Yeast transcription termination factor Rtt103 functions in DNA damage response

A Thesis submitted to the University of Hyderabad for the award of a Ph. D. degree in Department of Biochemistry, School of Life Sciences

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DECLARATION

I, I. Srividya, hereby declare that this thesis entitled **"Yeast transcription termination factor Rtt103 functions in DNA damage response"** submitted by me under the guidance and supervision of Dr. Krishnaveni Mishra, is an original and independent research work. I also declare that it has not been submitted previously in part or in full to this University or any other University or Institution for the award of any degree or diploma.

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List of Abbreviations

5-FOA	5-Fluoroorotic acid
ADE2	Adenine requiring
ADH1	Alcohol DeHydrogenase
ADH4	Alcohol dehydrogenase isoenzyme type IV
AMP	Ampicillin
BARD1	BRCA1-associated RING domain protein 1
BRCA1	Breast cancer type 1 susceptibility protein
BER	base excision repair
BSA	Bovine serum albumin
CEN	Centomere
ChIP	Chromatin immunoprecipitation
CstF	Cleavage stimulation factor
CHK1	CHeckpoint Kinase
CID	C-terminal domain interacting domain
CTD	C-terminal domain
CY3	Cyanine 3
DAPI	4', 6-Diamidino-2-phenylindole
DDR	DNA damage response
DMSO	Dimethyl sulfoxide
DNA	Deoxyribonucleic acid
DNA2	DNA synthesis defective
DSB	Double strand break
dNTP	deoxyribonukleotide
DUN1	DNA-damage UNinducible
DTT	Dithiothreitol
EDTA	Ethylenediaminetetraacetic acid
EST2	Ever Shorter Telomeres
GAL	Galactose
GLU	Glucose
HEPES	4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid
HO-endonuclease	Homothallic switching endonuclease
HR	Homologous recombination
HRP	Horseradish peroxidise
HIS	Histidine
HPR1	HyPerRecombination
HRQ1	Homologous to RecQ protein
I-Sce-1	Intron-encoded endonuclease found in *Saccharomyces cerevisiae*

IWR1	Interacts With RNA polymerase II
LacZ	Bacterial enzyme β-galactosidase
LB	Luria-Bertani broth
LEU	Leucine requiring
LiAC	Lithium Acetate
Lig4	DNA Ligase
Lif1	Ligase Interacting Factor
MEC1	Mitosis Entry Checkpoint
MFT1	Mitochondrial Fusion Targeting
MMS	Methyl methane sulfonate
MRX	Mre11, Rad50, Xrs2
MRE11	Meiotic REcombination
mRNA	Messenger RNA
NHEJ	Non-homologous end joining
NPC	Nuclear pore complex
NSP1	Nucleoskeletal-like protein 1
NSE3	Non SMC Element
OD	Optical density
ORC	Origin recognition complex
PCR	Polymerase chain reaction
PEG	Polyethylene glycol
PLM2	PLasmid Maintenance
PMA1	Plasma Membrane ATPase
Pol	Polymerase
PVDF	Polyvinylidene Fluoride
RAT1	Ribonucleic Acid Trafficking
RAI1	Rat1p Interacting Protein
RAP1	Repressor activator protein 1
RAD1	RADiation sensitive mutant 1
RAD9	RADiation sensitive mutant 9
RAD24	RADiation sensitive mutant 24
RAD51	RADiation sensitive mutant 51
RAD52	RADiation sensitive mutant 52
RAD53	RADiation sensitive mutant 53
RAD54	RADiation sensitive mutant 54
RIF1	RAP1-interacting factor 1
RIF2	RAP1-interacting factor 2
RNA	Ribonucleic acid
RNR2	RiboNucleotide Reductase
RTT103	Regulator of Ty1 Transposition
SC	Synthetic complete

SDS	Sodium dodecyl sulphate
SEN1	Splicing ENdonuclease
SDS-PAGE	Sodium dodecyl sulfate polyacrylamide gel electrophoresis
SGD	Saccharomyces genome database
SIR	Silent information regulator
SOB	Super optimal broth
SPS2	Sporulation specific 2
ssDNA	Single-stranded DNA
TCA	Trichloroacetic acid
Tel	Telomere
TEL1	TELomere maintenance
THO2	Suppressor of the Transcriptional defect of Hpr1 by Overexpression
THP1	Tho2/Hpr1 Phenotype
TLC1	TeLomerase Component
TPE	Telomere position effect
Tris	Tris(hydroxymethyl)-aminomethan
TRP1	Phosphoribosylanthranilate isomerise
ts	Temperature sensitive
URA3	Orotidine-5'-phosphate (OMP) decarboxylase
UV	Ultraviolet
WT	Wildtype
XRS2	X-Ray Sensitive
Yku70/80	Yeast KU 70/80
YPD	Yeast extract peptone dextrose
YPK	Yeast extract peptone potassium acetate

Chapter-1

General Introduction and Objectives of the study

1.1 Telomeres in yeast

The integrity and stability of the genome of an organism must be maintained in order to propagate successfully. One of the key elements that contribute to stability of linear chromosomes are telomeres. The term telomere was coined by Muller. Telomeres are the physical ends of linear eukaryotic chromosomes and consist of specialized non-nucleosomal DNA-protein complexes which have multiple functions. Telomeric DNA is composed of highly repetitive sequences, $(T_2AG_3)_n$ in humans which range from 5000-15000 nucleotides and $(C_{1-3}A/TG_{1-3})_n$ in yeast which is around 300 nucleotides (Zakian, 1996). There are two types of repetitive sequences namely Y' and X, associated with yeast telomeres. The Y' repeat is about 6.7 kilo bases (kb) long and is found in one or more copies at most of the yeast telomeres; the X repeat is about 2 kb in size and is found at the ends of all yeast chromosomes. The length of the telomeric tracts varies considerably among species and is heterogeneous. The concept of telomere function was developed by Muller and McClintock before DNA was known to be the genetic material. They reasoned, based on chromosome breakage studies, that telomeres function to prevent chromosome fusions as broken chromosome ends always fused with each other, but the ends of chromosomes (telomeres) never fused. So telomere provides a protective cap for the end of the chromosome (McClintock, 1938, 1941a, b, 1942, 1956; Muller 1938; Muller & Herskowitz 1954). Telomeres play multiple roles in the cell. They

are required for stable maintenance of chromosomes, involved in transcriptional silencing and for the organization of chromosomes within the nucleus.

Linear eukaryotic chromosomes require mechanisms in addition to the conventional DNA polymerases to complete the replication of the extreme termini, i.e., telomeres. During the semi-conservative replication leading strand synthesis is complete, whereas the lagging strand is not complete as the removal of the distal RNA primer leaves a 5′ terminal gap. This process will progressively shorten the daughter DNA. The inability of the conventional DNA polymerase machinery to fully replicate terminal sequences results in gradual erosion of telomeric repeats, leading to checkpoint activation and cellular senescence (Olovnikov, 1973). The end replication problem is overcome by cells through the action of the telomerase enzyme (Greider and Blackburn, 1985). Telomerase is a reverse transcriptase capable of synthesizing telomeric repeats onto chromosome ends using an intrinsic RNA template. Many eukaryotes utilize this enzyme consisting of a catalytic subunit, an RNA template and several accessory proteins for the regulated addition of telomere repeat sequence. Yeast telomerase consists of a catalytic subunit (Est2p), an RNA template (TLC1) and two additional protein subunits (Est1p and Est3p) (Smogorzewska and de Lange, 2004). In yeast, the absence of telomerase leads to replicative senescence after 60–80 doublings.

1.2 Telomere binding proteins

Number of proteins bind to the telomere and perform crucial functions. Proteins that bind to double-stranded DNA at telomeres include Rap1, Sir2, Sir3, Sir4, Rif1, Rif2 and YKu70/YKu80 in budding yeast as well as components of telomerase, DNA replication machinery and the capping proteins.

1.2.1 Rap1, Rif and Sir proteins

In budding yeast *Saccharomyces cerevisiae*, Rap1 directly binds the TG_{1-3} telomere sequences (Konig et al., 1996). Rap1 establishes a negative feedback loop on telomere elongation by telomerase. For this, Rap1 recruits Rif1 and Rif2 through its carboxy-terminal domain. Rif proteins are involved in negative regulation of telomerase (Bourns et al., 1998). Rap1 binds the promoters of a large fraction of genes expressed during exponential growth, where it seems to play an essential role in transcriptional activation (Lieb et al., 2001). Possibly because of its role in transcription, Rap1 is essential for viability in budding yeast. Rap1 also establishes transcriptional silencing on the adjacent chromatin by recruiting a different set of factors (Sir proteins) through the same carboxy-terminal domain. Genes placed near telomeres are transcriptionally repressed, a phenomenon called Telomere Position Effect (TPE). *SIR2*, *SIR3*, *SIR4* are essential for TPE as well as transcriptional silencing at internal loci. Rap1p directly binds duplex telomeric DNA, and through its interaction with the Sir

proteins and their interactions with histones H3 and H4, promotes TPE (Hecht et al., 1995).

1.2.2 Ku Complex

Another important protein binding to the very end of the telomeres is Ku complex. Ku was first identified as an autoantigen in the sera of patients with autoimmune diseases (Mimori et al., 1981). Ku is highly conserved from yeast to mammals. In eukaryotes, Ku consists of a heterodimeric complex of 70 and 80kDa subunits, namely Ku70 and Ku80, respectively. Ku binds double-stranded DNA (ds DNA) ends in a sequence-independent manner (Tuteja and Tuteja, 2000). Ku is a multifunctional protein and plays important roles at the telomeres (Figure 1) (Modified from Fisher and Zakian, 2005). Ku proteins are essential for non-homologous end joining (NHEJ) pathway of DNA repair and essential for telomere replication and end protection (Bertuch and Lundblad, 2003). Ku positively regulates the telomere length by interacting with *TLC1*, the telomerase RNA subunit. *yku70*Δ cells were shown to have shorter telomeres compared to wild type. As a consequence of their abnormal telomere end structure, cells lacking Ku are also temperature sensitive and cannot grow at 37°C. This temperature sensitivity is mostly due to defects in telomere maintenance, rather than a more generalized defect in DNA repair, because (1) overexpression of several telomerase subunits (e.g. *TLC1*, *EST1* and *EST2*) partially suppresses the temperature sensitivity of a Ku-deficient strain (Lewis et al., 2002; Nugent et al., 1998; Teo and Jackson, 2001), (2) the combination of

mutations in telomerase subunits and Ku results in a near-synthetic lethal phenotype and (3) *yku70*Δ cells accumulate single-stranded DNA (ss DNA) in subtelomeric regions at 37°C.

The Ku heterodimer is also essential for TPE. Although many genes affect TPE, both Ku and the SIR complex (*SIR2*, *SIR3* and *SIR4*) are essential for telomeric silencing. There are two other regions of the yeast genome that exhibit Sir-dependent epigenetic silencing, the silent mating type loci, *HMR* and *HML* and the ribosomal DNA, but Ku is essential for silencing only at telomeres, plays a minor role in HM silencing and does not affect rDNA silencing. Disruption of either *YKU70* or *YKU80* results in almost complete loss of TPE (Boulton and Jackson, 1998). yKu70p and yKu80p interact with Sir4p in the yeast two-hybrid system, and both Sir3p and Sir4p show reduced telomere binding in the absence of Ku. Reduced Sir3p and Sir4p telomere association is likely due to the loss of Ku-dependent recruitment and the reduction of Rap1-mediated recruitment of the Sir complex. Role of Ku in TPE is likely to be in recruitment or activation of Sir4 protein at telomere and overcome the inhibitory effect of Rif1 and Rif2. Deletion of *RIF1* or *RIF2* increases telomere length and improves telomere silencing and suppresses the *yku70/80* mediated loss in TPE (Mishra and Shore, 1999). Together, these studies suggest: (1) Ku aids in the recruitment of the SIR complex to telomere bound Rap1p, thereby facilitating TPE, (2) Ku helps establish TPE by antagonizing the effect of Rif proteins in competing with the Sir complex for binding Rap1p and (3) Rap1p mediated recruitment of the Sir

complex is sufficient for TPE independently of Ku when telomeres are either of sufficient length, or the Rif proteins are not present.

Ku directs telomeres to the nuclear periphery. Several proteins required for TPE (e.g. Rap1p, Sir and Ku) are concentrated in 6–8 foci near the periphery of the nucleus. Since 70% of Y' subtelomeric DNA colocalizes with these protein foci, these foci also contain clusters of telomeres. In Ku-deficient cells, these foci are disrupted, leading to the proposal that Ku helps tether telomeres to the nuclear periphery (Laroche et al., 1998). However, some telomeres remain associated with the periphery even in the absence of Ku. Thus, there may be at least one additional pathway that can direct telomeres to the nuclear periphery.

Even though Yku proteins perform multiple functions, the complete mechanism of action is not fully understood. For example it is responsible for joining of two broken DNA ends in the chromosome (interior regions) by non-homologous end joining, but does not do so at the telomeres. If it performs the same function at the telomeres, the ends will be joined which will be deleterious to the cell. So it plays an important role in maintaining genome stability by performing different functions at different chromosome loci.

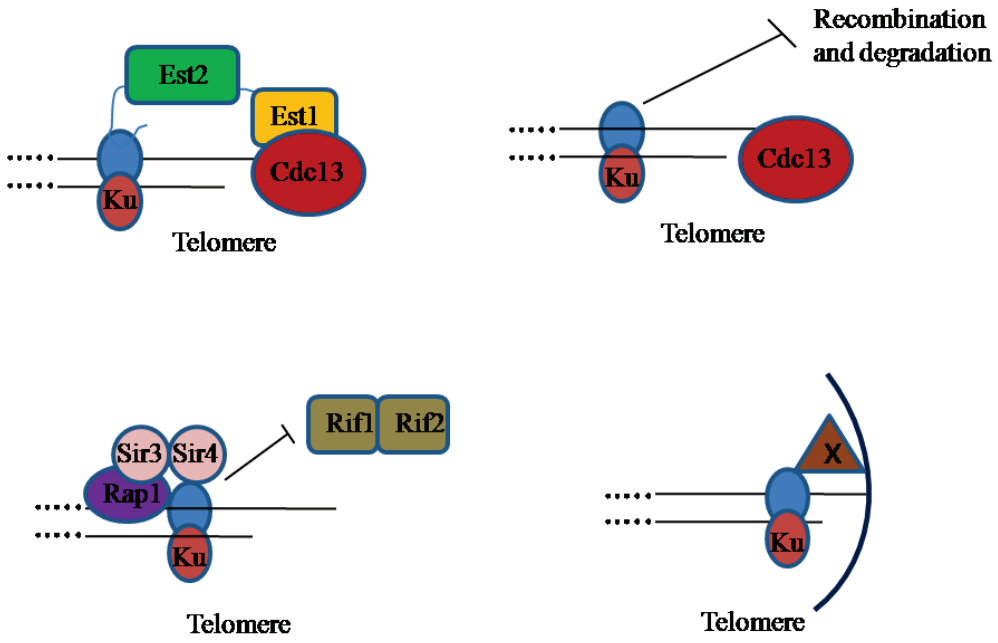


Figure 1: Multiple roles of Yku70/80 complex. a) recruitment of telomerase b) protection of telomeres from nucleases c) telomere position effect d) telomere anchoring to nuclear periphery

1.3. Genome stability

Genomes are constantly subjected to multiple forms of damage and if left unattended, can lead to mutations and chromosomal aberrations that result in cell death and diseases like cancer and premature ageing (Aguilera and Gomez-Gonzalez, 2008). Loss of genome stability may lead to point mutations or loss or gain of whole chromosomes or gross chromosomal rearrangements which include translocations, inversions, deletions and duplications. Consequently, cells have evolved several mechanisms to detect and repair damage to the genome, in order to maintain genome stability. As the genome is under constant

assault from exogenous and endogenous agents, loss of components of DNA damage response leads to increased basal DNA damage and a loss of genome stability.

