with a minimum of 30 repeats. Error bars indicate ±SEM.

5.6 Discussion

The activity of retrotransposons is commonly upregulated in response to environmental changes (Hunter *et al.*, 2013; Grandbastien, 2015; Negi *et al.*, 2016). One natural stress is a shortage of nutrients, such as sources of carbon and nitrogen. In budding yeast *S. cerevisiae*, nutrient deficiency was shown to activate *Ty* elements at both transcriptional and post-transcriptional levels (Morillon *et al.*, 2000; Dai *et al.*, 2007). However, the impact of changes in nutrient availability on the activity of *S. pombe Tf* retrotransposons has not been well studied.

Previous research has demonstrated that environmental factors such as temperature, oxidative stress, and hypoxia affect the expression of *Tf2* elements. Glucose concentration has also been shown to influence the modification state of the chromatin associated with *Tf2* elements (Chen *et al.*, 2003; Sehgal *et al.*, 2007; Yamanaka *et al.*, 2013; Murton *et al.*, 2016). However, the impact of culture media on *Tf2* has not been reported. EMM has been used for four decades and has been used in many plasmid-based assays of *Tf1/2* activity. (Hickey *et al.*, 2015; Sangesland *et al.*, 2016; Rai *et al.*, 2017). However an assessment of the impact of EMM medium upon the activity of an integrated *Tf2* mobilization reporter has not been performed. Interestingly, a quantitative analysis of the transcriptome in proliferating cells grown in EMM indicated that *Tf2* mRNA is present at ~1 copy per cell (Marguerat *et al.*, 2012). Under these conditions protein encoding genes produce a median of 2.4 transcripts per cell (Marguerat *et al.*, 2012). This suggests that when cells are cultured in EMM *Tf2* elements are not silenced.

The constitutive activation of the Sre1 transcription factor (*sre1-N*) increases *Tf2* expression and mobilization to a similar degree (~12 fold and ~20 fold respectively) (Murton *et al.*, 2016). In contrast, cell culture in EMM (as opposed to YE5S) increases expression ~6 fold while mobilization increases ~100 fold. This suggests that culture in EMM influences *Tf2* activity at both transcriptional and post-transcriptional levels. The expression of *Tf1* and *Tf2-11* was not induced by culture in EMM suggesting that these elements lack the necessary response element(s) in their LTRs. LTR sequence comparisons suggest that this element is most likely located between base pairs ~60 and ~136 in the *Tf2* LTR just upstream of the TATA box (Fig. 5.2). It would be interesting to perform a mutational analysis of this region in order to see whether an

'EMM' response element could be located. The data also suggested that the mechanism may involve the loss of HIRA mediated repression. However, more experiments on other HIRA complex proteins (Slm9, Hip3 and Hip4) are needed to reveal how the activity of the HIRA complex is regulated in response to culture of cells in EMM.

A major difference between YE5S and EMM is the availability of amino acids in the culture medium. YE5S is composed of yeast extract (YE), glucose, and five nucleobase and amino acid supplements. Commercial YE corresponds to the soluble fraction of molecules released after either yeast autolysis or controlled enzymatic lysis and contains about 4,600 different oligopeptides stretching from 6 to greater than 30 amino acids in length (Proust *et al.*, 2019). Therefore, it was hypothesized that high level of amino acids would repress *Tf2* expression particularly as amino acid starvation induces *Tf2* expression (Duncan *et al.*, 2018). However, amino acids (leucine and histidine) levels seemed only to have a modest effect on *Tf2* expression and none on mobility (Fig. 5.6AB). Also, changing the nitrogen source from ammonium to glutamate (another good nitrogen source) or proline (a poor source) had only little impact on both transcription and mobility. However, the discovery that phthalate has a great impact on *Tf2* overshadows the results of the swapping of nitrogen sources since the EMM medium used with different nitrogen sources contains potassium phthalate as a pH buffering agent. To confirm the effects of nitrogen sources on *Tf2*, the EMM medium with phthalate replaced by sodium acetate should be used.

Ammonium ions were shown to be important for the increased activity of *Tf2* when cells are cultured in EMM. In *S. cerevisiae*, the ammonium transporter Mep2, which is a member of the Amt/Mep/Rh family of transporters is responsible for the uptake and signal transduction of ammonium (van den Berg *et al.*, 2016). The low-capacity, high-affinity receptor/sensor Mep2, but not other ammonium transporters (Mep1 and 3), functions in fungal development and initiates a signaling cascade that leads to morphology changes to filamentous (pseudohyphal) growth under nitrogen limitation conditions (Lorenz and Heitman, 1998; van den Berg *et al.*, 2016). The same ammonium transporter/methylammonium permease (Amt/Mep) family was identified in *S. pombe* and is comprised of Amt1, Amt2 and Amt3 (Mitsuzawa, 2006). The characteristics of the three Amt proteins were briefly analysed and Amt1 is required for ammonium limitation-induced filamentous invasive growth (Mitsuzawa, 2006).

However, it is not clear whether Amt1 is the *S. pombe* ammonium transceptor. *Tf2-6 lacZ* expression and *Tf2-12natAI* mobilization assays revealed that the loss of *amt1*$^{+}$ had no effect on expression but slightly reduced mobilization (data not shown). Given the effect of ammonium ions on activity, it would be interesting to analyse *Tf2* expression and mobilization in *amt1*Δ *amt2*Δ *amt3*Δ double and triple mutants, but again the EMM medium in which phthalate is replaced by sodium acetate should be used to avoid the confounding effects of phthalate.

Potassium phthalate, is used as a pH buffering agent in EMM and replaced sodium acetate (Nurse, 1975). However, how phthalate induces *Tf2* expression is unclear as little is known about its impact on intracellular signaling pathways. Phthalates are widely used as plasticizers to soften vinyl and PVC compounds (Li and Suh, 2019). Because of their clear syrupy consistency, low water solubility, high oil solubility and low volatility, phthalates are present in a wide range of products such as household cleaners, food packaging, cosmetics and the enteric coatings of pharmaceutical pills and nutritional supplements. The use of phthalates is currently under debate due to the potential risk to health through environmental pollution (Esnault *et al.*, 2019; Li and Suh, 2019). Phthalates are classified as endocrine disruptors that interfere with normal hormonal mechanisms in humans and are linked to problems with male fertility, such as reduced quality of semen, DNA damage in sperm, decreased sperm motility and semen volume (Bansal *et al.*, 2018; Zamkowska *et al.*, 2018; Li and Suh, 2019). Phthalates have also been shown to be associated with DNA damage and oxidative stress in humans and earthworms (Zhao *et al.*, 2015; Huen *et al.*, 2016; Huang *et al.*, 2018; Song *et al.*, 2019). The impact of phthalates on retroelements is poorly understood but maternal exposure to phthalates influences the DNA methylation levels of *LINE-1* and *Alu* retroelements (Zhao *et al.*, 2015; Huen *et al.*, 2016; Zhao *et al.*, 2016; Huang *et al.*, 2018). The hypomethylation of *LINE-1* and *Alu* elements may potentially link phthalates to genome instability (Huang *et al.*, 2018).

The processes of DNA damage and oxidative stress signalling are well characterized in fission yeast, and so it would be interesting to determine how phthalate impacts on these processes. Furthermore, during this study, Levin and co-workers reported that phthalate along with other stress inducing agents such as caffeine and the heavy metal cobalt increased the mobilization of *Tf1* elements (Esnault *et al.*, 2019). It is clear that

phthalate induces the *Tf* retroelements in fission yeast, however the mechanisms remain to be determined.

Chapter 6 TOR system and retrotransposon

6.1 Introduction

Target **o**f **r**apamycin (TOR) was first discovered as the target of the immunosuppressive and anticancer drug rapamycin (Heitman *et al.*, 1991). TOR proteins are serine/threonine protein kinases which are structurally and functionally conserved from yeasts to human (Wullschleger *et al.*, 2006). Rapamycin inhibits TOR activity by forming a complex with FKBP12 which binds to and inhibits its activity (Harris and Lawrence, 2003). TOR proteins function as master regulators of cell growth in all eukaryotes, including worms, flies and plants, and mediate responses to changes environmental nutrient availability (Chiu *et al.*, 1994; Oldham *et al.*, 2000; Long *et al.*, 2002; Menand *et al.*, 2002; Alvarez and Moreno, 2006). In budding yeast *S. cerevisiae* and fission yeast *S. pombe*, there are two TOR proteins (Tor1 and Tor2) different from other eukaryotes that generally have only one TOR. Nonetheless, TOR proteins forms two multiprotein complexes which are structurally and functionally distinct: TOR complex 1 (TORC1) and TOR complex 2 (TORC2) (Wullschleger *et al.*, 2006; Avruch *et al.*, 2009). In *S. pombe*, TORC1 contains the essential catalytic subunit, Tor2, along with Mip1 (homolog of Kog1) and Wat1 (Lst8 homolog) while TORC2 contains Tor1, Sin1 (Avo1 homolog), Ste20 (Tsc1/Avo3 homolog) and Wat1 (Hayashi *et al.*, 2007; Matsuo *et al.*, 2007; Otsubo and Yamamato, 2008). As in other eukaryotes, TORC1 is the key sensor that in integrates environmental nutrient status with cell growth and proliferation. Fission yeast TORC2 is not essential for growth and is involved in the regulation of a range of processes including sexual differentiation, actin organization and heterochromatin (Otsubo and Yamamato, 2008)

6.2 Rapamycin stimulates *Tf2* expression and mobilization.

The TOR network is central to the co-ordination of growth and proliferation with the nutrient environment (Weisman, 2016). The finding that the activity of *Tf2* retrotransposons is markedly influenced by the culture medium prompted the examination of the impact of TOR signalling on these retrotransposons. To investigate whether *Tf2* retrotransposons are regulated by TOR, cells harbouring the *Tf2-12natAI* mobilization reporter were grown in rich medium YE5S either in the presence or absence of the drug, rapamycin. Rapamycin specifically reduces the activity of the TORC1 complex, which in all eukaryotes functions as the major nutrient sensor (Weisman, 2016). Strikingly, a huge increase in the frequency of ClonNAT resistant colonies was observed when cells were grown in the presence of rapamycin (100 nM) (Fig. 6.1), indicating that the activity of *Tf2* retrotransposons is upregulated following exposure to this drug.