1.3.1 DNA damage

DNA damage can occur either spontaneously by reactive oxygen species or can be induced by damage inducing agents. Detection of the damage is very important in dividing cells where replication or segregation of chromosomes with unrepaired lesions will affect genome integrity. So cells should assess the damage and either repair it or trigger an apoptotic program (Harrison and Haber, 2006). The various forms of DNA damage trigger common signal transduction pathways, termed DNA damage checkpoints. These checkpoints control the ability of the cells to arrest cell cycle progression after DNA damage and provide time for repair of the breaks. Checkpoint proteins are well conserved in all eukaryotes, indicating that the basic architecture of the DNA damage checkpoint pathways is conserved throughout evolution. When there is DNA damage, the abnormalities in the genomic DNA is detected by the sensors and a DNA damage signal is generated. Rad24, Ddc1, Mec3, Rad17, Tel1, Mec1 and Ddc2 are the sensors in *S. cerevisiae*. Mec1 dependent DNA damage checkpoint is activated when the resection of the DSB end yields long 3′ ended ssDNA tails or when ssDNA gaps arise by nucleotide excision repair (NER) or base excision repair (BER). Unresected, blunt-ended DNA activates a DNA

damage response, primarily through the Tel1 protein kinase and its associated MRX complex. The signal is received and amplified by transducers (Rad9). The effector kinases (Chk1 and Rad53) modulate the global cell response to DNA damage (Figure 2) (Marquez and Ibanez, 2003). Rad53 activates Dun1, which in turn leads to induction of transcription of DNA damage inducible genes. Rad53 also induces cell cycle arrest through phosphorylation of other substrates like Cdc5 and Cdc20. There are three important outputs of DNA damage signaling: phosphorylation of histone H2AX increases in some DSB repair events; arrest of the cell cycle prior to anaphase (G2/M arrest); and induction of damage-inducible genes as well as posttranslational regulation of ribonucleotide reductase (RNR). When the repair is complete the checkpoint arrest signal is extinguished and the cells resume the cell cycle.

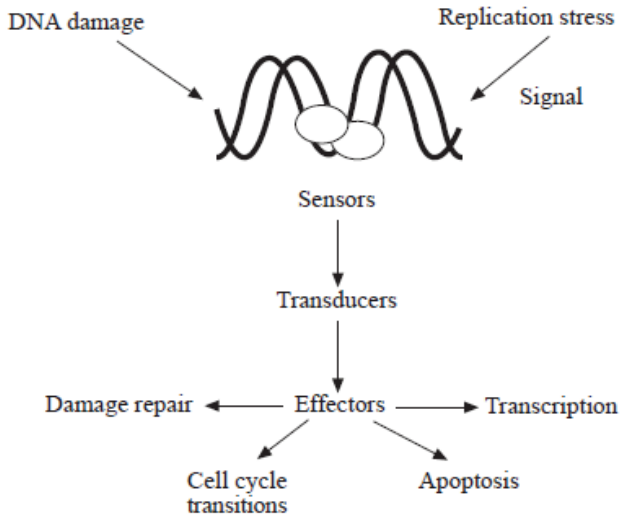


Figure 2: General architecture of DNA damage checkpoints.

1.3.2 NHEJ and HR

Of all the types of DNA damage DNA double strand break (DSB) is the most damaging lesion in the genome as they disrupt genetic information on both DNA strands. They may arise spontaneously by exposure to ionising radiations or genotoxic drugs. Improper repair or failure to repair DSBs can lead to gene deletions, duplications, translocations and missegregation of large chromosome fragments, which may be lethal**.** There are two major, evolutionarily conserved pathways that repair these kinds of breaks, one is non-homologous end joining (NHEJ) and the other is homologous recombination (HR). In NHEJ, the two broken ends with minimal or no homology are ligated and is generally error prone. HR is the more preferred pathway in lower eukaryotes. In HR the repair takes place by using the information present in the homologous sequence, which makes HR pathway of DNA repair error-free (Ataian and Krebs, 2006).

If homologous sequence is not present or found, the DNA will be repaired by NHEJ. NHEJ has been conserved through evolution and operates from bacteria to man. NHEJ requires Yku70/80 complex, the Lig4/Lif1 ligase complex, and the MRX complex. *YKU70* or *YKU80* gene disruption affects mating-type switching and spontaneous mitotic recombination (Mages et al., 1996). Since these proteins are required for NHEJ, deletions of these genes leads to sensitivity to bleomycin (Feldmann et al., 1996) and methyl methane sulphonate (Milne et al., 1996). Inactivation of *YKU70* or *YKU80* does not lead to

sensitivity towards agents such as ultraviolet (UV) light and hydroxyurea (HU) (Boulton and Jackson, 1996a). Cells expressing a functional Yku70/80 can precisely join cohesive ends of a transformed linearized plasmid. But cells deficient of these subunits display reduced recircularization efficiency and an increased frequency of imprecisely joined products. So Yku70/80 is considered to be an essential part of the *S. cerevisiae* NHEJ (Boulton and Jackson, 1996b; Milne et al., 1996; Moreau et al., 1999). In Yku70/80-deficient cells, plasmids are repaired in an error-prone way, yielding molecules that have undergone losses of up to several hundred base pairs at the joining site. During NHEJ, the ends of a DSB are detected and bound by the Ku70/Ku80 heterodimer. This binding protects the broken ends from degradation and marks the site of DNA damage. The MRX complex is recruited to the site of damage after binding of Ku70/Ku80 heterodimer. MRX complex promotes the end-processing of the cut sites. Finally the Lig4–Lif1 complex is recruited to the damage site and ligates the broken DNA ends.

HR requires genes in the *RAD52* epistasis group, which includes *RAD50*, *RAD51*, *RAD52*, *RAD54*, *RAD55*, *RAD57*, *RAD59*, *MRE11*, and *XRS2*. When there is a DSB, the ends are recognized by MRX complex which resects the DNA from 5′ to 3′. The resulting single stranded DNA is bound by RPA. Later the *RAD52* epistasis group proteins are recruited and search for the homologous sequences leading to repair of the damaged DNA.

1.3.3 Screens done to identify genes involved in genome stability

Although a lot is known about the different pathways that operate in repair of DNA lesions and the various checkpoint proteins that together contribute to arrest cell cycle and repair of damage, it is increasingly apparent that a large number of cellular processes are actually required for recovery from damage. Multiple screens have been done to identify components that affect genome stability, and with each new screen to understand responses to DNA damage, new proteins and pathways that are involved in this process are being discovered. Approaches involving the genome-wide measurement of transcriptional responses to DNA damage by UV or MMS show that over 25% of the genome showed changes in transcriptional status (Birrell et al., 2001; Fry et al., 2006; Jelinsky et al., 2000). In competitive fitness assays using the whole genome knock-out strains of yeast, several pathways including those involved in ubiquitination, gene silencing, and transport across the mitochondrial membrane were identified (Hanway et al., 2002). Similarly in protein localization based screens that look at the key damage sensors like H2Ax in mammals (Paulsen et al., 2009) or Rad52 in yeast (Alvaro et al., 2007), pathways involving nuclear transport, RNA processing, protein modification and chromosomal structural proteins were discovered. How all these responses contribute to recovery from DNA damage needs to be elucidated. An important outcome of these studies is that even though there are a few core damage response genes and repair pathways that do appear in most screens, there are a

large number of new and previously unidentified genes and molecular networks discovered with each new screen. Transcription associated factors are also shown to be involved in maintaining genome stability.

1.3.4. Transcription associated factors and genome stability

When there is damage to DNA, the RNA PolII which is transcribing the DNA is stalled and cannot move further which might potentially affect gene expression (Tornaletti and Hanawalt, 1999). These stalled transcription complexes mask the damaged DNA and do not allow repair to take place (Selby and Sancar, 1990). This kind of DNA damage is repaired by transcription coupled repair (TCR). There are recent reports in bacteria showing the involvement of transcription termination factor in genome stability. Rho, an RNA dependent ATPase is involved in terminating transcription. It was recently shown that Rho helps in clearing the stalled replication forks when there is DNA damage, so that the damaged DNA is available for repair (Washburn and Gottesman, 2011). In mammalian cells CstF, which is a polyadenylation factor, was shown to function in DNA repair response (Mirkin et al., 2008). CstF interacts with C-terminal domain of RNA PolII and participates in 3′ processing. Here it was shown that transcription and polyadenylation of mRNA precursors are affected after UV treatment. Cells with reduced levels of CstF show defects in repair of DNA damage. When the DNA is damaged, CstF interacts with BRCA1/BARD1 tumor suppressor

complex. After this binding, the BRCA1/BARD1 complex either ubiquitinates and degrades RNA PolII, so that the DNA is available for repair, or it recruits the transcription coupled repair machinery to the damage site so that the damaged DNA is repaired immediately and PolII resumes its function. In both these studies, they have shown that the RNA processing factors, Rho and CstF bind to the damaged DNA.

Link between transcription-coupled RNA processing and DNA repair has also been demonstrated. In yeast, Sen1 helicase, which is a transcription termination factor, has been shown to protect genome from transcription associated instability (Mischo et al., 2011). *SEN1* prevents transcription associated genome instability by preventing R-loop formation. R-loops are the structures in which the nascent RNA forms a RNA-DNA hybrid with the transcribed DNA strand and the non-transcribed strand remains single stranded (Gomez-Gonzalez and Aguilera, 2009). *THO* complex, which is a conserved complex containing Tho2, Hpr1, Mft1 and Thp2 proteins, has also been shown to maintain genome stability by preventing formation of R-loops (Figure 3) (Huertas and Aguilera, 2003). This complex coats the nascent RNA formed and promotes its export out of the nucleus. Thus in their absence, increased R-Loop formation leads to DNA breaks and subsequent genome instability.

From the available data it is clear that DNA repair is a complex process and new proteins and pathways are being identified to have roles in DNA damage response.

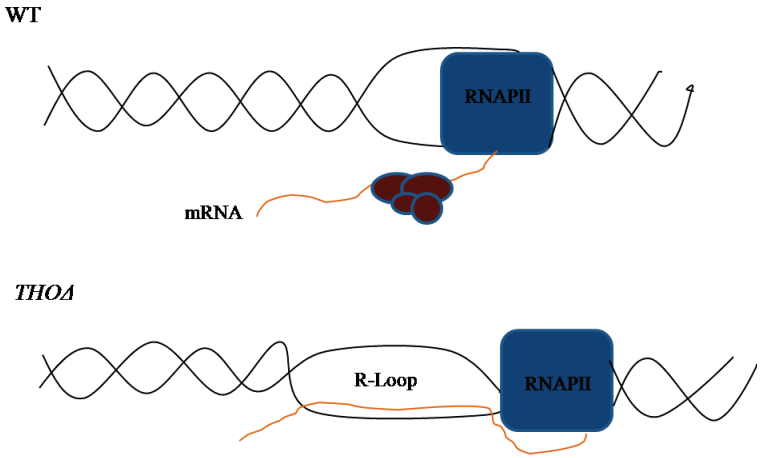


Figure 3: Formation of R-loops in *THO* null mutants

1.4 Objectives of the study

As mentioned above it is important to understand how the genome stability is maintained. This work was initiated to derive insights into the function of Yku70/80 heterodimer in genome integrity. Yku70/80 is a DNA binding heterodimer consisting of Yku70 and Yku80 proteins, that are conserved from yeast to mammals (Fisher and Zakian, 2005). They play a major role in repair of DNA breaks by NHEJ. Additionally, Yku70/80 are important for many telomeric functions, including loading of telomerase to telomeres, protecting telomeres from nucleolytic digestions, establishing stable silent chromatin at telomeres and also in anchoring telomeres to the nuclear periphery. *yku70/80* mutants are temperature sensitive and die at 37°C with large budded cells that contain more than G2 DNA content (Feldmann et al., 1996; Mages et al., 1996). In this study we have used the temperature sensitivity of *yku70* as a tool to study genome integrity. The following objectives were set.

- Multi-copy suppressor screen to identify the interacting partners that contribute to multiple roles of Yku70/80 complex
- Characterization of interacting partners
- Mechanism of action of the interacting partners

Chapter-2

Materials and Methods

2.1 Yeast methods

2.1.1 High efficiency yeast transformation

Yeast transformations with plasmids (including genomic library) or PCR products were done based on high efficiency LiAc protocol (Gietz and Woods, 2002). $5x10^6$ cells from the overnight incubated primary culture were added to the 25ml broth and incubated for 4-5 hrs at 30°C with constant rotation. $1x10^8$ cells from the secondary culture were used for transformation. The cell pellet was washed in 1ml of 0.1M LiAc and resuspended in 240µl of 50% Poly ethylene glycol (PEG). To this mixture, 36µl of 1M LiAc and 74µl of transformation mix containing 40µl of salmon sperm DNA and 34µl of both plasmid/DNA fragment and sterile MilliQ water were added. Cells were vortexed briefly and incubated at 42°C for 40 minutes. Then cells were spun at 13k for 15 sec and cell pellet was resuspended in 200µl of sterile water and plated on selective dropout media. If the selection was on G418 plates, the cell pellet was resuspended in 1ml of YPD broth and incubated at 30°C for 10-12 hrs to allow the expression of the gene and then plated on YPD media containing G418 drug (200 µg/ml).

2.1.2 Extraction of genomic DNA from yeast cells

2.1.2.1. Zymolyase method

Cells grown overnight in 5ml of YPD or in selective broth were harvested by centrifuging at 3k rpm for 5 min. The cell pellet was resuspended in 0.5ml of

1M Sorbitol and 0.1M Na_2 EDTA (pH 7.5) and transferred into 1.5ml microfuge tube. Cells were spheroplasted by incubating the cell suspension with 20 µl of Zymolyase 100,000U (2.5 mg/ml) at 37°C for 60min. Cells were centrifuged for 1 min at 13k rpm and the cell pellet was resuspended in 0.5ml of 50mM Tris-Cl (pH 7.4) and 20mM Na_2 EDTA (pH 8.0). 50µl of 10% SDS was added to the cell suspension, mixed well and then incubated at 65°C for 30 min. 200µl of 5M Potassium Acetate was then added to the cell suspension and placed in ice for 60 min. Cells were centrifuged for 5 min at 13k rpm and supernatant was transferred to a fresh microfuge tube. One volume (0.75ml) of 100% isopropanol was added to the supernatant, mixed and allowed to sit at room temperatue for 5 min. Then centrifuged very briefly for 2 min at 13k rpm and supernatant was poured off. DNA pellet was air dried and resuspended in 0.3ml of TE (pH 7.4) containing 20µg/ml of Rnase A. DNA was incubatded at 37°C for 30 min for degrading RNA. Then 30µl of 3M Sodium Acetate (pH 7.0) was added to the DNA solution and mixed. To this, 0.2ml of 100% isoproponal was added and mixed once again. DNA was recovered by centrifuging at 13k rpm for 2 min. The supernatant was poured off, DNA pellet was air dried and resuspended in 30µl of TE (pH 8.0).