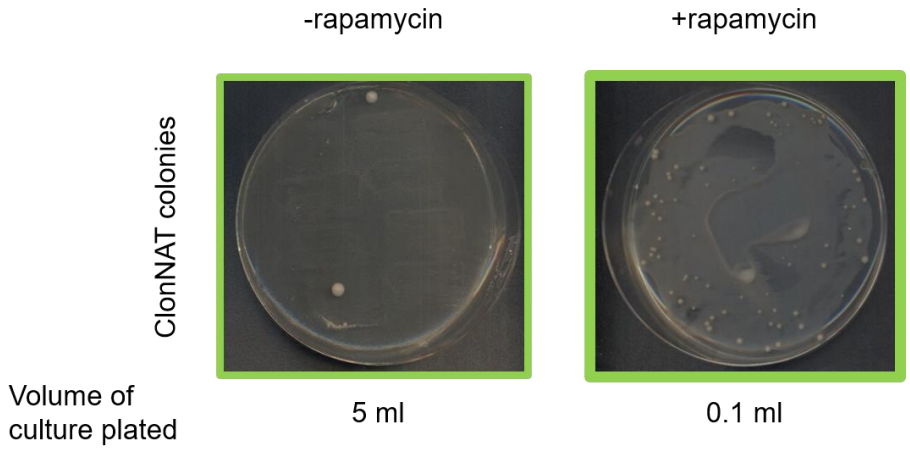
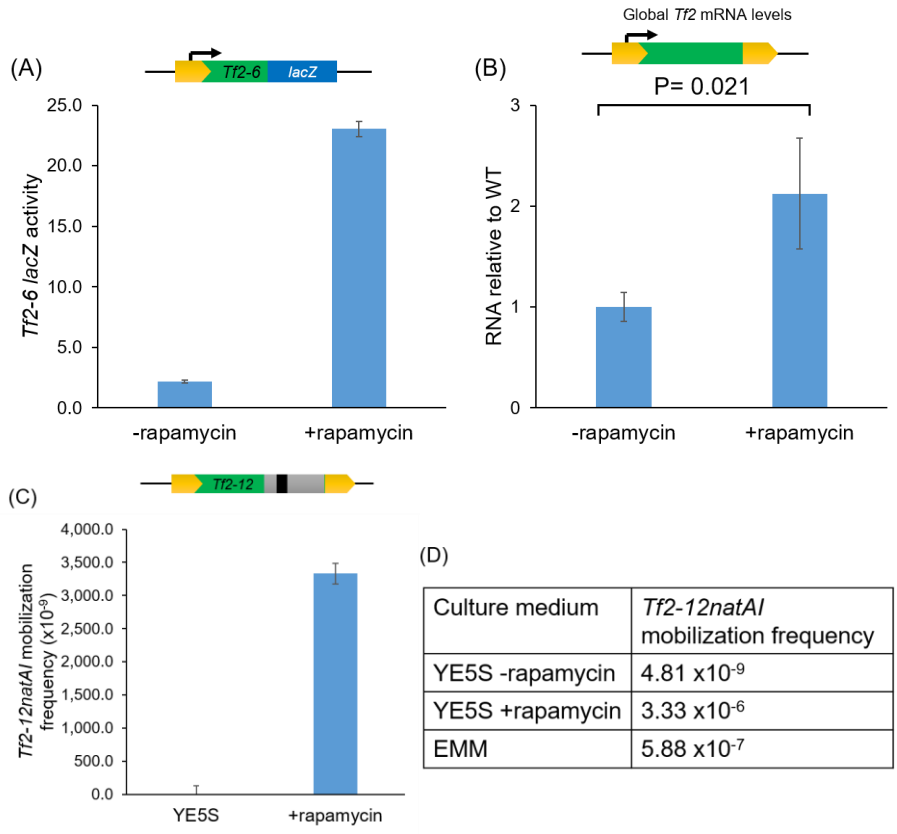


Figure 6.1 Rapamycin increases the activity of the *Tf2-12natAI* mobilization reporter.
Cells containing *Tf2-12natAI* were grown in 5 ml YE5S with or without rapamycin (100 nM) for 2 nights at 30°C. The indicated volumes of these saturated cultures was then harvested, resuspended in H_2O and spread onto YE5S agar containing 75 µg/ml ClonNAT. Plates were incubated at 30°C until colonies appeared.

The data described above demonstrate that rapamycin stimulates the activity of *Tf2* retrotransposons. In order to further investigate this the expression of *Tf2* elements was examined following the addition of rapamycin to the medium (Fig. 6.2A, B). β-galactosidase assays revealed that rapamycin resulted in a ~10 fold increase in expression from a *Tf2-6 lacZ* reporter. Furthermore, qRT-PCR assays demonstrated that *Tf2* mRNA levels increased ~3 fold, when rapamycin was present in the culture medium. Next, a fully quantitative mobilization assay was carried out using the *Tf2-12natAI* reporter (Fig. 6.2C). A huge increase in *Tf2* mobilization (~1000 fold) was observed in the presence of rapamycin (Fig. 6.2C). Indeed, growth in rich (YE5S) medium containing rapamycin results in a mobilization frequency that is even higher than when cells are grown in minimal (EMM) medium (Fig. 6.2D).



(D)

Culture medium	*Tf2-12natAI* mobilization frequency
YE5S -rapamycin	4.81×10^{-9}
YE5S +rapamycin	3.33×10^{-6}
EMM	5.88×10^{-7}

Figure 6.2 Rapamycin induces *Tf2* expression and mobilization

(A) *Tf2-6 lacZ* strains were grown at 30°C in YE5S either with or without 100 nM rapamycin to mid-log phase before being collected for use in quantitative β-galactosidase assays. Values shown represent the average of 8 biological repeats assayed in duplicate and error bars indicate ±SEM.

(B) RNA was extracted from cells cultured to mid log phase at 30°C in YE5S either with or without 100 nM rapamycin. *Tf2* mRNA levels were determined by RT-qPCR to detect transcripts from the 13 full length *Tf2* elements and normalised to *act1*$^{+}$ mRNA. Data is the mean of at least 3 biological repeats and error bars indicate ±SEM.

(C) *Tf2-12natAI* mobilization frequency was determined as the proportion of ClonNAT resistance colonies from the total number of viable colonies using the mean of medium method with a minimum of 30 repeats. Error bars indicate ±SEM.

(D) A table showing *Tf2* mobilization frequencies in the indicated growth medium.

6.3 Inhibition of TORC1 using caffeine does not induce the expression of *Tf2*

Although rapamycin inhibits the activity of the *S. pombe* TORC1 complex, this inhibition is incomplete (Takahara and Maeda, 2012). The addition of rapamycin in combination with caffeine was suggested to result in a more complete inhibition of TORC1 signalling that results in the induction of nitrogen starvation-responsive gene expression and autophagy (Takahara and Maeda, 2012). Moreover, caffeine was suggested to affect TORC1-dependent processes differently to rapamycin and augment the phenotypes associated with rapamycin (Rallis *et al.*, 2013). Interestingly, caffeine was also recently identified as one of the stresses that induces *Tf1* mobility in *S. pombe*, suggesting that caffeine is also a potential candidate to influence *Tf2* activity (Esnault *et al.*, 2019). However, quantitative β-galactosidase assays indicated that caffeine did not increase expression of a *Tf2-6 lacZ* reporter and moreover, the combination of caffeine and rapamycin in the growth medium actually reduced *Tf2* expression relative to the rapamycin alone condition (Fig. 6.3). This suggests that the 'partial' inhibition of TORC1 by rapamycin is sufficient to fully induce *Tf2* expression.

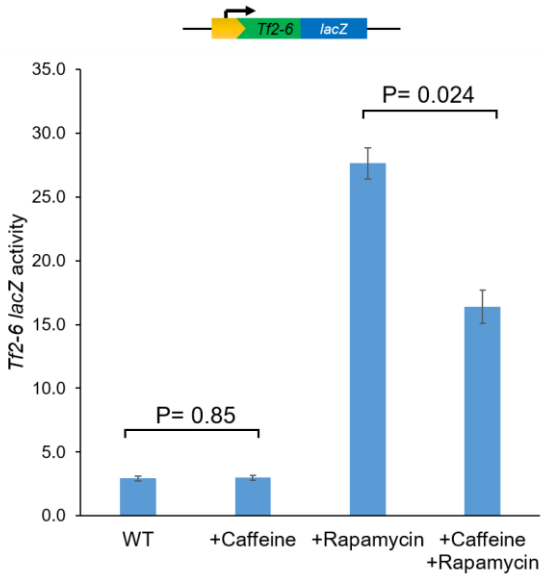


Figure 6.3 Caffeine does not influence *Tf2* activity

Tf2-6 lacZ strains were grown at 30°C in YE5S with the either 100 nM rapamycin, 10 mM caffeine or both to mid-log phase before being collected for use in quantitative β-galactosidase assays. Values shown represent the average of 8 biological repeats assayed in duplicate and error bars indicate ±SEM.

6.4 Rapamycin does not stimulate *Tf2* activity when cells are cultured in EMM

Since culture medium has big influence upon the activity of *Tf2* retrotransposons the impact of adding rapamycin to cells cultured in minimal EMM medium was determined. As EMM contains a good nitrogen source (ammonium) the TORC1 complex is active when cells are grown in this medium. Therefore it was surprising that the addition of rapamycin to EMM medium did not significantly increase the expression of a *Tf2-6 lacZ* reporter. One explanation could be an upper limit of *Tf2* expression has already been achieved when cells are grown in EMM. Another reason could be that the activity of the TORC1 complex is lower when cells are grown in EMM and that this is sufficient to induce *Tf2* retrotransposons. Consistent with the analysis of *Tf2* expression, there is almost no changes in mobilization frequency was observed when rapamycin was added to EMM, when compared to the ~ 1,000 fold increase by addition of rapamycin in rich medium (Fig. 6.4B). Therefore the effect of rapamycin seems to be limited to rich media.