2.1.2.2. Rapid isolation of genomic DNA from yeast cells

Cells grown overnight in 5ml of YPD or in selective broth were harvested by centrifuging at 3k rpm for 5 min. The cell pellet was washed in 0.5 ml of sterile distilled water and resuspended in 200µl of breaking buffer. Glass beads

(~200µl volume) were added to the cell suspension and then 200µl of phenol/chloroform/isoamylalchohol (25:24:1) was added and mixed. Cells were vortexed at high speed for 2 min. 200µl of TE (pH 8.0) was added and once again vortexed briefly for 10 to 15 sec. Then the sample was centrifuged at 13k rpm for 5 min at room temperature. The aqueous layer was transferred to a clean microfuge tube and 1ml of 100% ethanol was added and mixed by inversion. DNA was recovered by centrifuging at 13k rpm for 5 min. The supernatant was poured off, DNA pellet was air dried and resuspended in 30µl of TE (pH 8.0).

2.1.3 Extraction of whole cell protein from yeast cells by Trichloro Acetic acid (TCA) method

Cells grown overnight in 5ml of YPD or in selective broth were harvested by centrifuging at 3k rpm for 5 min. The cell pellet was resuspended in 200µl of 20% TCA and glass beads were added up to the meniscus and then cells were lysed by vorteing for 1 min. Cell suspension was transferred into a new microfuge tube. Glass beads were washed twice with 200µl of 5% TCA and the washes were added to the previous suspension. Cell pellet was collected by centrifuging at 3k rpm for 10 min and resuspended in 200µl of 1x laemmli buffer. The laemmli buffer turns red because of the low pH of cell pellet. Therefore, 50µl of 1M Tris base (no pH adjustment) was added to turn blue. The sample was boiled for 3 min and centrifuged again at 3k rpm for 10 min. Protein sample was transferred to a new microfuge tube and the pellet was discarded.

2.1.4 Spore enrichment

Diploid cells were grown on dextrose deficient YPK medium and incubated at 25°C for 4-5 days. Tooth pick full of spores were resuspended in 500µl of YPD broth. The cell suspension was vortex mixed and centrifuged at 13k rpm for 10 sec. The cell pellet was resuspended in 1ml of 100µg/ml of zymolyase (100,000U) and incubated at 30°C for 20 min. 500µl of cell suspension was aliquoted in separate microfuge tube and centrifuged at max speed for 30 sec. Cell pellet was washed in 1ml of sterile water and resuspended in 100µl of sterile water. Cells were then agitated for 2 min in upright position using vortex mixer at max speed. Aqueous cell suspension was discarded and the tube was rinsed with sterile water for several times. The spores were resuspended in 1ml of 0.01% Nonidet P-40. Appropriate volume of cell suspension was plated on YPD medium and incubated at 30°C for one day (refer Guthrie and Fink, 1991).

2.1.5 Silencing assay

Silencing assays were done to test the loss in silencing in yeast. For this, the yeast cells were initially grown in nutrient rich broth or selective broth dropped out for specific amino acids (for retaining plasmids) at 30°C with appropriate rotation for overnight and then the culture was subjected to 10-fold serial dilution for 5 times. 5µl of each dilution was spotted on complete medium to check the total number of cells grown and on selective medium for measuring the loss in silencing of reporter gene. In case, the reporter gene is *URA3*, then serially diluted cultures were spotted on the medium containing

1mg/ml of 5-FOA (5-Fluoro orotic acid). Expression of the *URA3* gene (Orotidine-5'-phosphate decarboxylase) leads to the conversion of 5-FOA into 5-fluorouracil, a toxic compound. This indicates that strains expressing *URA3* cannot grow in this medium and those repressing *URA3* can grow. Therefore, 5-FOA serves as a good indicator of the expression status of the *URA3* reporter gene. After spotting, plates are incubated at 30°C for 2-3 days and analyzed loss in silencing by observing growth of cells.

In case of *ADE2* marker the silencing was assayed by the colour of the colony. Cells with the gene in a repressed state accumulate the upstream substrate (5-aminoimidazole ribonucleotide carboxylase) which polymerizes forming a red pigment. As the colony forms through 20-25 rounds of division, the gene is stochastically repressed in some lineages and expressed in others resulting in red/white sectored colonies. Cells expressing the gene do not accumulate the substrate and are white. The cells were plated on YPD plates and incubated for 2-3 days at 30°C. Later they were kept at 4°C for about 4-5 days and analyzed for the loss of silencing by observing the colour of colony.

2.1.6 Construction of mutants and tags in yeast

Strains with gene deletion and tagging were done as described in (Longtine et al., 1998). For gene knockout, DNA fragment was PCR amplified so as to contain selectable marker flanked by around 50bp of DNA of the region of the gene of interest. This fragment was transformed into yeast by high efficiency LiAc protocol (Gietz and Woods, 2002). The flanking regions recombine with the

genomic region of the gene of interest by homologous recombination and insert the selectable marker in place of the gene. Therefore, the gene is replaced by selectable marker. For gene tagging, the forward primer was designed by taking sequence just upstream of the stop codon and in frame so that it does not disrupt the reading frame of the tag and selectable marker which are going to be inserted in the downstream of the gene. These insertions were confirmed by screening PCR which gives diagnostic fragment of approximately 500bp. The sequences of the primers used for gene deletion, tagging and screening PCR are given in table 3.

The strains that showed positive in screening PCR were further confirmed by genomic southern analysis. Genomic DNA from these strains was subjected to restriction digestion with appropriate enzymes that gives different fragments at gene locus of interest in wild type and deletion/tagged strains. The digested genomic DNA was transferred to nylon membrane and subjected to southern hybridization with radio labelled probe. The blot was then exposed to X-ray film to obtain the autoradiogram.

2.2 Recombinant DNA methodology

2.2.1 Preparation of ultra competent DH5 α cells

Ultra competent cells of *DH5a* strain of *E.coli* were prepared by Inoue method described in (Sambrook and Russell, 2001). A single bacterial colony was inoculated in 25ml of SOB/LB broth and incubated at 37°C with constant

rotation around 150-200rpm for 6-8 hrs. This primary culture was then inoculated (4ml, 3ml, 2ml, 1ml) into four 250ml conical flasks containing 100ml of SOB broth and incubated at 18-22°C with moderate shaking for overnight. Incubation was stopped when the OD reached 0.55 at 600nm and cells were harvested by centrifuging at 2500g for 10 min at 4°C. Supernatant was poured off and centrifuge tube was stored open on a stack of paper towel for 2 min to drain away the broth completely. Cell pellet was resuspended in 32µl of Inoue transformation buffer for 100ml of initial culture. Cells were harvested again by centrifuging at 2500g for 10 min at 4°C. Supernatant was discarded and centrifuge tube was stored open on a stack of paper towel for 2 min to drain away the solution completely. The cells were then suspended in 2ml of ice cold Inoue transformation buffer and 0.15µl of DMSO was added and mixed by swirling and stored in ice for 10 min. Bacterial suspension was aliquoted into microfuge tubes and immediately snap frozen by immersing the tightly closed tubes in liquid nitrogen. Then the tubes were stored at -70°C until needed.

2.2.2 Bacterial transformation

Ultra competent *E.coli* (*DH5a* strain) cells were thawed and aliquot into a sterile microfuge tube. 2.5µl of plasmid DNA of concentration around 1µg was added to 50µl of competent cells or 10µl of ligation mixture was added to 100µl of competent cells. The tubes were stored in ice for 30 min and then transferred to 42°C for exactly 90 sec. Then they were rapidly transferred to ice and kept for 1-2 min. 1ml of LB broth was added to each tube and incubated at 37°C for 45

min. The samples were centrifuged at 5000rpm for 1 min and the cell pellet was resuspended in 200µl of LB broth and plated on LB medium containing 100µg/ml of ampicillin. The plates were allowed to dry and incubated at 37°C for overnight.

2.2.3 Alkaline lysis miniprep for plasmid extraction from bacterial transformants

Plasmid DNA was isolated from bacterial transformants by alkaline lysis miniprep method described in (Sambrook and Russell, 2001). Single bacterial colony was inoculated in 5ml of LB broth containing 100µg/ml of ampicilin and incubated at 37°C and 160rpm for overnight. 1.5ml of bacterial culture was transferred in a microfuge tube and centrifuged at 13k rpm for 1 min. The cells were resuspended in 200µl of solution I and then solution II was added and mixed by gently inverting the tube for 5 times till the solution is turned clear and viscous. Ice cold 200µl of solution III was added and mixed immediately by inverting several times and left on ice for 5 min and the solution turns into a white precipitate. Then the sample was centrifuged for 8 min at 13k rpm at 4°C. Supernatant was gently pipetted and transferred to another clear microfuge tube. To this solution 420µl of 100% isopropanol (0.7 volume) was added and mixed by inverting. DNA was precipitated by centrifuging at 13k rpm for 10 min and the pellet was washed with 500µl of 70% ethanol and once again centrifuged at 13k rpm for 2 min. Supernatant was discarded, DNA pellet was

air dried and resuspended in 30-50µl of TE pH8.0 containing 30µg/ml of RNaseA.

2.2.4 Construction of plasmids

RTT103 clone in multi copy plasmid **(**CKM233) was constructed by digesting KM93 genomic library plasmid (CKM239) by *Hpa*I *and Nsi*I enzymes and DNA fragment of size 2.5kb containing full length *RTT103* gene along with its promoter region was ligated into *Pst*I *and Sma*I digested multi copy vector, YEplac181 (CKM6) listed in table 2. *RTT103* was then sub cloned into *Kpn*I and *Sph*I digested YCplac22 (CKM1) vector by digesting CKM233 plasmid with *Kpn*I and *Sph*I (CKM261). The other plasmids used in this work are listed in table.2. Plasmids used as templates in PCR to generate yeast knockouts and tags are described in (Longtine et al., 1998).

2.3 Methods in yeast cell biology

2.3.1 Western blot

WT (KRY105), *yku70* (KRY171), *rtt103* (KRY230), *mec1* (KRY443) and *rtt103mec1* (KRY445) cells were transformed with *RAD53-Myc* plasmid (gifted by Marco Foiani) (CKM272) for checking the phosphorylation (Pellicioli et al., 1999)). They were then treated with 0.03% MMS for 2hrs. For the dephophorylation experiment, after MMS treatment the cells were washed with equal amounts of SC-LEU broth and ice cold freshly prepared 10% sodium thiosulfate 3 times for removing the MMS and then incubated in SC-LEU broth

for 4 and 6hrs. Whole cell protein form the treated and untreated cells were extracted using standard TCA protocol described in (Lewis et al., 2007). Equal amounts of protein were loaded in 8% polyacrylamide gel. After electrophoresis, proteins were transferred to PVDF membrane and blocked with 5% non fat dry milk in TBST (150 mM NaCl, 10mM Tris-HCl pH8.0 and 0.1% Tween 20) buffer for one hour at RT. Then the membrane was incubated with Abcam anti myc antibody, ab9106 (1:30,000 in 1% BSA in TBST buffer) for Rad53-myc for 2 hours at RT. The membrane was washed thrice in TBST buffer for 10 min and incubated with secondary anti-rabbit-HRP antibody (1:10,000 in 1% BSA in TBST buffer) for Rad53p for 1 hour at RT. Membrane was washed thrice with TBST for 10 min. BioRad detection reagents and BioRad versadoc instrument were used for detecting the protein of interest as directed by manufacturers instructions. Same blot was probed for Sir2 using anti-Sir2 antibody (1:2,000 in 1% BSA in TBST buffer) for 2 hours at RT and anti-Rabbit-HRP antibody (1:10,000 in 1% BSA in TBST buffer) for 1 hour at RT to check the loading consistency.

2.3.2 Immunofluorescence

Immunofluorescence was done as described in (Gotta et al., 1996). Briefly, diploid yeast strain KRY184 (Rtt103-13xMyc) was grown in YPD broth. Overnight grown 5ml culture was fixed with 0.5ml of formaldehyde and incubated at 30°C in a shaker for 20 min. Cells were then washed thrice with sterile water and resuspended in 200µl of 0.1M EDTA-KOH and 10mM DTT

and incubated at 30°C for 10 min. Cell suspension was centrifuged at 3k rpm for 5 min. The cells were spheroplasted by resuspending in 200µl of YPD broth containing 1.2M sorbitol and one-tenth volume of zymolyase (2mg/ml) and incubated at 30°C for 15 min. Spheroplasts were washed thrice with 500µl of YPD sorbitol and resuspended in 100µl of YPD sorbitol. Spheroplasts were spotted on multi-well slides coated with polylysine. They were further permeabilized with methanol and acetone by incubating for 5 min and 1 min respectively at -20°C. Spheroplasts were blocked with 1% ovalbumin and incubated with appropriate primary antibody dilutions (mouse NSP1 antibody 1:500, rabbit myc antibody 1:200) for overnight at 4°C. Cells were then thoroughly washed thrice with PBST buffer for 5 min each and incubated with recommended dilutions of fluorescently labelled secondary antibody (Alexa Fluor 488 tagged secondary anti mouse antibody 1:500, Cy3 tagged secondary anti rabbit antibody 1:500) in dark at room temperature for 45 min. Cells were thoroughly washed thrice with PBST buffer for 5 min each. After washes, slides were mounted in mounting medium containing DAPI and then viewed and photographed in a Leica confocal microscope. Images were processed using the same software.

2.4 Chromatin Immunoprecipitation

ChIP experiments were done by following the method described in (Xu et al., 2007). Yeast strains KRY447 and KRY448 were grown in 50ml of SC-Raffinose minimal broth till OD_{600} reached 0.5-1.0. These cells were then

transferred to 2 flasks, one containing SC-Raffinose (uninduced for *Sce*-I endonuclease) and the other SC-galactose (induced for *Sce*-I endonuclease) and incubated for 3hrs. These cultures were cross linked with 1.4ml of 37% formaldehyde and cross links were quenched with 3.4ml of 2M glycine. Then cells were pelleted and washed with ice cold TBS (150mM NaCl and 20mM Tris-HCl pH 7.6) buffer for two times and centrifuged at 3,000rpm for 5 min. Cells were lysed with 400µl of ice cold lysis buffer with protease inhibitor (0.1% Deoxycholic acid, 1mM EDTA, 50mM HEPES/KOH, pH 7.5, 140mM NaCl and 1% Triton X-100) equal volume of glass beads were added and vortexed at max speed for 10 min at 4°C. Lyaste was collected in a fresh tube and the beads were washed with 400µl of lysis buffer and vortexed again for 2 min at 4°C and wash was added to the lysate. Lysate was sonicated in Biorupter Sonifier at power setting of 15 sec pulse on and 2 min off to shear the chromatin to 500-800bp of average length. Then sample was clarified by centrifuging at max speed for 15 min at 4°C. The supernatant was pre-cleared by adding 30µl bed volume of Protein A Sepharose beads (Amersham Biosciences) and incubated for 1 hr at 4°C with constant rotation. Sample was centrifuged at 7,500 rpm for 5 min at 4°C and supernatant was aliquoted into fresh tubes. At this point 50µl of sample was taken in fresh tube and used as input DNA. 1µl of 1:5 dilution primary antibody against myc epitope of Rtt103p and Yku80p (Abcam) was added to the sample and incubated for overnight at 4°C with constant rotation. 30µl of Protein A sepharose beads were added to the chromatin antibody mix and

incubated for 2 hr at 4°C with constant rotation. Protein A Sepharose beads were washed with 1ml each of lysis buffer, lysis-500 (0.1% deoxycholic acid, 1mM EDTA, 50 mM HEPES/KOH, pH 7.5, 500 mM NaCl and 1% Triton X-100) buffer, LiCl/detergent solution (0.5% deoxycholic acid, 1mM EDTA, 250 mM NaCl and 0.5% NP-40, 10 mM Tris-HCl pH 8.0) and TBS buffer. Chromatin imunoprecipitate was eluted first with 100 µl of 1% SDS in TE (10 mM Tris-HCl pH8.0 and 1 mM EDTA pH8.0) and then with 150µl of 0.67% SDS in TE buffer by incubating at 65°C for 10 min. DNA from bound and unbound chromatin (input sample) was purified by phenol:chloroform:isoamylacohol extraction and ethanol precipitation.

2.5 Real Time PCR for analyzing ChIP DNA

DNA sample from ChIP experiments were analysed by Real-Time PCR using SYBR green master mix (Aplied Biosystems) according to manufacturer's instructions on an Applied Biosystems 7500 HT fast Real-Time PCR system. The primers for real time PCR were designed by taking sequences at different regions in and around the cut site. The sequences of these primers are given in Table 4. Relative quantification of immunoprecipitated DNA was done based on comparative C_T value method using sequence detection software. Data were calculated according to the formula.

$$\Delta Ct_{(\text{telomere primers})} = (Ct_{(\text{telomere primers and ChIP DNA})}) - (Ct_{(\text{telomere primers and Input DNA})})$$
$$\Delta Ct_{(\text{SPS2 internal control})} = (Ct_{(\text{SPS2 internal control and ChIP DNA})}) - (Ct_{(\text{SPS2 internal control and Input DNA})})$$

Fold in enrichment: $2^{(\Delta Ct(\text{SPS2 internal control}) - \Delta Ct(\text{telomere primers}))}$

Δ Ct values are calculated by subtracting the Ct values of the input DNA with the Ct values of the ChIP DNA. This Δ Ct value is further normalised to an internal control by subtracting with the Δ Ct value of the *SPS2* locus. This gives the relative amount of precipitated chromatin at telomeres with respective to internal *SPS2* locus.

2.6. DNA damage assays

2.6.1 Plasmid rejoining assay (Milne et al., 1996)

pRS313 (CKM32) DNA (100µg) was digested with *Eco*RI to completion. This linearised DNA was then used to transform yeast by the lithium acetate method. Parallel transformations with the same competent cells were performed with an equivalent amount of uncut plasmid to enable normalisation for minor differences in transformation efficiencies between strains and between experiments. Following transformation, cells were plated and colonies arising on selective media (SC-HIS) after 3–4 days were counted. The data are represented as fraction of linear plasmid recovered relative to the supercoiled plasmid

2.6.2 MMS assay

Yeast strains were grown overnight to mid log phase in YPD medium. 10 fold serial dilutions of the strains were done and 5µl of the sample was spotted on YPD plates and YPD containing different concentrations of MMS

(YPD containing MMS plates were prepared by first autoclaving the YPD agar and then after slight cooling, required concentrations of MMS were added to the media and poured into plates). The plates were incubated for 2-3 days at 30°C.

2.6.3 I-*Sce*I endonuclease assay

For I-*Sce*I endonuclease assay KRY304 strain was used which has two I-*Sce*I sites inserted in opposite orientation on each side of the *URA3* gene on chromosome V (Marcand S et al., 2008). The sequence encoding the nuclease was inserted at a different locus and placed under the control of a galactose-inducible promoter. The other strains which were used for this assay were derived from KRY304. Strains were grown overnight in SC broth and spread on SC-GLU (When the cells were grown on glucose medium the endonuclease will not be produced) and SC-GAL (When I-*Sce*I endonuclease is induced by galactose, breaks are generated on either side of *URA*3 gene) plates. Colonies were counted after incubation at 30°C for 4 days. The graphs indicate the relative survival on galactose versus glucose.

2.6.4 Hyper-recombination assays

2.6.4.1 Chromosome hyper-recombination assay

The strains used for this study have *ADE2* and *URA3* genes flanked by *leu2-k* repeats (KRY615) (Santos-Rosa and Aguilera, 1995). All the strains used for this study are isogenic to KRY615. Recombination between the *leu2-k* repeats

results in loss of *ADE2* and *URA3.* Cells were picked from the plate and suspended in 200µl water. 5 fold serial dilution was done and 10µl was spotted on SC and SC-FOA plates. The growth on SC-FOA plates indicate the loss of *URA3.* The number of recombinants were calculated by plating the cells from the dilutions done and counting the number of colonies on SC+ FOA plates after 2-3 days. The total number of survivors were calculated from SC plate. The recombination was also confirmed by the appearance of red colonies showing loss of *ADE2.* Number of recombinants per 1,00,000 cells were calculated and plotted.

2.6.4.2 Plasmid hyper-recombination assay

This was done using a plasmid LLac (CKM 328) which has *TRP* marker (Mischo et al., 2011) . This plasmid has *leu2* alleles flanking the LacZ gene under the control of *LEU2* promoter. *LEU2* is normally not functional in this plasmid. If recombination occurs between the *leu2* repeats, LacZ gene will be lost, the repeats will come together and *LEU2* will be functional. This plasmid was transformed into the respective strains and the transformants were selected on SC-TRP plates. The colonies were restreaked on SC-TRP plate. From this individual colonies were picked, serial diluted and then equal number of cells (cells were counted in haemocytometer) were plated on SC-TRP (total number of colonies) and SC-LEU (number of recombinants) plates. Recombination frequency per 10,000 cells was calculated.

Tables

Table 1: List of the yeast strains used in this study

Name	Genotype	Source/Reference
KRY2	MATa leu2-3,112 his3-11,15 ura3-1 ade2-1 trp1-1 can1-100 rad5-535	Rodney Rothstein
KRY60	MATa leu2-3,112 his3-11,15 ura3-1 ade2-1 trp1-1 can1-100	Hannah Klein
KRY61	MATα leu2-3,112 his3-11,15 ura3-1 ade2-1 trp1-1 can1-100	Hannah Klein
KRY105	MATa leu2-3,112 his3-11,15 ura3-1 ade2-1 trp1-1 can1-100 *adh4::ADE2* TEL VII L	This Study
KRY162	MATa *rif2::His3*, *adh4::ADE2* TEL VII L	This Study
KRY171	KRY105 except *yku70*::*KanMx*	This Study
KRY172	KRY193 except Matα *yku70::KanMx*	This Study
KRY193	MATa *adh4::URA3* TEL VII L	This Study
KRY225	Matα *yku80::KanMx adh4::ADE2* TEL VII L	This Study
KRY227	KRY193 except Matα *yku80::KanMx*	This Study
KRY230	KRY105 except MAT α *rtt103::KanMx*	This Study
KRY231	MATa *rtt103::KanMx adh4::ADE2* TEL VII L rad5-535	This Study
KRY284	KRY193 except *rtt103::KanMx*	This Study
KRY285	KRY193 except MATα *rtt103::KanMx*	This Study
KRY286	KRY193 except *rtt103::KanMx yku70::LEU2*	This Study
KRY290	KRY105 except MATα *rtt103::KanMx yku70::LEU2*	This Study
KRY304	MATa *Δ lys2::pGAL-ISCEI ISceI::URA3::ISceI*	Stéphane Marcand

KRY365	KRY105 except Matα *rad9::URA3*	This Study
KRY366	KRY105 except *rad24::TRP1*	This Study
KRY367	KRY105 except *yku70::LEU2 rad24::TRP1*	This Study
KRY369	KRY105 except *yku70::LEU2 rad9::URA3*	This Study
KRY371	KRY105 except Matα *rtt103::KanMx rad9::URA3*	This Study
KRY372	KRY105 except *rtt103::KanMx rad24::TRP1*	This Study
KRY375	KRY 371 except *rtt103::KanMx*	This Study
KRY376	KRY371 *except yku70::LEU2*	This Study
KRY379	KRY371 Matα *rtt103::KanMx yku70::LEU2*	This Study
KRY440	KRY105 except MATα *tel1::His3*	This Study
KRY441	KRY105 except MATα *yku70::Leu2 tel1::His3*	This Study
KRY442	KRY105 except MATα *rtt103:;kanMx tel1::His3*	This Study
KRY477	KRY193 except MATα *rad52::TRP1*	This Study
KRY448	KRY304 except *RTT103* 13Myc	This Study
KRY473	W303 MATa *rad1::LEU2*	Hannah Klein
KRY482	W303 *rtt103* diploid	This Study
KRY615	Mat a ade2-1 trp1, his3, ura3 can1-100 leu2-k::ADE2 URA3::leu2-k rad5-535	Andre Aguilera
KRY617	W303 MATα *rad51::KanMx* rad5-535	Andre Aguilera
KRY622	MAT α *pGALHO::LEU2* rad5-535	David Shore
KRY624	MATa *pGALHO::LEU2 rai1::KanMx* rad5-535	This study
KRY631	MAT α RAI1 his3Δ1 leu2Δ0 ura3Δ0 (BY4739)	Arlen Johnson

KRY632	KRY 631 except rai1::KamMX	Arlen Johnson
KRY633	MATa ura3-52 leu2Δ1 trp1Δ63 (FY23)	Arlen Johnson
KRY634	KRY 633 except MAT α *rat1-1* ts	Arlen Johnson
KRY646	MAT α *pGALHO::LEU2 rtt103::KanMx* rad5-535	This study
KRY650	KRY 615 except MAT α *rtt103::KanMx*	This Study
KRY652	KRY 615 except MAT α *hpr1Δ3::HIS3*	This Study
KRY676	Mat a *rtt103::KanMx rad52::TRP1*	This Study
KRY681	KRY617 except Mata *rtt103::KanMx adh4::URA3* TEL VII L	This Study

Table 2: List of the plasmids used in this study

Name of the plasmid	Old Name	Brief Description
CKM1	YCplac22	*CEN* plasmid containing *TRP1* marker
CKM6	YEplac181	2μ plasmid containing *LEU2* marker
CKM32	pRS313	*CEN* plasmid containing *HIS3* marker
CKM67	E335	pFA6a-kanMx6 (gene deletion)
CKM90	E358	pFA6a-13Myc-kanMx6 (C-terminus tagging)
CKM239	KM93	Genomic library plasmid
CKM240	KM95	Genomic library plasmid
CKM233	YEplac181 + *RTT103*	*Hpa*1/*Nsi*1 cut *RTT103* from KM 93 inserted into *Pst*1/*Sma*1 of CKM6
CKM261	YCplac22+ *RTT103*	*Kpn*I/*Sph*I cut *RTT103* from CKM 233 inserted into *Kpn*I/*Sph*I of CKM1
CKM271	YCpGal	EcoRI endonuclease under Gal Promoter (*CEN4, URA3*)
CKM272	pCH10	RAD53-9 myc in Ycplac111(*CEN4, ARS1, LEU2*)
CKM	pLLacZ	LacZ gene under control of *LEU2* Promoter (*CEN, TRP1*)

Table 3: List of primers and their sequences used for PCR in this study

Name of the primer	Primer sequence (5′ to 3′)	Purpose
F1 YKU70	gatttgttaagtgactctaagcctgattttaaaacgggaatattatg CGGATCCCCGGGTTAATTAA	*YKU70* gene deletion
R1 YKU70 Cor	gtcgtgcataaatatcttgctaatagttgtacagtacaacgtttagc acgGAATTCGAGCTCGTTTAAAC	*YKU70* gene deletion
YKU70 Scr	GGCAAACACTTGGCGTGGTT	Screening PCR
F2 YKU80	ggtgaacaacacagtaggggaagtccaaacaatagcaataatC GGATCCCCGGGTTAATTAA	*YKU80* gene C-tagging
R1 YKU80	ggtaacaatgcaaatcagtagtatgacaattatttacccgcGAA TTCGAGCTCGTTTAAAC	*YKU80* gene C-tagging
YKU80 Scr	CTTTCACGTAAGTTCCCCACG	Screening PCR
F1 RTT103	catgttgttcaacaggctaaaggtcaaaaaattattcaatttcaag attccCGGATCCCCGGGTTAATTAA	*RTT103* gene deletion
R1 RTT103	atatatttgtataagttatctccttgttttctttttactcaaccatcata GAATTCGAGCTCGTTTAAAC	*RTT103*gene deletion/C-tagging
F2 RTT103	ccggaggggtttcttctagtatacaagacttgttaagtaagcttgc aaatCGGATCCCCGGGTTAATTAA	*RTT103* gene C-tagging
RTT103 Scr	gtcagattggaggagaaatc	Screening PCR
Scr F	GTAATATCATGCGTCAATCG	Screening PCR
Yep13 F	GCTACTTGGAGCCACTATC	DNA Sequencing
Yep13 R	CCAGCAACCGCACCTGT	DNA Sequencing

Table 4: List of primers and their sequences used for Real Time PCR to check the binding of Rtt103p and Yku80p

Chromosome	Primer	Sequence of the primer in (5′ to 3′)
V	ChIP Sce 1 ChIP Sce 2 (1kb from I-*Sce*-Ia)	GGATCAGACGGAGTACTTGTCC GCTCTCGCTCTGCTCTTTCATT
	ChIP Sce3 ChIP Sce4 (0.5kb from I-*Sce*-Ia)	AACGCTAGAGCAGACGCTCATC CGTTTCGTTGCTTGTCTTCCCT
	ChIP Sce 5 ChIP Sce 6 (At I-*Sce*-Ia)	GAAGGTTAATGTGGCTGTGG GTCTGTGCTCCTTCCTTCGT
	ChIP Sce 7 ChIP Sce 8 (5′ region of *URA3*)	AGGAACGTGCTGCTACTCATCC CCTTTAGCGGCTTAACTGTGCC
	ChIP Sce 9 ChIP Sce 10 (3′ region of *URA3*)	CAGAATTGTCATGCAAGGGCTC CCAATGCGTCTCCCTTGTCATC
	ChIP Sce 11 ChIP Sce 12 (At I-*Sce*-Ib)	TATTTGAGAAGATGCGGCCAGC CTTGGTTCTGGCGAGGTATTGG
	ChIP Sce 13 ChIP Sce 14 (0.5kb from I-*Sce*-Ib)	CTTGGGCCGATAAGGTGTACTG GCTGTTCCAGCCCATATCCAAC