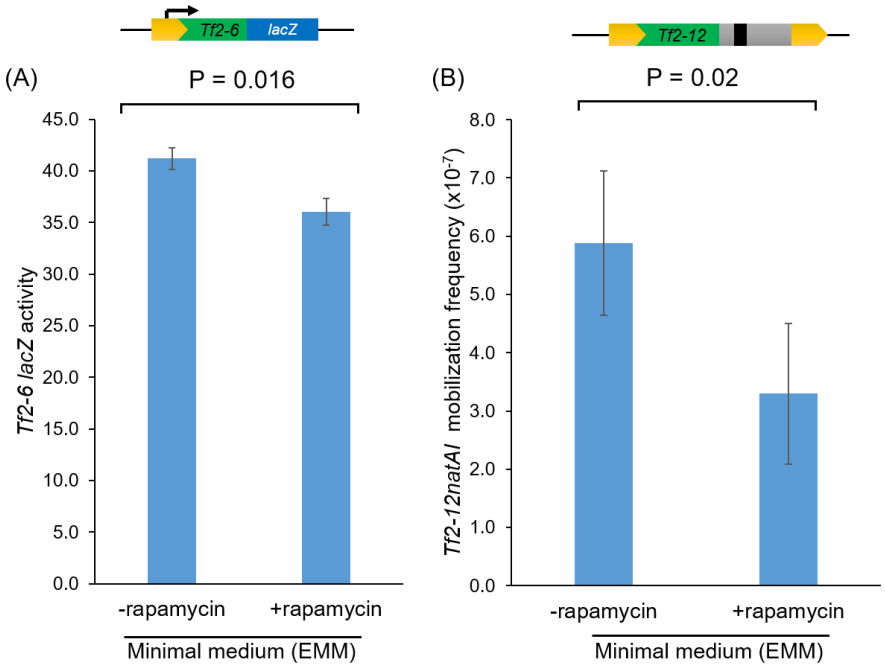


Figure 6.4 Rapamycin cannot stimulate *Tf2* activity when cells are grown in EMM.

(A) *Tf2-6 lacZ* strains were grown at 30°C in EMM with or without 100 nM rapamycin to mid-log phase before being collected for use in quantitative β-galactosidase assays. Values shown represent the average of 8 biological repeats assayed in duplicate and error bars indicate ±SEM. p value was calculated using t test.

(B) *Tf2-12natAI* mobilization frequency was determined as the proportion of ClonNAT resistant colonies from the total number of viable colonies using the mean of medium method. Average values determined by mean of medium analysis with a minimum of 30 repeats. Error bars indicate ±SEM. p value was calculated using t test.

6.5 Rapamycin does not activate the expression of *Tf1* and *Tf2-11*

As described previously (Chapter 5), portions of the LTR (promoter) regions differ between the majority of the *Tf2* elements and the *Tf2-11* and *Tf1* elements (Fig. 5.2). To determine whether the expression of *Tf1* and *Tf2-11* is also rapamycin-responsive, β-galactosidase assays were carried out on strains containing integrated *Tf1* and *Tf2-11 lacZ* reporters (Fig. 6.5). Expression from the *Tf1* reporter was only slightly increased by the presence rapamycin and no increase in expression from *Tf2-11* was observed. Therefore the *Tf* family of elements are differentially expressed in the presence of rapamycin. Furthermore, the data suggest that the DNA elements in the *Tf2* LTR that mediate the response to rapamycin are likely to be located between base pairs 60 and 160 (Fig. 5.2A).

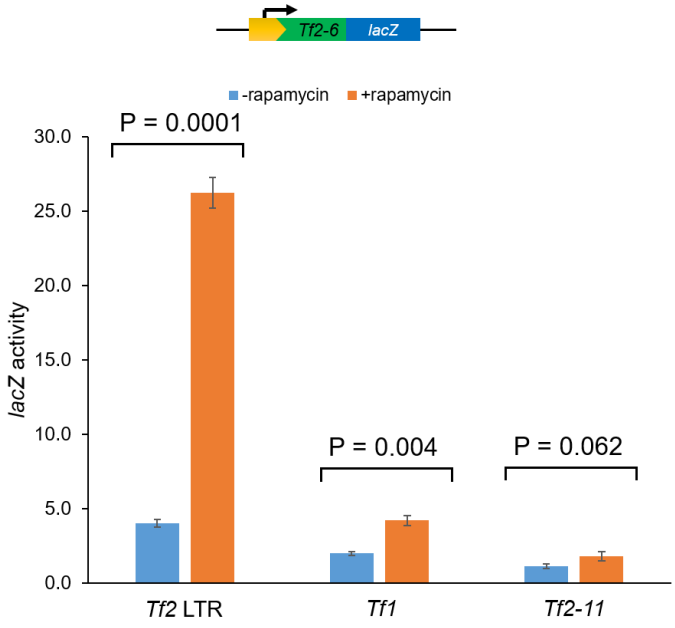


Figure 6.5 Rapamycin strongly activates *Tf2*, but not *Tf1* or *Tf2-11*.

Strains with the indicated *lacZ* reporters were grown at 30°C in YE5S with or without rapamycin (100 nM) to mid-log phase before being collected for use in quantitative β-galactosidase assays. Values shown represent the average of 8 biological repeats assayed in duplicate and error bars indicate ±SEM. p values were calculated using a t test.

6.6 The inactivation of Tor2 using a conditional allele or a direct chemical inhibitor does not induce *Tf2* expression

Rapamycin binds to the cellular protein FKBP12 in *S. cerevisiae* and this FKBP12-drug complex inhibits TORC1 (Heitman *et al.*, 1991). In fission yeast, rapamycin binds to the FKBP12 homologue, Fkh1 to form a protein-drug complex that inhibits the activity of Tor2 in TORC1 (Weisman and Choder, 2001; Weisman *et al.*, 2001; Ikai *et al.*, 2011). The results described above suggest that inhibiting TORC1 signaling using rapamycin results in increased activity of *Tf2* retrotransposons. To further investigate this, alternative methods for inactivating Tor2 (and thus TORC1 signalling) were employed. As *tor2*$^{+}$ is an essential gene in *S. pombe,* a temperature sensitive mutant allele (*tor2-51*) (Alvarez and Moreno, 2006; Matsuo *et al.*, 2007) was used. Wild type and *tor2-ts* cells containing the *Tf2-6 lacZ* reporter were grown to mid log phase at a permissive temperature and then shifted to a restrictive temperature (34°C). The expression of *Tf2-6 lacZ* in the *tor2-ts* mutant did not increase even after 8 hours at the restrictive temperature (Fig. 6.6A). This was surprising as this treatment has previously been shown to result in the increased expression of genes that are repressed by TORC1 (Matsuo *et al.*, 2007). One possible explanation is that β-galactosidase is not stable at higher temperatures in the absence of functional Tor2. Therefore, the global level of *Tf2* mRNA was monitored by RT-qPCR (Fig. 6.6B). Consistent with the *lacZ* assays, *Tf2* mRNA levels did not increase following the inactivation of Tor2 protein using the temperature sensitive allele.

In order to confirm that the temperature shift of the *tor2 ts* strain was sufficient to inactivate Tor2, *isp4*$^{+}$ mRNA levels were measured by RT-qPCR. The *isp4*$^{+}$ gene encodes an OPT oligopeptide transmembrane transporter and expression of this gene is known to be induced by inactivation of Tor2 (Fig. 6.6C) (Matsuo *et al.*, 2007). This analysis clearly showed that in the *tor2-ts* background *isp4* mRNA levels were massively increased by incubation at the restrictive temperature (Fig. 6.6C). As expected, the growth of the *tor2-ts* strain was severely inhibited by incubation at the restrictive temperature and as a result *Tf2* mobilization assays following Tor2 inactivation were not possible. Nonetheless, the results suggest that the loss of Tor2 function does not result in the increased expression of *Tf2*. Given the impact of rapamycin on *Tf2* elements this was very surprising.

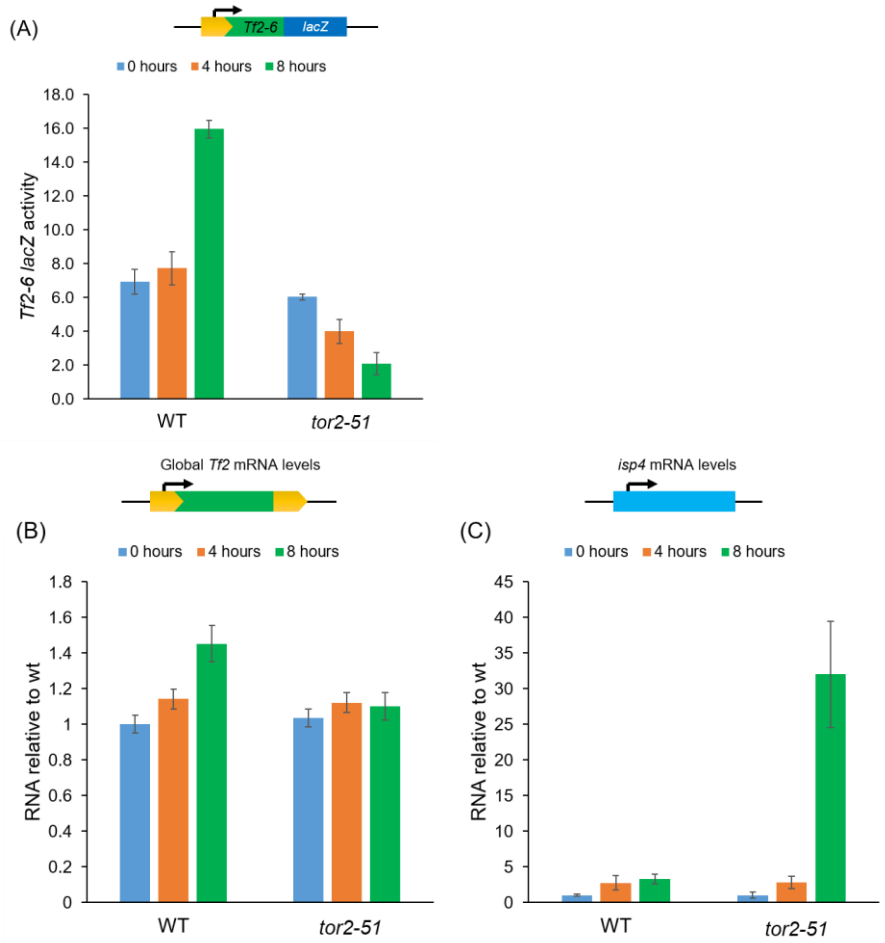


Figure 6.6 Inactivation of Tor2 does not affect *Tf2* expression

(A) *Tf2-6 lacZ* strains were grown at 25°C in YE5S to mid-log phase before being collected and resuspended in fresh YE5S medium prewarmed to 34°C for the indicated times (0, 4 and 8 hours). Samples were then processed for quantitative β-galactosidase assays. Values shown represent the average of 8 biological repeats assayed in duplicate. Error bars indicate ±SEM

(B-C) RNA was extracted from cells treated as described in (A). RNA samples were analysed by RT-qPCR. *Tf2* and *isp4* mRNA levels were normalised using *act1*$^{+}$ mRNA and are expressed relative to the *wt* time 0 control. Data is the mean of at least three biological repeats and error bars indicate ±SEM.

Next, an alternative chemical inhibitor of Tor kinases called Torin1 (Torin) was employed to inhibit TORC signalling. Unlike rapamycin, Torin1 is a direct inhibitor and blocks the active site of Tor kinases. High concentrations of this drug completely arrests growth without cell death in *S. pombe* (Atkin *et al.*, 2014). Torin1 binds to the glycine at position 2040 within the ATP-binding pocket of the kinase domain of Tor2 to inhibit its activity which provides an FKBP12 protein independent mode of inhibition towards TORC1 (Atkin *et al.*, 2014).

As expected the addition of Torin, resulted in increased levels of *isp4*$^{+}$ mRNA levels consistent with the inhibition of TORC1 (Fig. 6.7). However, the expression of a *Tf2-lacZ* reporter was not induced by the addition of Torin1 to the culture medium (Fig. 6.7). Also, there was a no significant increase in *Tf2* mRNA levels as measured by RT-qPCR. Therefore, neither the inactivation of Tor2 using a *ts* allele or a direct chemical inhibitor induces the expression of *Tf2* retrotransposons.

The mobility of *Tf2* was also investigated. Surprisingly, the mobilization frequency was massively (~500 fold) increased when Torin was added to the culture medium (Fig. 6.8). This suggests that TORC signalling suppresses steps in the lifecycle of *Tf2* retrotransposons at a post-transcriptional level.

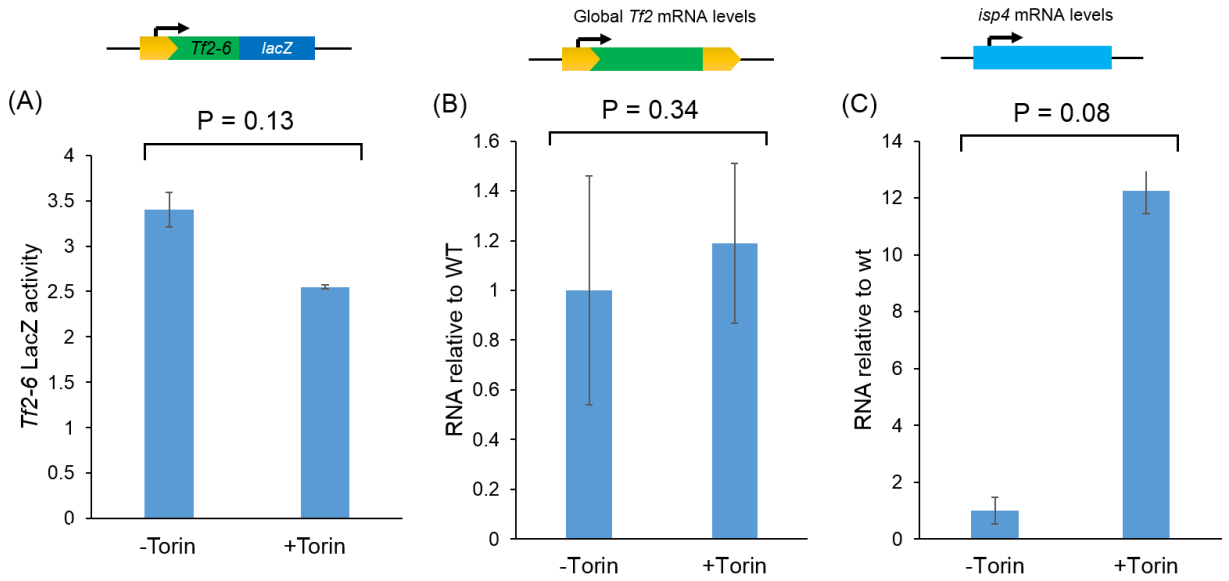


Figure 6.7 Direct inhibition of Tor2 does not influence *Tf2* expression

(A) *Tf2-6 lacZ* strains were grown at 30°C in YE5S with or without Torin1 (25 nM) to mid-log phase before being collected for use in quantitative β-galactosidase assays. Values shown represent the average of 8 biological repeats assayed in duplicate and error bars indicate ±SEM.

(B-C) RNA was extracted from cells cultured at 30°C in YE5S with or without Torin1 (25 nM) to mid log phase and subjected to qRT-PCR to detect the indicated mRNA. Values shown represent the average of 8 biological repeats assayed in duplicate and error bars indicate ±SEM. p values were calculated using t test.

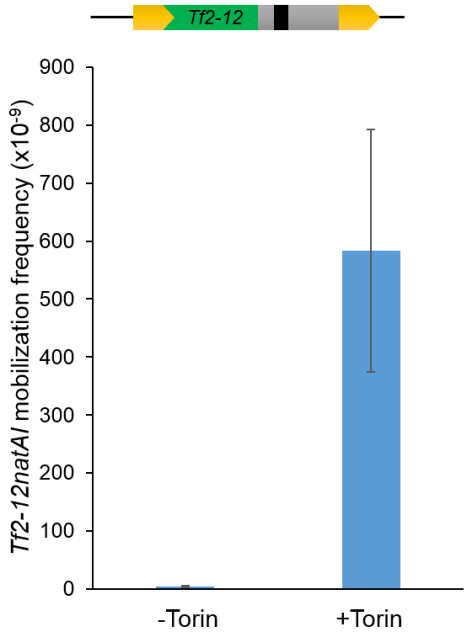


Figure 6.8 Torin stimulates the mobility of *Tf2* elements.

Cells with the *Tf2-12natAI* mobilization reporter were grown in YE5S for two days at 30°C either in the absence or presence of Torin (25 nM). Mobilization frequency was determined as the proportion of ClonNAT resistant colonies in the total number of viable colonies using the mean of medium method with a minimum of 30 repeats. Error bars indicate ±SEM.

6.7 Expression of *Tf2* is not TORC2 dependent

Studies of global gene expression indicated that Torin1 inhibits the function of both TORC1 and TORC2 complexes (Lie *et al.*, 2018). The rapamycin insensitive TORC2 complex is composed of the kinase Tor1, the homologue of mammalian Rictor, Ste20, Sin1 and Wat1/Pop3 and functions in opposition to TORC1 (Wullschleger *et al.*, 2006; Matsuo *et al.*, 2007; Ikai *et al.*, 2011; Rallis *et al.*, 2013). To investigate the influence of TORC2 on the activity *Tf2* retrotransposons, strains with mutations in genes encoding the Tor1 kinase (*tor1Δ*) and Rictor Ste20 (*ste20Δ*) were employed. Relative to wild type no change in was observed in *tor1Δ* and *ste20Δ* mutant strains (Fig. 6.9A) suggesting that the removal of TORC2 protein has no influence on rapamycin induced *Tf2* expression. However, the frequency of mobilization of *Tf2* in the presence of rapamycin was reduced in *tor1Δ* and *ste20Δ* relative to wild type with a slight increased base mobility rate (Fig. 6.9B). This indicates that the stimulation of *Tf2* mobilization that occurs after the addition of rapamycin is partially dependent upon TORC2.

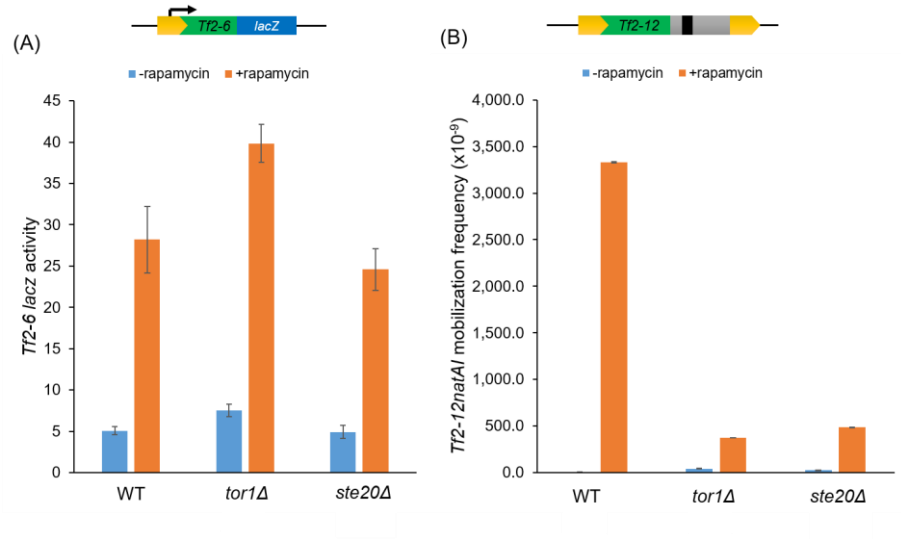


Figure 6.9 Influence of TORC2 on *Tf2* activity.

(A) *Tf2-6 lacZ* strains were grown at 30°C in YE5S with or without rapamycin (100 nM) to mid-log phase before being collected for use in quantitative β-galactosidase assays. Values shown represent the average of 8 biological repeats assayed in duplicate and error bars indicate ±SEM.