	ChIP Sce 15 ChIP Sce 16 (1kb from I-*Sce*-Ib)	AGCGACATTAACCCGGAGGAC GCACATGGTACGCTGTGGTG
	ChIP Sce 17 ChIP Sce 18 (3kb from I-*Sce*-Ia)	CCTTAGGAATGCTGCACAATC CTTCAATGCCATCGTCCTCGC
	ChIP Sce 19 ChIP Sce 20 (2kb from I-*Sce*-Ia)	ATTGGTCTCCCTAGTCGATG GGAGTCGGAAGCAATGAAAC
	ChIP Sce 21 ChIP Sce 22 (2kb from I-*Sce*-Ib)	ACGCTCAAGCCTATATCTCC CAGACTCTTCTCCACTGAAC
	ChIP Sce 23 ChIP Sce 24 (3kb from I-*Sce*-Ib)	CTTGGCGATCTTACACCTTAG CTTGTCCTATCACCTGCAAAC
	ChIP PMA1 3′ FP ChIP PMA1 3′ RP	ATAATAGTTCCTGCCCAGCTC CAGTAAAGGTATTTCGCGGAG
	ChIP ADH1 3′ FP ChIP ADH1 3′ RP	CTTATTGACCACACCTCTACC GACGATGAAGATAGAGCCCAA
IV	SPS F' SPS R'	ACTGTCCCGTCATTGATGCGTCTC GGGATCGTTGCATTAGTGTTAACC
Telomere VI R	10.0Kb F' 10.0KbR'	TCATCCGTACACACACAGAGACCA TCCAATTGTCAATGAGCAGGTTGA

Chapter-3

Multi-copy suppressor screen to identify the interacting partners that contribute to multiple roles of Yku70/80 complex

3.1 Introduction

A multi-copy suppressor screen was done to identify the interacting partners of Yku70. *yku70*Δ cells have various phenotypes like shorter telomere length, loss in telomere position effect, sensitive to DNA damaging agents and sensitive to temperature. In our study we have used the temperature sensitive (ts) phenotype of *yku70* as a tool to identify its interacting partners. *yku70*Δ/ *yku80*Δ cells are sensitive to temperature at 37°C (Barnes and Rio, 1997; Boulton and Jackson, 1996; Feldmann and Winnacker, 1993) and die at this temperature. They grow slowly at 35°C. At 37°C the *yku70*Δ cells arrest as an almost equal mixture of large-budded cells and unbudded cells. At 30°C the DNA content of *yku70* is similar to WT cells. After shifting to 37°C, while WT cells showed normal distribution between G_1 and G_2 DNA content, *yku70* showed DNA content greater than the normal G_2 level. *yku70Δ* cells activate the DNA damage signalling pathway when grown at 37°C and this is suppressed partly by overexpression of *EST2* and *TLC1* (telomerase components) or by deletion of *RAD9* and *RAD24*, thus allowing growth at elevated temperatures (Teo and Jackson, 2001). The reason for this ts phenotype of *yku70* is not completely understood although it appears to be linked to DNA damage at the telomeres.

3.2 Results

3.2.1 Multi-copy suppressor screen

Multi-copy suppressor screen was done for genes, that when over expressed suppress the *yku70* ts phenotype at 35°C. We have constructed *yku70* knock-out by replacing the complete *YKU70* ORF with *KanMx* by PCR based homologous recombination method (Appendix 2.1, Longtine MS *et al*., 1998). *yku70* were transformed with a genomic library in YepLac181 (gift from K. Nasmyth) and plated at 35°C and incubated for 2 days. *yku70* form small colonies at this temperature. Any large colonies that were formed indicate that the ts of *yku70* might be suppressed. Schematic representation of the screen is shown in figure 4.

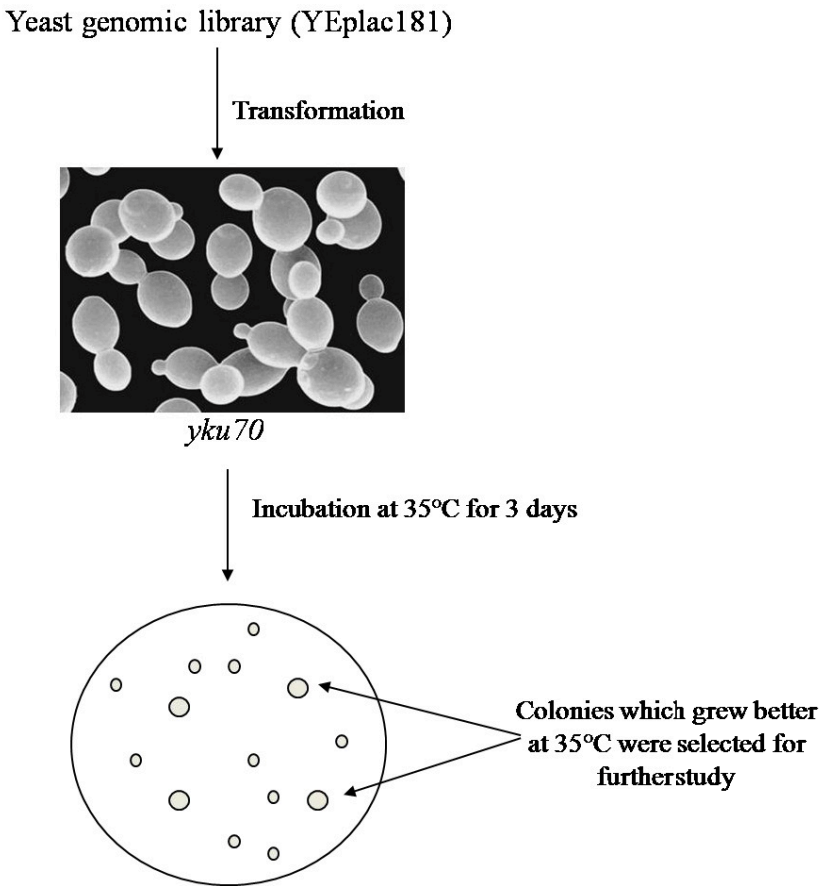


Figure 4: Schematic representation of multi-copy suppressor screen

From the screen four large colonies that appeared were picked up. In order to further confirm that the suppression of *yku70* ts phenotype is due to overexpression of library plasmids, we extracted the library plasmids from these four transformants that showed improved growth and re-transformed into *yku70* and WT strains. They were named KM93, KM94, KM95 and KM96. These transformants were grown overnight and the liquid cultures were serially diluted ten-fold. They were tested for growth by spotting on synthetic complete medium lacking leucine (SC-LEU) and incubated at 35°C for 2-3 days (Figure 5).

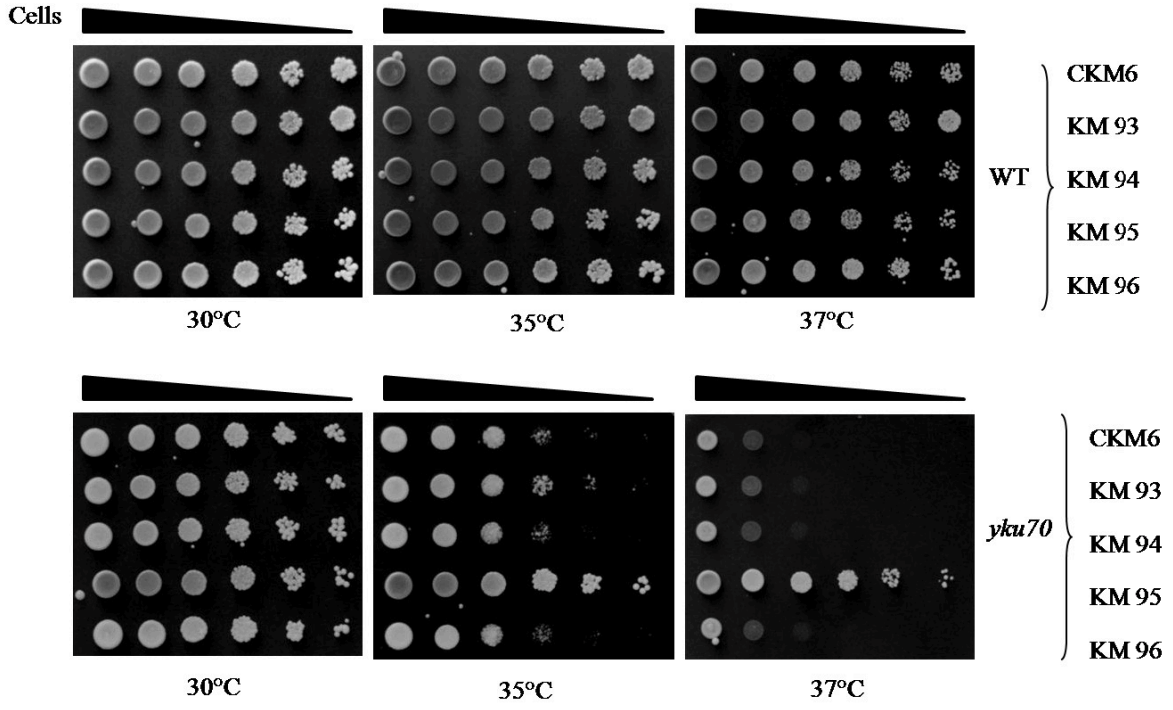


Figure 5: Suppressors of the *yku70* ts phenotype. WT (KRY193) and *yku70* (KRY172) were transformed with empty vector, KM93, KM94, KM95 and KM96 plasmids. 5µl of 10-fold serial dilutions of yeast cultures were plated on SC-LEU plates and incubated at 30°C, 35°C and 37°C for 2-3 days.

As seen in figure 5 WT cells grow normally at 30°C, 35°C and 37°C. *yku70* cells transformed with empty vector grow slowly at 35°C and died at 37°C. Upon overexpression of KM94 and KM96, there is no improvement in growth in *yku70* compared to empty vector overexpression (compare lanes 3 and 5 with lane 1). When KM93 is overexpressed, we can see partial suppression of ts phenotype of *yku70* at 35°C and this suppression is not seen at 37°C (compare lane 2 with lane 1). KM95 overexpression in *yku70* completely suppressed the ts phenotype at 35°C and 37°C (compare lane 4 with lane 1). Since KM94 and KM96 did not show any improvement in growth in *yku70* upon overexpression,

we did not pursue them. KM93 and KM95 plasmids were used for further study.

3.2.2 Identification of genes in the plasmids

In order to find out the gene(s) in the genomic insert of the plasmids that improved the growth of *yku70* at 35°C, we sequenced KM93 and KM95 plasmids using primers listed in Table 3. The DNA sequence thus obtained was compared with the yeast genome sequence in *Saccharomyces cerevisiae* genome database (SGD) (Cherry et al., 1998). The sequence in KM95 was found to contain the region of the chromosome XIII encompassing *YKU70*. So it is the *YKU70* gene present in KM95 plasmid that completely complemented the ts phenotype of *yku70.* The sequence in KM93 corresponded to the region of the chromosome IV encompassing C-terminal region of *NSE3, RTT103* and N-terminal region of *HRQ1* genes (Figure 6).

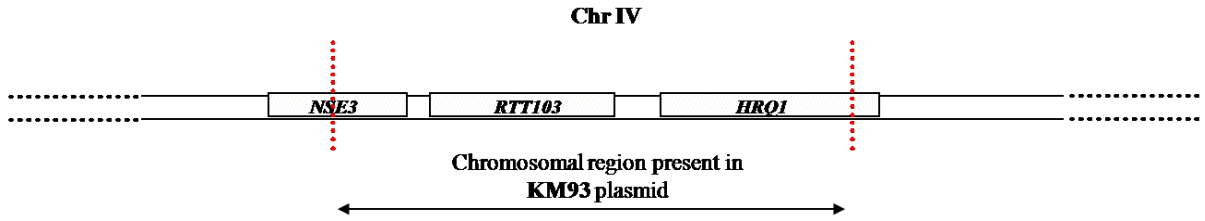


Figure 6: Schematic representation of chromosomal region present in KM93

3.2.3 Effect of overexpression of *RTT103* on ts phenotype of *yku70*

Since *RTT103* is the only complete gene present in the KM93 plasmid we tested whether *RTT103* overexpression leads to improved growth of *yku70* at 35°C. To this end, we first sub-cloned the genomic DNA fragment containing only *RTT103* in Yeplac181 (CKM6), a multi copy expression vector (Appendix 1.1). Temperature sensitivity was tested by transforming WT and *yku70* strains with either empty vector CKM6, KM93 or *RTT103* multi copy plasmid. The transformants were initially selected on leucine drop out medium to retain the plasmid in the strain. These transformants were then tested for temperature sensitivity assay by growing in SC- LEU broth overnight and then the liquid cultures were ten-fold serial diluted and 5µl was spotted on medium lacking leucine to test for improved growth of *yku70* upon overexpression of *RTT103*. The result of the experiment is shown in figure 7.

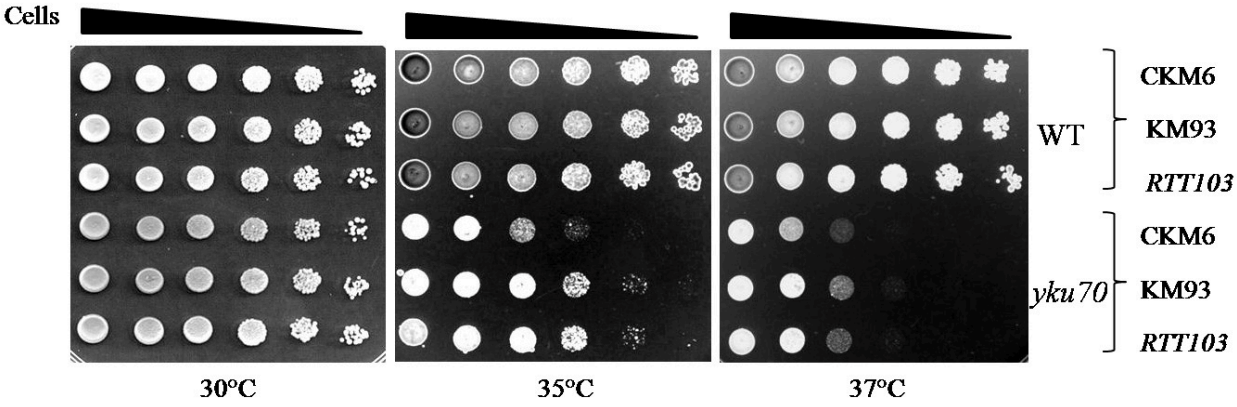


Figure 7: Suppression of the *yku70* ts phenotype by overexpression of *RTT103*. WT (KRY193) and *yku70* (KRY172) were transformed with empty vector (CKM6), KM93 and *RTT103* 2µ plasmids. 5µl of 10-fold serial dilutions of yeast cultures were plated on SC-LEU plates and incubated at 30°C, 35°C and 37°C for 2-3 days.