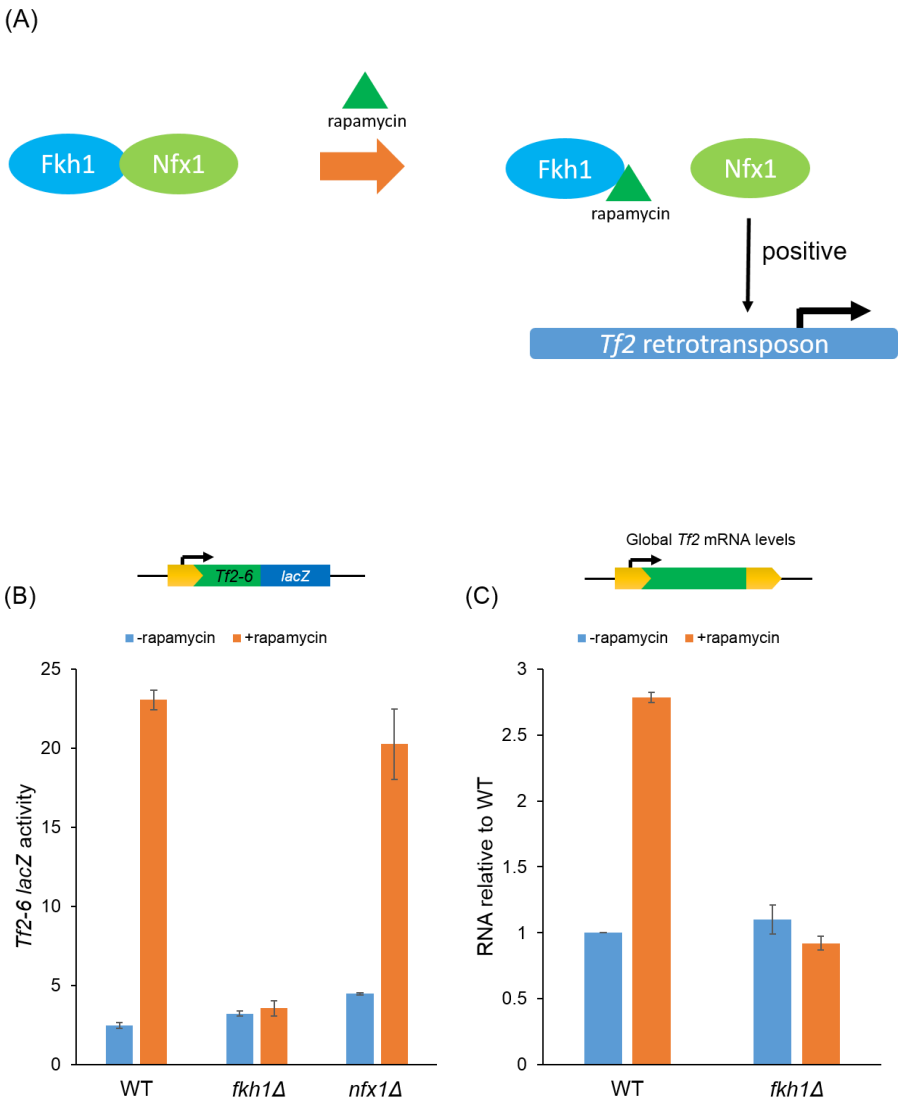
(B) *Tf2-12natAI* mobilization frequency was determined as the proportion of ClonNAT resistance colonies from the total number of viable colonies using the mean of medium method. Average values determined by mean of medium analysis with a minimum of 30 repeats. Error bars indicate ±SEM.

6.8 The FKBP12 homologue, Fkh1 is required for the transcriptional response to rapamycin

The results described above indicate that while rapamycin induces the expression of *Tf2* retrotransposons, other methods of inhibiting TORC1 do not. Rapamycin functions by binding to FKBP12, which in *S. pombe* is called Fkh1 (Weisman et al 2001 ref). To investigate the role of FKBP12 the expression of a *Tf2-6 lacZ* reporter was analysed in an *fkh1Δ* background. This revealed that the loss of Fkh1 abolished the induction of *Tf2* expression in response to rapamycin (Fig. 6.10A). To confirm that the loss of *Tf2* induction under rapamycin conditions is not due to instability of β-galactosidase, *Tf2* mRNA levels were analysed by RT-qPCR (6.10B). No increase of *Tf2* mRNA was observed in *fkh1Δ* confirming that the induction of *Tf2* expression in response to rapamycin is FKBP12 dependent.

In *S. cerevisiae*, screening for rapamycin resistance identified a novel yeast gene *FAP1*, which encodes a homologue of human transcription factor NFX1 (Kunz *et al.*, 2000). Kunz and co-workers also showed that Fap1 interacts physically with FKBP12 and this interaction was abolished by rapamycin or by mutation that alter the drug binding/active site of FKBP12 or surface residues (Kunz *et al.*, 2000). In *S. pombe*, the homologue of *S. cerevisiae* Fap1 and human NFX1 has not been characterised. However BLAST searches identified an ORF (SPCC18.03) that encodes a homologue of these proteins which was named *nfx1*$^{+}$ with a low degree of sequence homology. Based on the data from *S. cerevisiae* it was hypothesized that rapamycin binding to the FKBP12 protein would release the putative Nfx1 transcription factor which could then activate *Tf2* expression (Fig. 6.10C) However, analysis of an *nfx1*Δ strain showed no defect in the induction of *Tf2* expression in response to rapamycin.

The mobility of *Tf2* in *fkh1Δ* was also investigated using the *Tf2-12natAI* reporter (Fig. 6.10D). Unsurprisingly, the loss of rapamycin binding protein Fkh1 abolished the stimulatory effect of rapamycin on mobilization. Overall, these results indicate that the effect of rapamycin on both expression and mobilization *Tf2* is dependent upon the FKBP12 protein Fkh1.


(A)
Fkh1
Nfx1
rapamycin
Fkh1
rapamycin
Nfx1
positive
Tf2 retrotransposon
(B)
Tf2-6
lacZ
-rapamycin
+rapamycin
Tf2-6 lacZ activity
25
20
15
10
5
0
WT
fkh1Δ
nfx1Δ
(C)
Global Tf2 mRNA levels
-rapamycin
+rapamycin
RNA relative to WT
3
2.5
2
1.5
1
0.5
0
WT
fkh1Δ

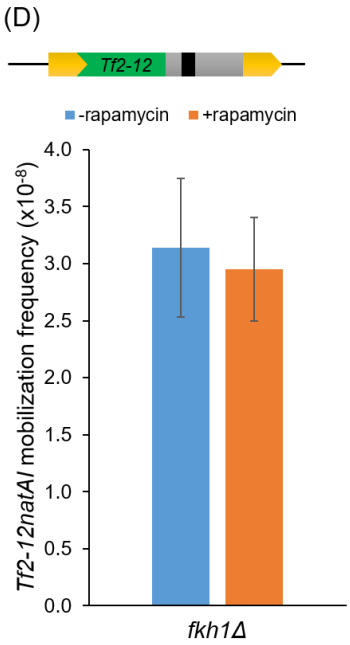


Figure 6.10 Role of the FKBP12 protein, Fkh1 on *Tf2* activity

(A) A hypothetical model for the interaction of Fkh1-Nfx1 in the presence of rapamycin.

(B) *Tf2-6 lacZ* strains were grown at 30°C in YE5S with or without rapamycin (100 nM) to mid-log phase before being collected for use in quantitative β-galactosidase assays. Values shown represent the average of 8 biological repeats assayed in duplicate and error bars indicate ±SEM.

(C) RNA was extracted from cells cultured at 30°C in YE5S with or without rapamycin (100 nM) to mid log phase and subjected to qRT-PCR to detect *Tf2* transcripts. Values shown represent at least three biological repeats and error bars indicate ±SEM.

(D) *Tf2-12natAI* mobilization frequency was determined as the proportion of ClonNAT resistance colonies from the total number of viable colonies using the mean of medium method with a minimum of 30 repeats. Error bars indicate ±SEM.

6.9 The GATA transcription factor Gaf1 does not activate *Tf2* expression in response to rapamycin.

In order to identify proteins that mediate the rapamycin-dependent increase in *Tf2* expression transcription factors that function downstream of TORC1 were analysed. In nitrogen-rich conditions, TORC1 positively regulates the phosphorylation and maintains the cytoplasmic status of the GATA transcriptional factor Gaf1 (Laor *et al.*, 2015). When TORC1 is inactivated, Gaf1 undergoes dephosphorylation and translocates into nucleus (Laor *et al.*, 2015). However, the rapamycin dependent increase in expression of *Tf2* was not affected by the loss of Gaf1 (Fig. 6.11).

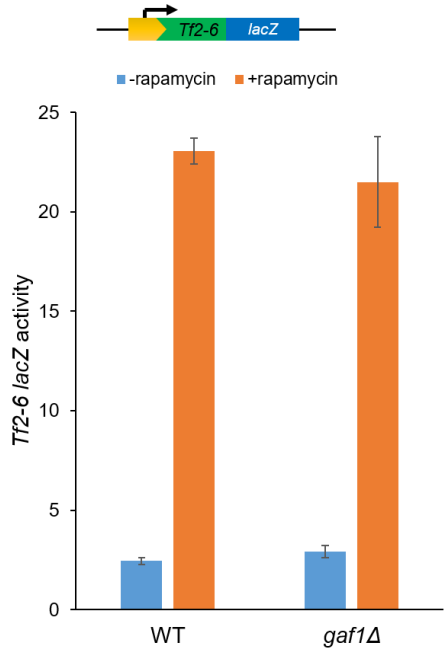


Figure 6.11 The loss of Gaf1 does not influence *Tf2* expression

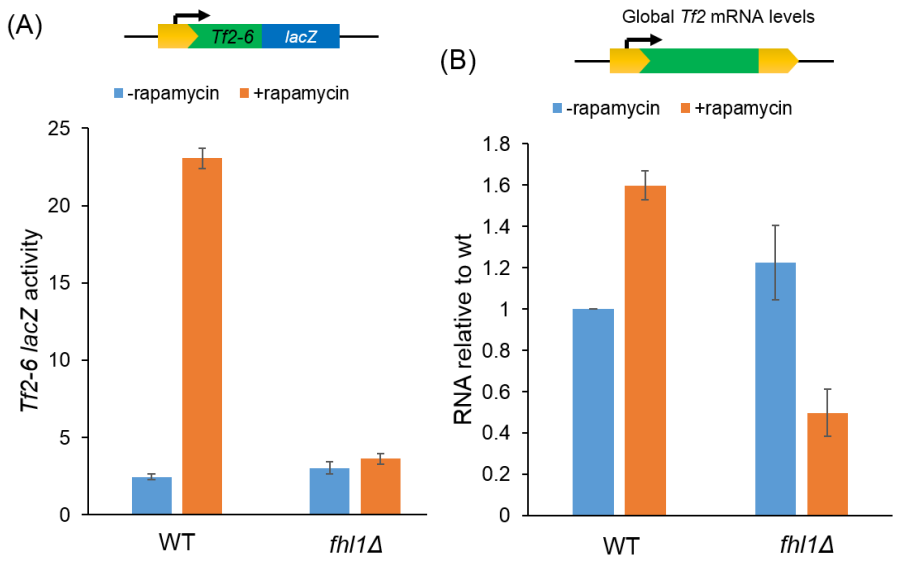
(A) *Tf2-6 lacZ* strains were grown at 30°C in YE5S with or without rapamycin (100 nM) to mid-log phase before being collected for use in quantitative β-galactosidase assays. Values shown represent the average of 8 biological repeats assayed in duplicate and error bars indicate ±SEM.

(B) *Tf2-12natAI* mobilization frequency was determined as the proportion of ClonNAT resistance colonies from the total number of viable colonies using the mean of medium method with a minimum of 30 repeats. Error bars indicate ±SEM.

6.10 Forkhead transcription factor Fhl1 and its putative co-activator Crf1 are essential for *Tf2* activity

Four forkhead type proteins called Sep1, Mei4, Fkh2 and Fhl1 have been identified in *S. pombe* (Pataki *et al.*, 2017). Although *S. pombe* Fhl1 is a homologue of *S. cerevisiae* Fhl1 that controls ribosomal gene expression, its function is relatively poorly understood. Nonetheless, Pataki and co-workers have suggested that Fhl1 lies downstream of TORC1 (Tor2) in *S. pombe* because the expression of *fhl1*$^{+}$ from a strong promoter can suppress the rapamycin and temperature sensitivity of *tor2-ts* mutant cells (Pataki *et al.*, 2017), Therefore, a *fhl1Δ* strain was employed to investigate whether Fhl1 is involved in regulating *Tf2* activity in response to rapamycin. Interestingly, the expression of *Tf2* elements was not induced after rapamycin addition in an *fhl1Δ* strain (Fig. 6.12A and B) suggesting that *Tf2* expression is under the either direct or indirect control of Fhl1, supported by the positive control of *isp4* (Fig. 6.12C).

A high level of *Tf2* expression is observed when rapamycin is present and also when cells are cultured in minimal medium (Chapter 5). To determine whether this is the result of a common mechanism, *Tf2-6 lacZ* expression was determined in an *fhl1*Δ mutant grown in EMM. No difference in expression relative to wild type cells was observed suggesting that the transcriptional response of *Tf2* to rapamycin and EMM is mediated by distinct mechanisms (Fig. 6.12D).


(A)
Tf2-6
lacZ
-rapamycin
+rapamycin
Tf2-6 lacZ activity
25
20
15
10
5
0
WT
fhl1Δ
(B)
Global Tf2 mRNA levels
-rapamycin
+rapamycin
RNA relative to wt
1.8
1.6
1.4
1.2
1
0.8
0.6
0.4
0.2
0
WT
fhl1Δ

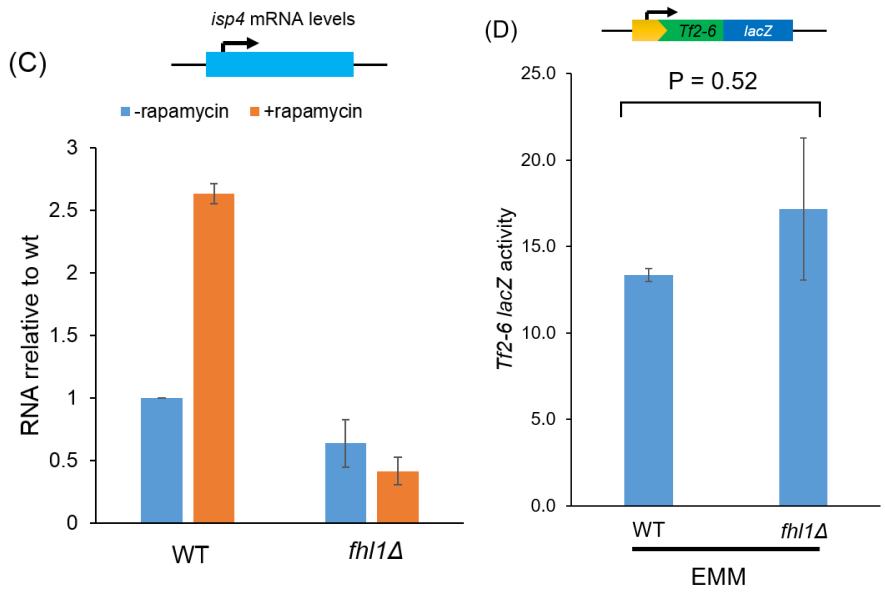


Figure 6.12 The forkhead transcription factor, Fhl1 is required for *Tf2* activity

(A) *Tf2-6 lacZ* strains were grown at 30°C in YE5S with or without rapamycin (100 nM) to mid-log phase before being collected for use in quantitative β-galactosidase assays. Values shown represent the average of 8 biological repeats assayed in duplicate and error bars indicate ±SEM.

(B-C) RNA was extracted from cells cultured at 30°C in YE5S with or without rapamycin (100 nM) to mid log phase and subjected to qRT-PCR to detect the indicated transcripts. Values shown represent at least three biological repeats and error bars indicate ±SEM.

(D) *Tf2-6 lacZ* strains were grown at 30°C in EMM to mid-log phase before being collected for use in quantitative β-galactosidase assays. Values shown represent the average of 8 biological repeats assayed in duplicate and error bars indicate ±SEM. p value was calculated using a t test.

In budding yeast *S. cerevisiae*, both TORC1 and Fhl1 participate in the regulation of ribosomal protein gene (RPG) transcription (Fig. 6.13) (Lempiainen and Shore, 2009; Albert *et al.*, 2016). When TORC1 is active, Fhl1 forms a complex with Rap1, the split finger protein Sfp1 and the co-activator, Ifh1 and activates RPG expression. When TORC1 is inactive a co-repressor protein called Crf1 (which is an Ifh1 paralogue) translocates to the nucleus, competes with Ifh1 and represses transcription (Fig. 6.13) (Xiao and Grove, 2009; Albert *et al.*, 2016). Counterparts of Ifh1 and Crf1 in *S. pombe* have not been functionally characterized, however an ORF (SPAC22H10.11c) has been designated *crf1*$^{+}$ in Pombase based on its similarity to *S. cerevisiae CRF1/IFH1.* Therefore, the expression of a *Tf2-6 lacZ* reporter was monitored in ***crf1Δ*** background (Fig. 6.14A). This showed that the increase in *Tf2* expression in response to rapamycin is dependent upon Crf1. This suggests that Crf1 functions as co-activator with Fhl1.

To further investigate the roles of other proteins that are part of the RP gene transcription pathway that has been defined in *S. cerevisiae*, the function of Rap1 (SPBC1778.02) and Sfp1 (SPAC16.05c) homologues was characterized. Loss of Rap1 had only a very minor effect on *Tf2* expression while loss of Sfp1 resulted in an increase in *Tf2* expression both in the absence and presence of rapamycin (Fig. 6.14A).

In *S. cerevisiae* the HMG protein Hmo1 binds to the FKBP12 protein Fpr1 (Dolinski and Heitman, 1999) and is also involved in the regulation of RP genes with Fhl1 (Xiao and Grove, 2009). Therefore the impact of deleting the *S. pombe* Hmo1 homologue was also investigated. The *hmo1*Δ mutant showed increased in *Tf2* expression in the absence of rapamycin and decreased expression in the presence of rapamycin. This suggests that this HMG protein is required for the proper regulation of *Tf2* retrotransposons (Fig. 6.14B).

The mobilization of *Tf2* elements was also monitored in both *fhl1*Δ and *crf1*Δ mutants. Surprisingly, when cells were grown in the absence of rapamycin no mobilization of the *Tf2-12natAI* reporter was detectable in either mutant. Mobilization was also undetectable in a *crf1*Δ mutant when rapamycin was present. In the *fhl1*Δ mutant rapamycin did increase mobilization to a detectable level, however the observed frequency was around 500-fold lower than in wild type cells (Fig. 6.14C). These results indicate that both Fhl1 and Crf1 are critical for the life cycle of *Tf2* elements.

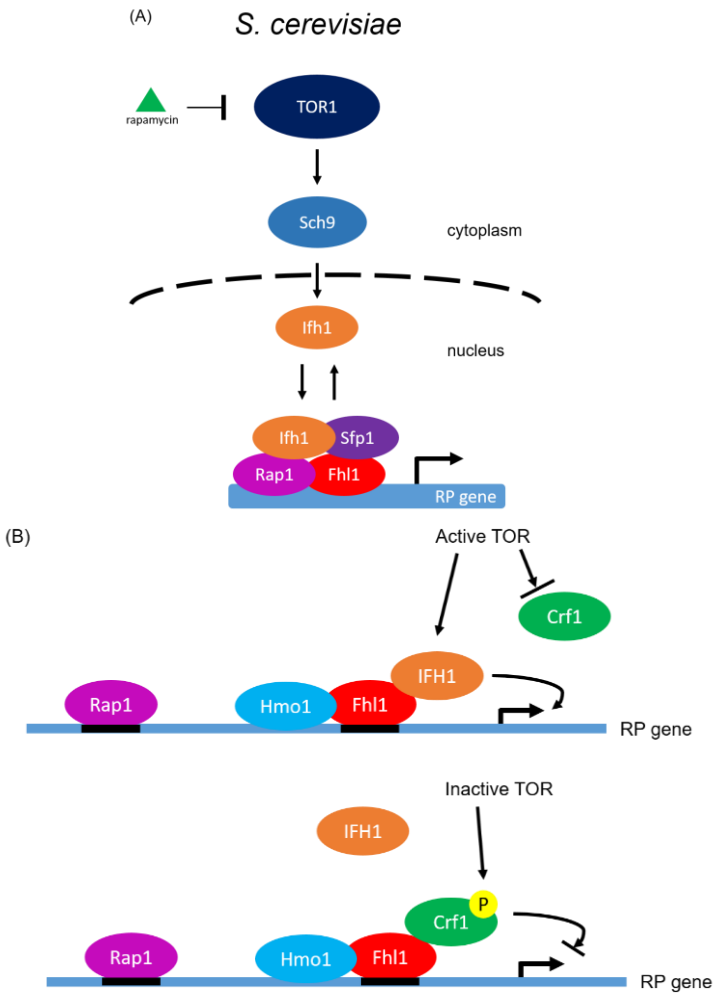
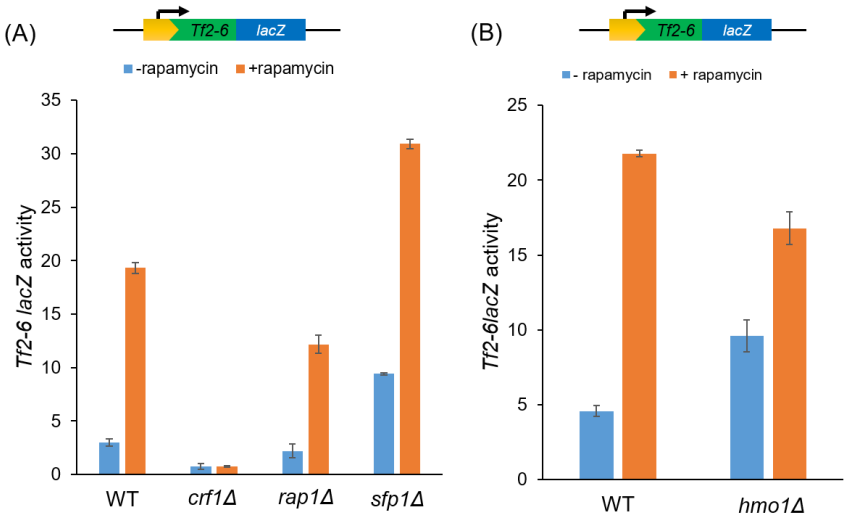


Figure 6.13 A schematic representation of the regulation of RP genes by Fhl1 and TORC1 signalling in *S. cerevisiae*

(A) In *S. cerevisiae*, during exponential growth, Sch9 kinase activity promotes Ifh1 binding on RP gene promoters to stimulate transcription. The association of Rap1 was suggested to be required for the subsequent cooperative binding of Hmo1 and Fhl1 at the IFHL site. (B) When TORC1 is active, the coactivator Ifh1 binds to Fhl1 to activate RP gene expression. Phosphorylated co-repressor, Crf1 translocates into the nucleus and competes with Ifh1 for binding to Fhl1 to repress gene transcription when TORC1 is inactive. (Adapted from Xiao and Grove, 2009; Albert *et al.,* 2016).