As seen in figure 7 overexpression of *RTT103* gene improved the growth of *yku70* at 35°C and this improved growth was equivalent to overexpression of library plasmid KM93 (lanes 5 and 6) as seen by better growth of transformants on leucine drop out medium in rows 5 and 6. This data confirms that *RTT103* partially suppresses the ts of *yku70*.

3.2.4 Quantification of temperature sensitivity of *yku70* upon overexpression of *RTT103*

Suppression of ts phenotype of *yku70* was quantified by plating around 200 cells from serially diluted cultures on SC- LEU medium. After 3 days of incubation at 30°C and 35°C, the colonies grown on both plates were counted. The fold sensitivity at 35°C for WT cells was taken as 1 and the sensitivity of *yku70* was calculated by dividing the number of colonies/ml at 35°C for WT by the number of colonies/ml at 35°C for *yku70* at 35°C. Table 5 shows the quantification values of three different experiments. The average value of these three experiments shows around 10-fold improvement in growth of *yku70* cells upon *RTT103* overexpression. Therefore *RTT103* overexpression suppressed the ts phenotype of *yku70*.

Strain	No of colonies/ml at 30°C	No of colonies/ml at 35°C	Fold sensitivity at 35°C
WT + CKM6	150 x10^7	124x10^7	1
	146 x10^7	120x10^7	1
	135 x10^7	119x10^7	1
WT + 2µ *RTT103*	135 x10^7	116x10^7	1
	142 x10^7	121x10^7	1
	143 x10^7	120x10^7	1
yku70 + CKM6	158 x10^7	3.5x10^7	35.4
	152x10^7	3x10^7	41.3
	145 x10^7	2.5x10^7	48
yku70 + 2µ *RTT103*	121 x10^7	25x10^7	4.6
	140 x10^7	26x10^7	4.6
	135 x10^7	30x10^7	3.8

Table 5: Quantification of temperature sensitivity of *yku70*. Quantification was done by plating around 200 cells on SC-LEU medium from the silencing assay experiment (Figure 7). After 3 days of incubation at 30°C and 35°C the colonies grown were counted and temperature sensitivity was quantified.

3.3 Summary

Yku70 is a multifunctional protein which is important protein for non-homologous end-joining of DNA repair and it plays multiple roles at the telomeres. Although it performs multiple functions, the mechanism of action is still poorly understood. We designed a genetic screen to isolate interacting partners that contribute to the multiple roles of Yku proteins.

In the screen *RTT103* gene was identified which when overexpressed leads to improved growth of *yku70* at 35°C. This improved growth is not seen at 37°C. So *RTT103* is able to partially overcome the defect of *yku70*Δ.

Chapter-4

Overexpression studies in *yku70/80*

4.1 Introduction

YKU70 is involved in several processes including DNA repair, telomere metabolism and gene silencing. Deletion of *YKU* subunits result in telomere shortening. In *yku*Δ telomeres are about one third the telomere length of WT cells. In *yku70/80*Δ cells ~65% of the $C_{1-3}A$ terminal telomeric repeat sequences are lost (Boulton and Jackson, 1996). When WT strain is shifted to 37°C telomere length does not change, but when *yku70/80* were shifted to the restrictive temperature it leads to further loss of telomeric repeats. So the death of *yku70/80* at 37°C might be due to the loss of telomeric repeats (Boulton and Jackson, 1998). Ku positively regulates telomere length by interacting with *TLC1*, which is the RNA subunit of telomerase (Stellwagen et al., 2003).

Ku also protects the telomeres from degradation and recombination. One of the important roles of Ku is to protect the telomeres from C-strand degradation by limiting the nuclease activity. It is reported that WT cells have long G-tails only in late S-phase, but *yku70/80* have long G-tails throughout the cell cycle (Gravel et al., 1998; Polotnianka et al., 1998).

Ku is required for telomere position effect (TPE). In many eukaryotes the adjacent genes of the telomeres are repressed, a phenomenon called TPE. Both Ku and *SIR* complex (*SIR2, SIR3, SIR4*) are essential for telomeric silencing, in addition to many other genes (Aparicio et al., 1991). Disruption of *YKU70/80* results in almost complete loss of TPE (Boulton and Jackson, 1998).

Ku is one of the important components of NHEJ pathway of DNA repair. When there is DNA DSB KU70/80 binds to the broken DNA ends. Disruption of *YKU70* or *YKU80* genes affects spontaneous recombination (Mages et al., 1996). *yku70/80* are sensitive to methyl methane sulphonate (MMS) (Feldmann et al., 1996; Milne et al., 1996), a drug which induces DNA DSBs directly or indirectly. Inactivation of *YKU70* or *YKU80* does not result in any detectable increase in sensitivity towards ultraviolet (Boulton and Jackson, 1996).

As *RTT103* is shown to suppress the ts phenotype of *yku70* (Chapter 3), we tested if multiple copies of *RTT103* also suppressed any of these phenotypes (Telomere length, telomere position effect and DNA damage sensitivity)

4.2 Results

4.2.1 Effect of overexpression of *RTT103* on telomere length of *yku70/80*

yku70/80 have shorter telomere length compared to WT. We wished to check if the telomere length of *yku70/80* will be altered when *RTT103* is overexpressed. *yku70* (KRY171), *yku80* (KRY225), *rif2* (KRY162) (used as a control which has longer telomere lengths compared to WT) and WT strains were transformed with either CKM6 or 2µ *RTT103*. The transformants were selected on leucine drop-out medium to retain the plasmids in the strain. The cultures were grown overnight in leucine dropout medium and genomic DNA was extracted from them. This DNA was then digested with the restriction enzyme *Xho*I and was subjected to southern blot-hybridisation using the

radiolabelled oligonucleotide poly(dG-dT)$_{20}$ that hybridises to the telomeric repeat elements (consensus $C_{1-3}A$) (Figure 8a) (Boulton and Jackson, 1996). *Xho*I cleaves within the sub-telomeric Y′ region that is found in many *S.cerevisiae* telomeres. In wild-type strains this digestion generates a chromosomal fragment of ~1.3 kb that hybridises to poly(G-T)$_{20}$.

The result of the experiment is shown in figure 8.

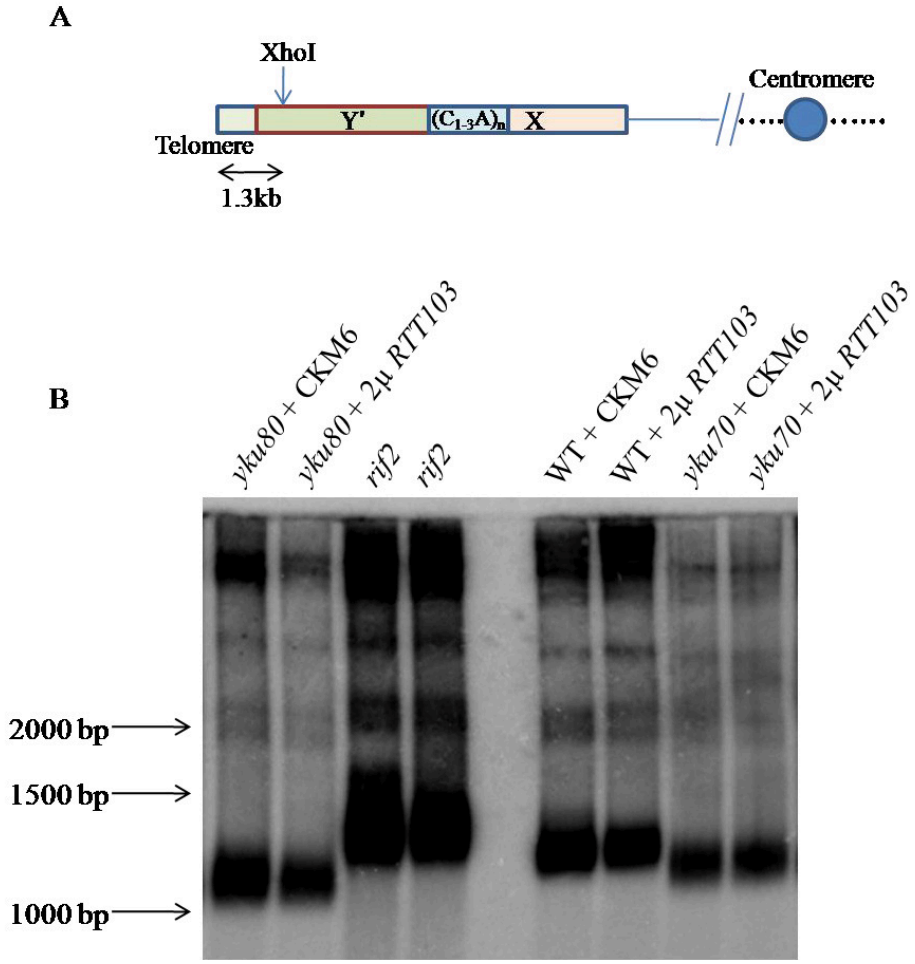


Figure 8: Effect of overexpression of *RTT103* on telomere length of *yku70/80*. **8a.** Schematic representation of a yeast chromosome showing the locations of the centromeric and telomeric regions, and the relative positions of the ($C_{1-3}A$) repeats, and the X and Y′ elements. **8b.** Southern blot showing telomere lengths of *yku70/80* under overexpression of *RTT103*.

As described previously and as shown in figure 8b, WT cells showed telomere length of approximately 1300bp, *yku70* and *yku80* strains have shorter telomere lengths than the WT cells. *rif*2 have longer telomeres compared to WT. When *RTT103* was overexpressed in either *yku70* or *yku80* there was no change in telomere lengths of these strains. So *RTT103* has no effect on the telomere length of *yku70/80.*

4.2.2 Effect of overexpression of *RTT103* on TPE of *yku70/80*

We examined the effect of overexpression of *RTT103* on transcriptional silencing at the telomeres of *yku70/80*. To test this we used yeast strains containing *URA3* gene integrated into the telomeric region of chromosome VII (Figure 9a) (Boulton and Jackson, 1998). WT, *yku70* and *yku80* strains were transformed either with empty vector CKM6 or with *RTT103* 2µ plasmids. The transformants were selected on leucine drop-out medium to retain the plasmids in the strain. Overnight grown cultures of these transformants were subjected to silencing assay by spotting on the medium containing 5-FOA to test the expression of *URA3* reporter at telomere VIIL. Expression of *URA3* makes the cell sensitive to 5-FOA and serves as a tool to test the status of silencing at telomeres.

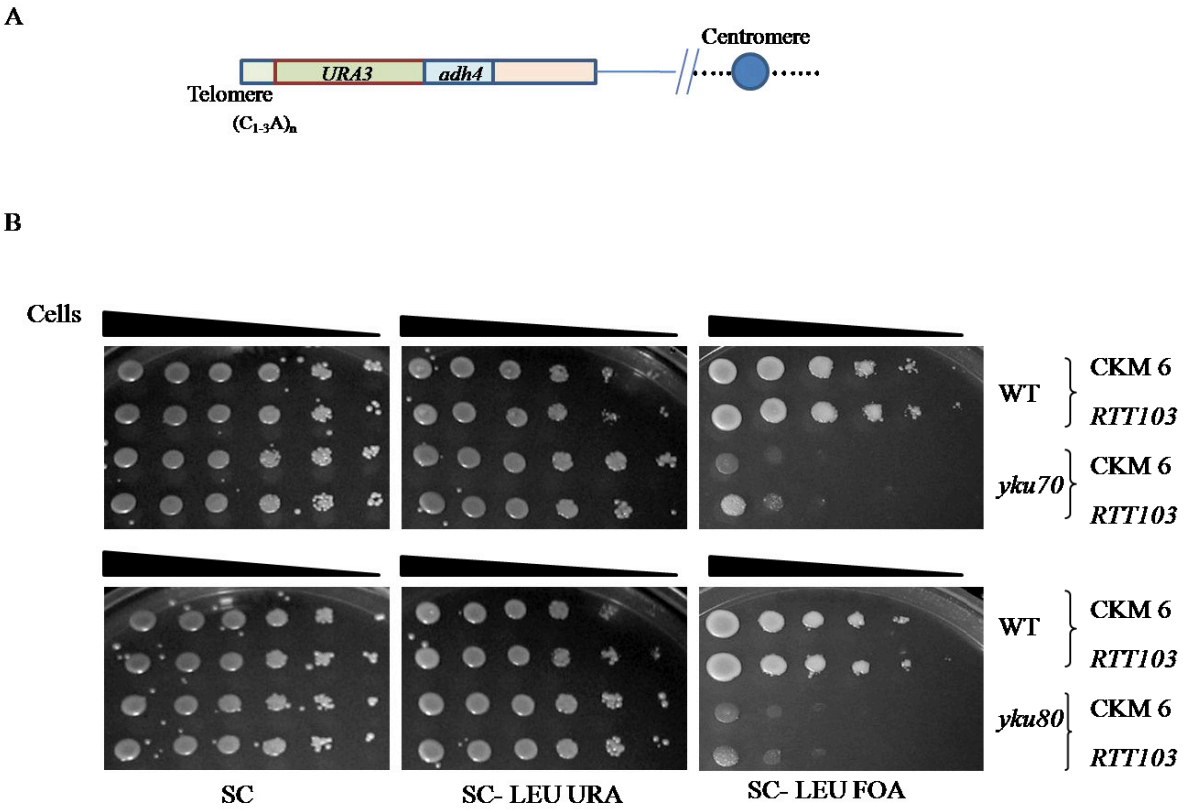


Figure 9: Effect of overexpression of *RTT103* on TPE of *yku70/80*. (9a) Schematic representation of the organization of the *URA3*-marked telomere **(9b)** WT **(**KRY193), *yku70* (KRY172) and *yku80* (KRY227) strains were transformed with CKM6 (empty vector) or *RTT103* 2µ plasmids. The transformants were initially grown in SC-LEU broth and 5µl of 10 fold serial dilution was spotted on SC, SC-LEU URA (control) and SC-LEU + 5-FOA (to test loss in TPE) plates.

The result in figure 9b shows that WT cells exhibit TPE and do not express *URA3*. So they grow either in the presence or absence of 5-FOA (lanes 1 and 2). Disruption of either *YKU70* or *YKU80* leads to loss in telomeric silencing as described earlier. So *URA3* is expressed, which makes the *yku70/80* sensitive to 5-FOA (lane 3), but the cells grow normally on SC-LEU URA plates. When *RTT103* was overexpressed in *yku70/80* there was no improvement in TPE and the growth on 5-FOA plate is similar to overexpression of empty vector

(compare lane 3 with lane 4). So *RTT103* overexpression does not have any effect on telomeric silencing in *yku70/80.*

4.2.3 Effect of overexpression of *RTT103* on DNA damage sensitivity of *yku70*

YKU70/YKU80 is involved in NHEJ pathway of DNA repair. It is known that *yku70/80* mutants are sensitive to MMS. MMS is a monofunctional DNA alkylating agent and a known carcinogen. It is referred to as IR-radiomimetic because the effect of this drug is similar to ionizing radiation (Snow and Korch, 1970). It primarily methylates DNA on *N*7-deoxyguanine and *N*3-deoxyadenine. Although the *N*7-methylguanine adduct may be nontoxic and non-mutagenic, *N*3-methyladenine is a lethal lesion that inhibits DNA synthesis and needs to be actively repaired. It induces DNA double-strand breaks during repair. We checked the MMS sensitivity of *yku70* upon *RTT103* overexpression.