(C)

	Tf2-12natAI mobilization frequency	
Strain	-rapamycin	+rapamycin
WT	4.81×10^{-9}	3.33×10^{-6}
crf1Δ	0	0
fhl1Δ	0	2.21×10^{-9}

Figure 6.14 The putative co-activator, Crf1 is essential for Tf2 activity.

(A&B) *Tf2-6 lacZ* strains were grown at 30°C in YE5S with or without rapamycin (100 nM) to mid-log phase before being collected for use in quantitative β-galactosidase assays. Values shown represent the average of 8 biological repeats assayed in duplicate and error bars indicate ±SEM.

(C) *Tf2-12natAI* mobilization frequency was determined as the proportion of ClonNAT resistance colonies from the total number of viable colonies using the mean of medium method with a minimum of 30 repeats. Error bars indicate ±SEM.

6.11. The Pka pathway is critical for the transcriptional response of *Tf2* retrotransposons to rapamycin.

In *S. cerevisiae* the activity of Fhl1 is under the control of the Pka signalling pathway (Martin *et al.*, 2004). Therefore, the impact of deletion of *pka1*$^{+}$ upon *Tf2 lacZ* expression was investigated. In the *pka1*Δ strain no increase in expression was observed in the presence of rapamycin. Therefore, the induction of *Tf2* transcription in response to rapamycin is dependent upon the Pka signalling pathway (Fig. 6.15A). Next, the potential role of the Sty1 stress activated MAPK kinase pathway was assessed (Fig. 6.15B). This revealed that Sty1 was not required for the upregulation of *Tf2* expression in response to rapamycin. The mobility of *Tf2* elements was also determined in a *pka1*Δ background and when rapamycin was present in the medium an approximately 2 fold reduction in *Tf2-12natAI* mobilization frequency was observed. Therefore, although the Pka pathway is required for the rapamycin-dependent transcriptional upregulation of *Tf2*, it is only partially required for the stimulation of posttranscriptional step(s) in the life cycle (Fig. 6.15C).

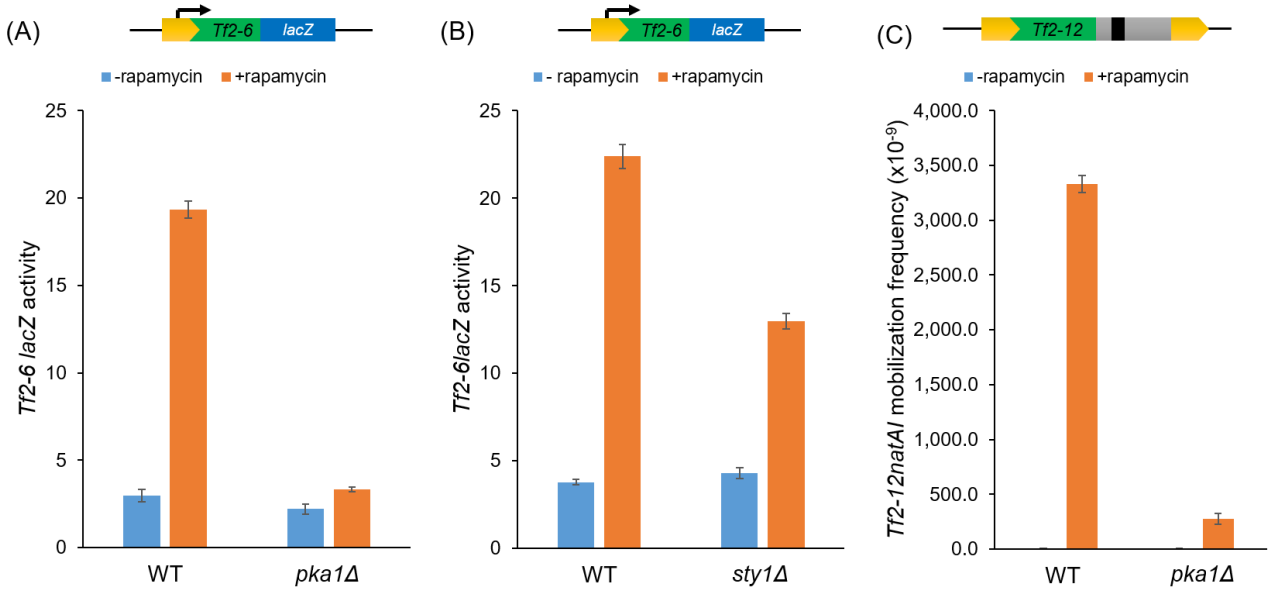


Figure 6.15 The Pka pathway regulates *Tf2* activity.

(A&B) *Tf2-6 lacZ* strains were grown at 30°C in YE5S with or without rapamycin (100 nM) to mid-log phase before being collected for use in quantitative β-galactosidase assays. Values shown represent the average of 8 biological repeats assayed in duplicate and error bars indicate ±SEM.

(C) *Tf2-12natAI* mobilization frequency was determined as the proportion of ClonNAT resistant colonies from the total number of viable colonies using the mean of medium method with a minimum of 30 repeats. Error bars indicate ±SEM.

6.12 Discussion

In the course of this study, rapamycin was identified as a drug which activates both the expression and mobilization of *Tf2* elements. Rapamycin inhibits TORC signalling cascades but only TORC1 is rapamycin-sensitive while TORC2 is rapamycin-insensitive (Zheng *et al.*, 1995). Therefore, the results described in this chapter initially suggested that the activity of *Tf2* elements is suppressed by the TORC1 signalling cascade. However, the inactivation of TORC1 using a temperature sensitive *tor2* allele (*tor2-51*) (Alvarez and Moreno, 2006) did not result in increased *Tf2* expression. Furthermore the use of the direct inhibitor, Torin1 also did not activate *Tf2* expression. This suggested that rapamycin increases *Tf2* expression in a TORC1-independent manner. This was unexpected as the cellular effects of rapamycin are believed to operate through inhibition of TORC1 signalling (Nakashima *et al.*, 2010; Ikai *et al.*, 2011). However TORC1-independent effects of rapamycin are not without precedent. Rapamycin has been shown to have a TORC-independent function in *S. cerevisiae.* While transcription-coupled nucleotide excision repair (TC-NER) is inhibited by rapamycin, a *tor1*Δ *tor2*^{ts} mutant grown at the non-permissive temperature (which essentially lacks both TORC1 and TORC2) has wild-type rates of repair. The inhibitory influence of rapamycin on TC-NER was abolished in cells lacking the FKBP12 homologue, Fpr1 (Limson and Sweder, 2010). Similarly, the influence of rapamycin on *Tf2* expression was found to be dependent upon the *S. pombe* FKBP12 homologue, Fkh1.

Several lines of evidence suggest that, in addition to increasing expression, rapamycin also stimulates other steps in the *Tf2* life cycle. Firstly, rapamycin increases *Tf2* expression by ~10 fold, but increases mobilization frequency by about three orders of magnitude. Secondly, while treatment with the direct inhibitor Torin1 does not increase expression, it does increase *Tf2* mobilization. Taken together, the results suggest rapamycin induces *Tf2* expression through a TORC1-independent mechanism but its influence on mobilization is at least partly dependent upon its inhibitory effect on TORC1 (Fig. 6.16).

Deletion of the TORC2 genes, *tor1*$^{+}$ and *ste20*$^{+}$ had no impact on the expression of *Tf2* elements following the addition of rapamycin. However, the *tor1Δ* and *ste20Δ* mutants showed a significant decrease in the induction of *Tf2* mobilization relative to

wild type cells after rapamycin. Thus the high level of retrotransposon mobility in the presence of rapamycin is at least partly dependent upon the integrity of the TORC2 pathway. These findings are in good agreement with the results of the mobility assays using the drug Torin1. This drug inhibits both Tor1 and Tor2 kinase activities by direct binding (Atkin *et al.*, 2014) and would therefore be predicted to inhibit both TORC1 and TORC2 signalling pathways. Consistent with this finding Torin1 increases *Tf2* mobilization but not to the same degree as rapamycin (which would only inhibit TORC1) (Fig. 6.16)

In *S. cerevisiae*, rapamycin resistance can be conferred by overexpressing a FKBP12 (Fpr1) binding protein called Fap1, which competes with rapamycin for Fpr1 binding (Kunz *et al.*, 2000). Fap1 is an evolutionarily conserved protein which physically interacts with Fpr1 and blocks the drug-binding/active site of Fpr1 (Kunz *et al.*, 2000). Fap1 contains a cysteine-rich DNA-binding motif and the mammalian homologue NFX1 functions as a transcription factor. Interestingly, the TORC1-independent effect of rapamycin on TC-NER in *S. cerevisiae* requires Fap1. This prompted the examination of the fission yeast homologue of *S. cerevisiae* Fap1 and mammalian NFX1 which is encoded by an uncharacterised ORF, SPCC18.03 (herein named *nfx1*$^{+}$). However the results showed that the loss of Fkh1 but not Nfx1 abolished the effects of rapamycin on *Tf2* transcription. Therefore, therefore there is no evidence at the moment to indicate that Nfx1 either acts as a transcriptional regulator or interacts with the FKBP12 protein Fkh1.