WT and *yku70* strains were transformed either with empty vector (CKM6) or with *RTT103* 2µ plasmids. The transformants were selected on leucine drop-out medium to retain the plasmids in the strain. Overnight grown cultures of these transformants were subjected to MMS assay by spotting on medium lacking leucine and the plates containing increasing concentrations of MMS.

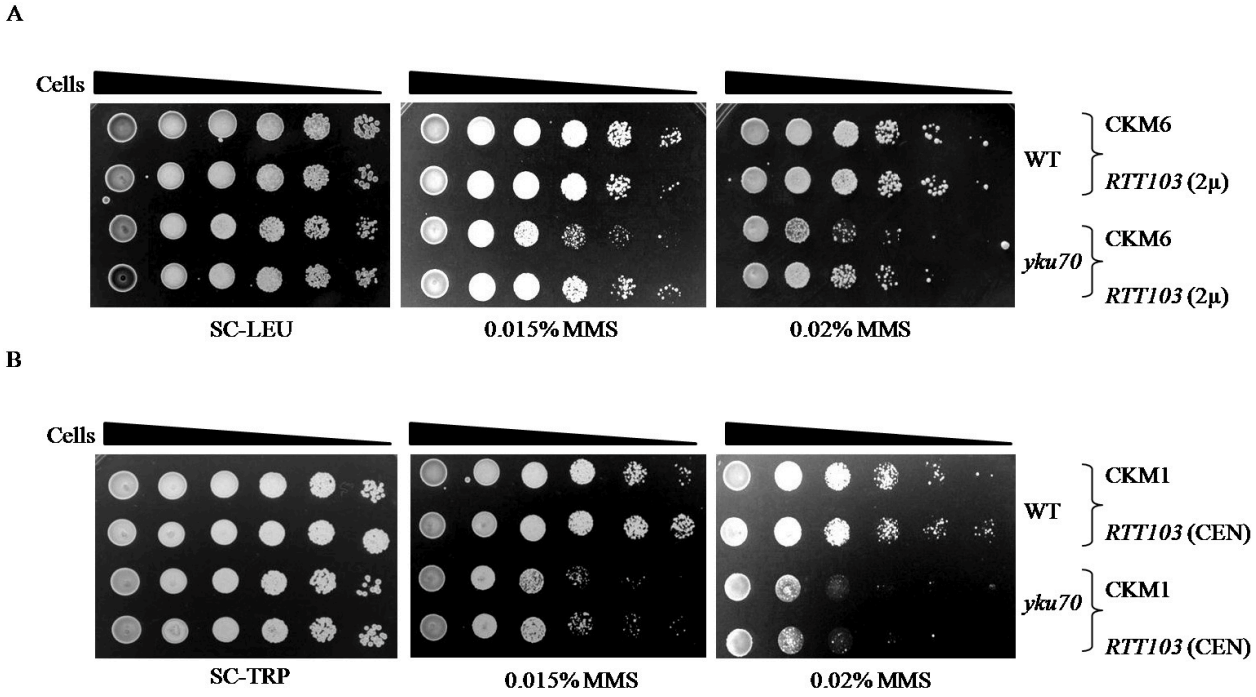


Figure 10: Effect of overexpression of *RTT103* on DNA repair of *yku70*. (10a) WT **(**KRY 193) and *yku70* (KRY172) strains were transformed with CKM6 (empty vector) or *RTT103* 2μ plasmids. The transformants were initially grown in SC-LEU broth and 5μl of 10 fold serial dilution was spotted on SC-LEU, SC-LEU + MMS plates with increasing concentrations of MMS. **(10b)** WT **(**KRY 193) and *yku70* (KRY172) strains were transformed with CKM1 (empty vector) or *RTT103* CEN plasmids. The transformants were initially grown in SC-TRP broth and 5μl of 10 fold serial dilution was spotted on SC-TRP, SC-TRP + MMS plates with increasing concentrations of MMS.

As seen in figure 10a, WT cells with empty vector and *RTT103* overexpression grow normally on leucine drop out plate and on plates containing increasing concentrations of MMS (lanes 1 and 2 of 10a). *yku70*Δ cells are sensitive to MMS compared to WT with empty plasmid overexpression at both 0.015% and 0.02% MMS concentration (compare lane 3 with lane 1 of 10a). When *RTT103* was overexpressed in *yku70* we can see an improvement in

growth at 0.015% and 0.02% MMS concentrations (compare lane 4 with lane 3 of 10a). So *RTT103* overexpression suppresses the DNA damage sensitive phenotype of *yku70.*

We now tested if multiple copies of *RTT103* are required for this suppression or single copy is enough. To this end, we first sub-cloned *RTT103* in YCplac22 (CKM1), a CEN vector (Appendix 1.2). WT and *yku70* strains were transformed either with empty vector (CKM1) or with *RTT103* CEN plasmids. The transformants were selected on tryptophan drop-out medium to retain the plasmids in the strain. Overnight grown cultures of these transformants were subjected to MMS assay by spotting on the medium lacking tryptophan and the plates containing increasing concentrations of MMS.

As seen in figure 10b WT cells transformed with empty vector and *RTT103* on CEN plasmid grow normally on tryptophan drop out plate and on plates containing increasing concentrations of MMS (lanes 1 and 2 of 10b). *yku70*Δ cells were sensitive to MMS compared to WT with empty plasmid overexpression at both 0.015% and 0.02% MMS concentrations (compare lane 3 with lane 1 of 10b). When *RTT103* was expressed on CEN plasmid in *yku70* we can see no improvement in growth at 0.015% and 0.02% MMS concentrations (compare lane 4 with lane 3 of figure 10b). The growth was similar with both empty vector and *RTT103* on CEN plasmid. So single copy *RTT103* could not suppresses the DNA damage sensitive phenotype of *yku70.*

4.2.4 Quantification of MMS sensitivity of *yku70* upon overexpression of *RTT103*

Suppression of MMS sensitivity phenotype of *yku70* was quantified by plating around 200 cells from serially diluted cultures on SC- LEU medium. After 3 days of incubation on SC-LEU and 0.015% MMS plates at 30°C, the colonies grown on both plates were counted. The fold sensitivity to MMS for WT cells was taken as 1 and the sensitivity of *yku70* was calculated by dividing the number of colonies/ml on 0.015% MMS plate for WT by the number of colonies/ml on 0.015% MMS plate for *yku70* at 30°C. Table 6 shows the quantification values of three different experiments. The average value of these three experiments shows around 8 fold improvement in resistance of *yku70* cells upon *RTT103* overexpression. Therefore *RTT103* overexpression suppressed the MMS sensitive phenotype of *yku70*.

Strain	No of colonies/ml at 30°C	No of colonies/ml at 0.015% MMS	Fold sensitivity compared to WT
WT + CKM6	152 x10^7	57x10^7	1
	128 x10^7	51x10^7	1
	134 x10^7	50x10^7	1
WT + 2µ *RTT103*	134 x10^7	54x10^7	1
	138 x10^7	59x10^7	1
	126 x10^7	53x10^7	1
yku70 + CKM6	159 x10^7	1.01x10^7	56
	146 x10^7	0.9x10^7	63
	154 x10^7	1.05x10^7	54
yku70 + 2µ *RTT103*	123 x10^7	7.2x10^7	7
	136 x10^7	8.1x10^7	7.3
	139 x10^7	7.9x10^7	7.4

Table 6: Quantification of MMS sensitivity of *yku70*. Quantification was done by plating around 200 cells on SC-LEU medium from the silencing assay experiment (Figure 10a). After 3 days of incubation at 30°C on 0.015% MMS plates the colonies grown were counted and sensitivity was quantified.

4.2.5 Effect of overexpression of *RTT103* on temperature sensitivity of *yku70*

As discussed earlier in chapter 3 *yku70*Δ cells are sensitive at 35°C and grow slowly. Upon overexpression of *RTT103* on 2µ vector this sensitivity was suppressed. We also wanted to check the ts of *yku70* when *RTT103* is expressed on CEN plasmid.

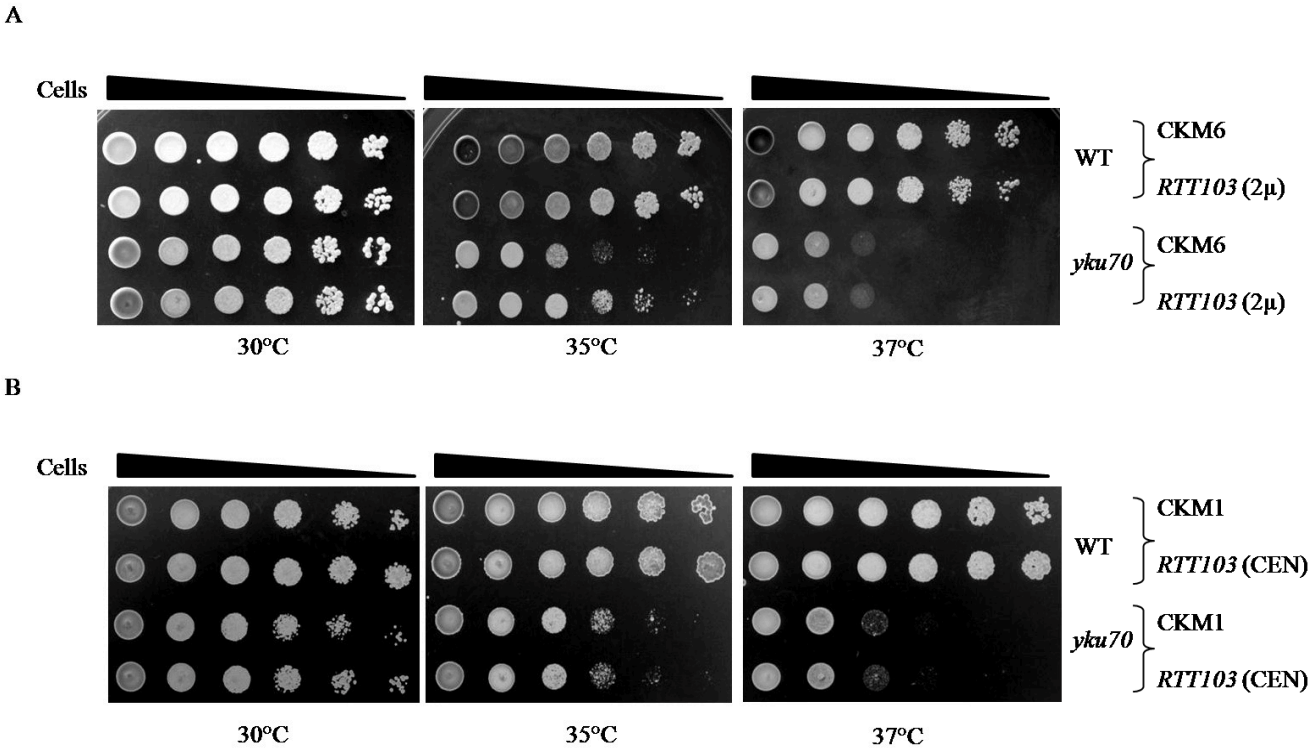


Figure 11: Effect of overexpression of *RTT103* on temperature sensitivity of *yku70*. (11a) WT **(**KRY193) and *yku70* (KRY172) strains were transformed with CKM6 (empty vector) or *RTT103* 2μ plasmids. The transformants were initially grown in SC-LEU broth and 5μl of 10 fold serial dilution was spotted on SC-LEU and incubated at 30°C, 35°C and 37°C for 2-3 days. **(11b)** WT **(**KRY193) and *yku70* (KRY172) strains were transformed with CKM1 (empty vector) or *RTT103* CEN plasmids. The transformants were initially grown in SC-TRP broth and 5μl of 10 fold serial dilution was spotted on SC-TRP and incubated at 30°C, 35°C and 37°C for 2-3 days.

As shown in chapter 3 and as seen in figure 11a, *RTT103* overexpression suppresses the ts phenotype of *yku70* (compare lanes 3 and 4 of 11a). Temperature sensitivity (with CEN vector) was tested by transforming WT and *yku70* strains with either empty vector (CKM1) or *RTT103* CEN plasmid. The transformants were initially selected on tryptophan drop out medium to retain

the plasmid in the strain. These transformants were then used to do temperature sensitivity assay by growing in SC- TRP broth overnight and then the liquid cultures were ten-fold serial diluted and 5µl was spotted on medium lacking tryptophan to test for improved growth of *yku70* upon expression of *RTT103* on CEN plasmid. The result of the experiment was shown in figure 11b. WT cells transformed with either empty vector or *RTT103* grow normally at all three temperatures (lanes 1 and 2). *yku70* with empty vector grow slowly at 35°C and inviable at 37°C. When *RTT103* was expressed on CEN plasmid improvement in growth was not observed in *yku70* at 35°C (compare lanes 3 and 4). So expression of *RTT103* on CEN vector did not suppress the ts phenotype of *yku70.*

4.3 Summary

In chapter 3 it was shown that *RTT103* overexpression suppressed the ts phenotype of *yku70*. As *YKU70* is involved in multiple functions, we have checked the other phenotypes of *yku70.*

*yku70*Δ cells have short telomeres and lose TPE. We have observed that upon overexpression of *RTT103* on multicopy vector, there is no change in either telomere length or TPE of *yku70*. *yku70*Δ cells are sensitive to DNA damaging agents like MMS, as *YKU70* is involved in NHEJ pathway of DNA repair. When *RTT103* is expressed on multicopy vector, we observed that the

resistance of *yku70* to MMS is increased. But when *RTT103* is expressed on CEN vector there is no change in the sensitivity of *yku70* to MMS. The improvement in growth of *yku70* at 35°C is also not seen upon expression of *RTT103* on CEN vector. So we conclude that the suppression of ts and MMS sensitivity of *yku70* requires multiple copies of *RTT103* and single copy is not enough for this suppression.