FKBP12 proteins such as (*Sc*Fpr1 and *Sp*Fkh1) are peptidyl-prolyl cis-trans isomerases. These proteins were isolated in mammalian cells in a drug screen for factors that bind FK506 (Kino *et al.*, 1987; Tanaka *et al.*, 1987). Although FKBP12 and rapamycin form a complex with Tor kinases there is no strong evidence that FKBP12 proteins are natural regulators of TORC signalling. Indeed very little is understood about the normal *in vivo* function of FKBP12 proteins. *Sc*Fpr1 interacts with calcineurin even in the absence of FK506, and there is some genetic evidence that it is a negative regulator of calcineurin function (Cardenas *et al.*, 1994). In mammalian cells FKBP12 interacts with several intracellular signal transduction proteins such as type I TGF-β receptors and calcium release channels such as the skeletal muscle ryanodine receptor (Siekierka *et al.*, 1989; Michnick *et al.*, 1991; Van Duyne *et al.*, 1991). However functional information is lacking.

In this work the forkhead transcription factor Fhl1 was identified as a regulator of *Tf2* activity. Fhl1 is a homologue of *S. cerevisiae* Fhl1 and was recently identified in *S. pombe* as a transcription factor downstream of the TORC1 signalling cascade (Pataki *et al.*, 2017). Fhl1 targets ~75 genes in *S. pombe* cells and mainly regulates genes that are responsible for the starvation responses, mating and sporulation (Pataki *et al.*, 2017). Whether *Sp*Fhl1 is directly phosphorylated by the TORC1 pathway remains to be determined. Furthermore, the *in vivo* binding sites of *Sp*Fhl1 are also yet to be characterised and no genome-wide or gene-specific chromatin immunoprecipitation (ChIP) experiments have been performed. As a result it is not clear whether *Sp*Fhl1 binds directly to *Tf2* LTRs. To address this issue epitope tagging experiments were initiated, however due to time constraints no ChIP experiments were completed. Therefore it remains possible that Fhl1 indirectly regulates *Tf2* expression, particularly as no obvious forkhead transcription factor binding sites are located in *Tf2* LTRs (data no shown). It is very likely that Fhl1 influences *Tf2* activity at multiple levels because loss of Fhl1 totally abolished *Tf2-12natAI* mobilization when cells are grown in rich (YE5S) medium in the absence of rapamycin (Fig. 6.14C). This may reflect a role for *Sp*Fhl1 in the regulation of ribosomal protein genes, which could in turn impact upon ribosomes and thus *Tf2* protein levels.

In budding yeast, *Sc*Fhl1 regulates ribosomal protein (RP) genes and forms a complex with Ifh1 or Crf1. Although these proteins are share sequence similarity, Ifhl1 functions as a co-activator protein while Crf1 is a co-repressor (Albert *et al.*, 2016). After rapamycin treatment, Ifhl1 is replaced with Crf1 and removed from ribosomal protein gene promoters (Schawalder *et al.*, 2004). Prior to this work, co-activator/co-repressors of *Sp*Fhl1 had not been identified. BLAST searches of the *S. pombe* genome database revealed a single uncharacterised ORF (SPAC22H10.11c) with homology to *Sc*Ifh1/Crf1 which was named *Sp*Crf1. The results strongly suggest that *Sp*Crf1 acts as a co-activator for *Sp*Fhl1. No rapamycin dependent increase in *Tf2* expression was observed in a ***crf1Δ*** background. Furthermore, like loss of *Sp*Fhl1, the loss of *Sp*Crf1 abolished *Tf2* mobilization when cells were cultured in YE5S (Fig. 6.14C). It will be interesting to determine if there is a direct physical interaction between *Sp*Fhl1 and *Sp*Crf1 and if this interaction is regulated by TORC1 signalling.

While both the forkhead transcription factor, Fhl1 and the FKBP12 protein, Fkh1 are required for the rapamycin dependent increase in *Tf2* expression the nature of connection between these proteins remains to be determined. Interestingly, in *S. cerevisiae* Berger and co-workers showed an interaction between Fpr1 (FKBP12) and the high mobility group (HMG) protein, Hmo1 (Berger *et al.*, 2007). This is interesting as Hmo1also interacts with transcription factor Fhl1 and functions in the regulation of RP genes in *S. cerevisiae* (Hall *et al.*, 2006). The connections between Fhl1, Hmo1 and Fpr1 (FKBP12) in *S. cerevisiae* prompted an examination of the role of the *S. pombe* homologue of Hmo1. Loss of *Sp*Hmo1 had a distinct impact on *Tf2* expression: relative to wild type, expression was increased in the absence of rapamycin and decreased in its presence. These results suggest that it would be important to determine whether Hmo1 physically interacts with Fhl1, Fkh1 and *Tf2* LTRs.

During the course of this work, another study that connected the expression of *Tf2* elements to TORC signalling was published (Nakase and Matsumoto, 2018). In this study the authors demonstrated that *Tf2* mRNA was induced after nitrogen starvation in *tsc2*Δ mutants or in cells harbouring an up-mutant of the RHEB GTPase (Nakase and Matsumoto, 2018). This indicates that the constitutive activation of TORC1 under nitrogen starvation induces the expression of *Tf2*. This induction could be suppressed by overexpression of members of the cAMP/glucose signalling pathway Pka1 and the TORC2 pathway (Nakase and Matsumoto, 2018).

The main finding is that the loss of the Tsc1-Tsc2 complex constitutively activates TORC1, inhibits autophagy and induces *Tf2s* expression even in nitrogen poor-conditions (Nakase and Matsumoto, 2018). The results are difficult to reconcile with the findings of this study which essentially indicate that the TORC1 complex represses *Tf2* mobilization. However, it is important to note that the experiments of Nakase and co-workers were performed using minimal (EMM) medium and under conditions of nitrogen starvation. Interestingly, the work in this thesis indicates that the composition of the growth medium has a marked influence on *Tf2* retrotransposons. Relative to rich medium, *Tf2* activity is significantly higher when cells are cultured in EMM and rapamycin addition to EMM has no effect on *Tf2* expression. It is worth noting that the high level of *Tf2* expression that is observed when cells are cultured in EMM is not dependent upon Fhl1. As such the signalling pathways that stimulate *Tf2* expression in response to ‘rapamycin’ and ‘EMM’ are likely to be distinct. Nonetheless, the results

in this thesis indicate that both expression and mobilization of *Tf2* LTR retrotransposons is subject to control by multiple environment sensing signalling pathways.

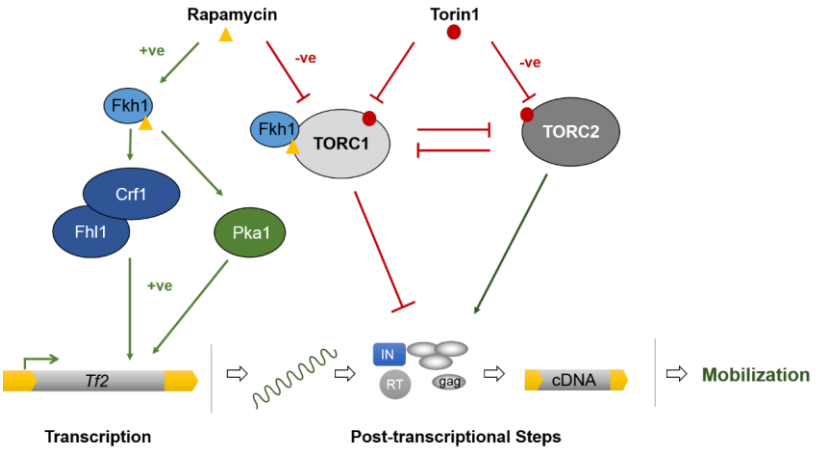


Figure 6.16 Model for the control of *Tf2* expression and mobilization via TOR signalling cascade.

The expression of *Tf2* highly relayed by the activity of Fkh1-Fhl1 signal. The inhibition effect of rapamycin showed a significant induction of *Tf2* mobility. High level of *Tf2* mobility requires the activity of TORC2 for post-transcriptional regulation.

Chapter 7 Final Discussion

The aim of this study was to identify new host and environmental factors that contribute to the regulation of the *Tf2* LTR retrotransposons of *S. pombe*. Such studies are important as retrotransposons have helped shape the structure and function of eukaryotic genomes. LTR retrotransposons also provide a useful model for understanding the propagation of infectious retroviruses. Indeed, the analysis of *Tf2* could reveal novel host factors that are essential for retroelement propagation and may provide new targets for therapeutic intervention. Furthermore, comparison of the *Tf* family with the *Ty* elements of *S. cerevisiae* is insightful as it allows conserved and species-specific controls to be elucidated.

Systematic screens in *S. cerevisiae* have proved highly informative for identifying the host cell machinery that control *Ty* elements. Unfortunately, the basal mobilization frequency of the *Tf2-12natAI* element proved to be too low to allow high throughput screening (Chapter 4). However, the findings outlined in Chapter 5 indicate that the basal level of *Tf2* mobilization is greatly increased in minimal (EMM) medium. This suggests that it may be worth investigating the feasibility of systematic screens using EMM as the growth medium. Alternatively, a smaller scale targeted screen could be undertaken. For example and Rai and co-workers have identified a set of 61 factors that are required for completion of the Tf1 lifecycle (Rai *et al.*, 2017) and so it would be informative to determine how many of these are also required for successful *Tf2* propagation.

The results of Chapter 5 suggest a copy number control (CNC) mechanism is not present in *S. pombe* for *Tf* elements. However, the results in this Chapter clearly demonstrate that *Tf2* can 'highjack' another retroelement to replicate itself. The results also show the potential for crosstalk between retrotransposons. This is potentially advantageous for the element because it expands the repertoire of signals that it can respond to. Such crosstalk may help explain why a particular stress can lead to a global upregulation of retrotransposable elements in certain cases (Negi *et al.*, 2016).

Analysis of the composition of EMM identified two components (ammonium and phthalate ions) that can stimulate the expression and mobilization of *Tf2* elements. It will be interesting to determine to what extent the signalling pathways for these stimuli overlap. The discovery of phthalates stimuli towards retrotransposon activity is

important that we convey the relevance of our work on health, well-being and economic development.

Finally, the work in Chapter 6 identified rapamycin as drug that can activate *Tf2* elements. Drugs that activate retroviral elements are potentially useful for 'flushing out' reservoirs of latent virus (Marsden and Zack, 2013). The results in this chapter indicate that rapamycin acts upon *Tf2* activity via TORC-independent and –dependent mechanisms. Further characterisation of these mechanisms will be important for a detailed understanding of the biology of *Tf* LTR retrotransposons and their impact on the genome.

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