Chapter-5

Characterization of *RTT103*

5.1 Introduction

RTT103 (**R**egulator of **T**y1 **T**ransposition) was initially isolated in a screen for mutants that enhance the transposition of Ty1 elements (Scholes et al., 2001). Ty elements of *Saccharomyces cerevisiae* are retrotransposons whose life cycles are similar to those of retroviruses. These transposons transpose via an RNA intermediate which is converted to dsDNA and then integrated into the genome of the host. In *rtt103* there is approximately 13 fold increase in the mobility of Ty1 elements compared to wt. *RTT103* has an RPR domain (also known as C-terminal domain interacting domain or CID) which is present in several proteins involved in regulation of nuclear pre mRNA (Doerks et al., 2002). An *RTT103* deletion strain is viable. *RTT103* has homologues in higher eukaryotes, and a mouse homologue (BC021395/Q8VD54) was also found. It was reported that Rtt103 copurifies with Rat1 and Rai1proteins (Kim et al., 2004). Rat1 is a nuclear 5′ to 3′ exoRNase. Rai1 is a non-essential protein that co-purifies with Rat1 and enhances Rat1 activity in vitro. While *rat1-1* and *rai1* show transcription termination defects, *rtt103*Δ do not show any termination defects. However, it was shown that Rtt103, Rat1 and Rai1 crosslink strongly at 3′ ends of protein coding genes. Rtt103 interacts with ser2 phosphorylated CTD of RNA PolII and through its interaction with Rat1 and Rai1 is thought to promote transcription termination (Kim et al., 2004). *rtt103* were also reported to increase the number of Rad52 foci in unperturbed cells (Alvaro et al., 2007). In response to DNA damage, Rad52 proteins relocalize into discrete subnuclear

foci. *rtt103* is synthetically sick with *dna2*, a mutant that is sensitive to multiple forms of DNA damage (Budd et al., 2005). *rtt103* was also found to produce growth defects in synthetic combinations with condensins (Waples et al., 2009). *rtt103* is synthetically sick with *orc2-1* and *orc5-1* (Suter et al., 2004). Rtt103 is shown to genetically interact with Iwr1, an RNA PolII interacting factor (Krogan et al., 2006).

All the above mentioned reports indicate that *RTT103* plays a role in maintaining genome integrity. Following our observation that *RTT103* suppressed the ts phenotype and improved the resistance of *yku70* to DNA damaging agent, MMS, we further investigated if *RTT103* is essential for recovery from DNA damage.

5.2 Results

5.2.1 Telomere length of *rtt103*

We have constructed *rtt103* knock-out by replacing the complete *RTT103* ORF with *KanMx* by PCR based homologous recombination method (Appendix 2.2, (Longtine et al., 1998). *rtt103yku70* strain was also constructed by crossing the single deletion strains. WT, *rtt103, yku70* and *rtt103yku70* strains were grown overnight in YPD medium and genomic DNA was extracted from them. This DNA was then digested with the restriction enzyme *Xho*I and was subjected to southern blot-hybridisation using the radiolabelled oligonucleotide

poly(dG-dT)$_{20}$ that hybridises to the telomeric repeat elements (consensus $C_{1-3}A$). The result of the experiment is shown in figure 12.

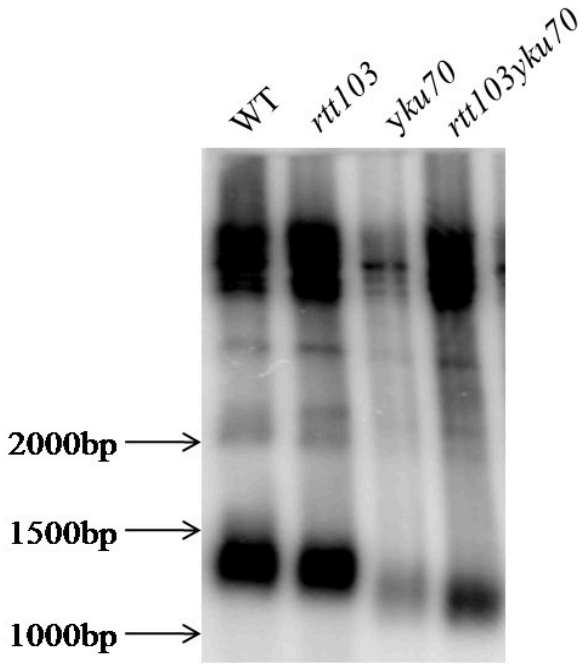


Figure 12: Telomere length of *rtt103* and *rtt103yku70*. Southern blot showing telomere lengths of WT (KRY105), *rtt103* (KRY230), *yku70* (KRY171) and *rtt103yku70* (KRY290).

As described previously and as shown in figure 12, WT cells have telomere length of approximately 1300bp. *rtt103* have telomere lengths similar to that of WT strain and *rtt103yku70* have length similar to *yku70*. So telomere length is not altered when *RTT103* is deleted.

5.2.2 Telomere silencing in *rtt103*

We examined the effect of *RTT103* deletion on transcriptional silencing at the telomeres. To test this we have used yeast strains containing *URA3* gene integrated into the telomeric region of chromosome VII. WT, *yku70, rtt103* and

rtt103yku70 strains were grown overnight in YPD and the grown cultures were subjected to silencing assay by spotting on the medium containing 5-FOA to test the expression of *URA3* reporter at telomere VIIL. The result of the experiment is shown in figure 13.

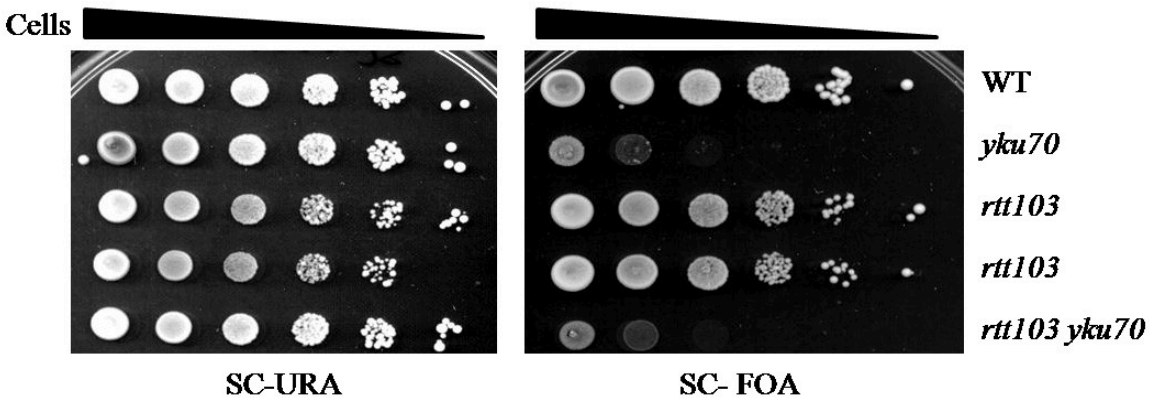


Figure 13: Telomeric silencing in *rtt103*. WT **(**KRY193), *yku70* (KRY172), *rtt103* (KRY284, KRY285) and *rtt103yku70* (KRY286) strains were grown in YPD broth and 5µl of 10 fold serial dilution was spotted on SC- URA (control) and SC + 5-FOA (to test loss in TPE) plates.

The result in figure 13 shows that WT cells exhibit TPE and do not express *URA3*. So they will grow either in the presence or absence of 5-FOA (lane 1). Disruption of *YKU70* leads to loss in telomeric silencing as described earlier. So *URA3* will be expressed which makes the *yku70* sensitive to 5-FOA (lane 2), but the cells will grow normally on SC-URA plates. *rtt103*Δ cells showed growth similar to WT (lanes 3 and 4). The growth of *rtt103yku70* is similar to *yku70* (compare lane 5 with lane 2). This experiment shows that loss of *RTT103* has no effect on silencing at the telomeres.

We also assayed telomeric silencing by *ADE2* marker. To test this we have used yeast strains containing *ADE2* gene integrated into the telomeric region of chromosome VII (Figure 14a).

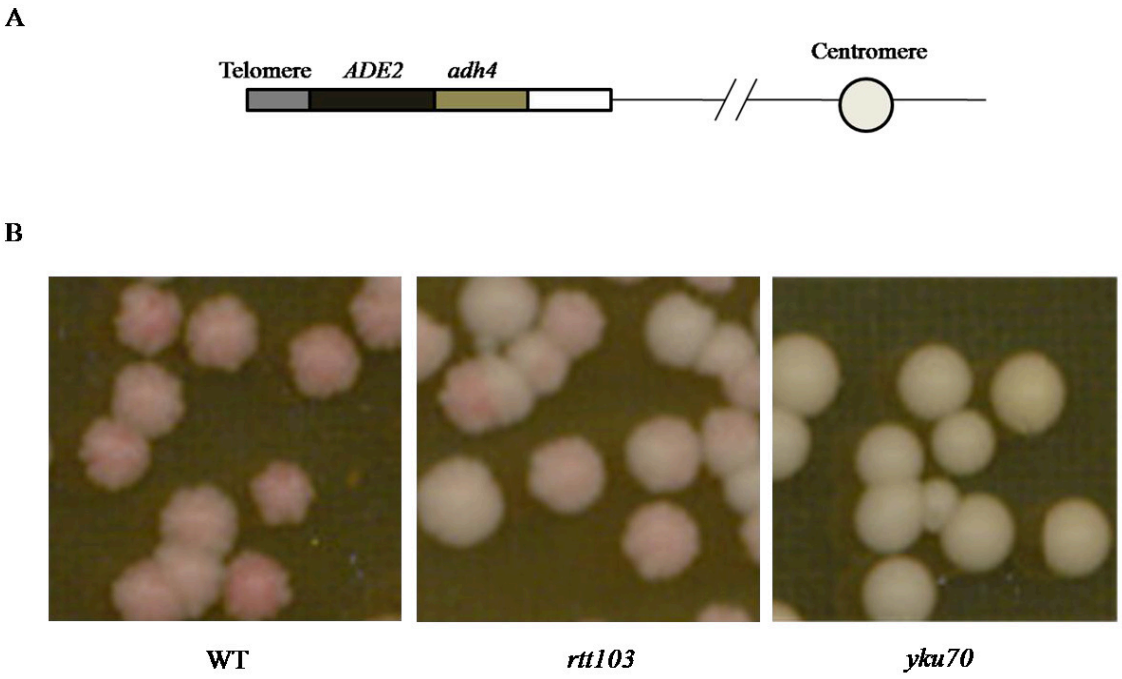


Figure 14: Telomeric silencing in *rtt103*. (14a) Schematic representation of the organization of the *ADE2*-marked telomere. **(14b)** WT **(**KRY105), *rtt103* (KRY230) and *yku70* (KRY171) strains were inoculated and plated on YPD plates to get individual colonies.

WT, *rtt103* and *yku70* strains were inoculated in YPD and about 200 cells of each strain were spread on YPD plates. The plates were then incubated at 30°C for about 4-5 days. Then they were kept at 4°C for one week and pictures were taken. As seen in figure 14b, WT cells showed variegated expression of *ADE2* which results in the formation of red and white sectored colonies. WT cells exhibit TPE due to which *ADE2* gene which is placed near the telomeres is

silent. When *ADE2* is not expressed it leads to the accumulation of an upstream substrate which polymerizes to form a red pigment. *yku70* was taken as control, which forms white colonies due to the loss of TPE. Red pigment does not accumulate in these cells as *ADE2* was expressed. *rtt103* showed variegated expression and showed pink and white colonies. But these cells were light pink in colour compared to WT. Although the previous experiment (Figure 13) showed that *rtt103*Δ cells did not have any affect on telomere silencing, this experiment shows that there is a slight loss in silencing in *rtt103*. The difference in two markers is that the 5-FOA selection, being a growth assay, is less sensitive to small changes in TPE whereas ADE2, where growth is not affected, is more sensitive to slight changes in TPE. Therefore we conclude that *rtt103* leads to very slight reduction in TPE.

5.2.3 Temperature sensitivity in *rtt103*

Following our observation that elevated *RTT103* dosage could partially suppress temperature sensitivity of *yku70,* we wanted to check if *rtt103* will show sensitivity to temperature. WT, *yku70*, *rtt103* and *rtt103yku70* strains were grown overnight in YPD and then the liquid cultures were ten-fold serial diluted and 5µl was spotted on YPD medium. The result of the experiment is shown in figure 15. WT cells grew normally at all three temperatures (lanes 1 and 2). *yku70* grew slowly at 35°C and was inviable at 37°C (lanes 3 and 4). *rtt103* did not show any sensitivity to temperature either at 35°C or 37°C (lanes 5 and 6). *rtt103yku70* double deletion strain showed sensitivity to temperature at

35°C as reflected by the poor growth and this sensitivity is more than *yku70* (compare lanes 7 and 8 with 3 and 4). So *rtt103* exacerbates the temperature sensitivity of *yku70*.

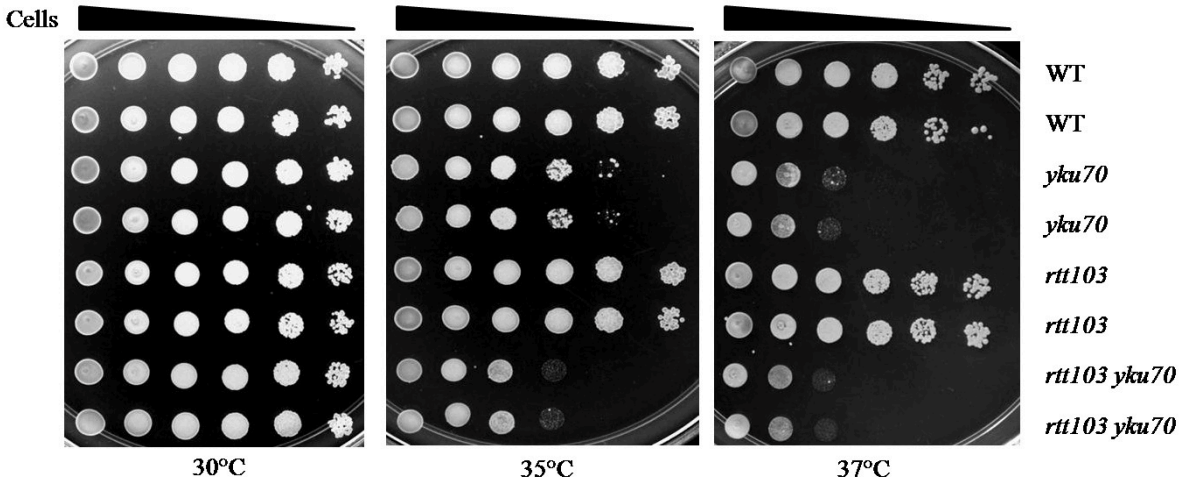


Figure 15: Temperature sensitivity of *rtt103*. WT **(**KRY193), *yku70* (KRY172), *rtt103* (KRY284) and *rtt103yku70* (KRY286) strains were grown in YPD broth and 5µl of 10 fold serial dilution was spotted on YPD in duplicates and incubated at 30°C, 35°C and 37°C for 2-3 days.

5.2.4 DNA damage sensitivity in *rtt103*

5.2.4.1 Sensitivity to MMS

In chapter 4 it was shown that *RTT103* overexpression suppressed the MMS sensitivity of *yku70*. *YKU70/YKU80* is involved in NHEJ pathway of DNA repair. So *yku70/80* are sensitive to MMS. From the data available in the literature and from the overexpression studies it can be said that *RTT103* is involved in maintaining genome stability. In order to check if *RTT103* has any

role in DNA damage response we directly tested the sensitivity of *rtt103* to DNA damage. So we checked the MMS sensitivity of *rtt103*.

WT, *rtt103*, *yku70* and *rtt103yku70* strains were grown overnight and cultures were subjected to MMS assay by spotting on YPD plates and the plates containing increasing concentrations of MMS.

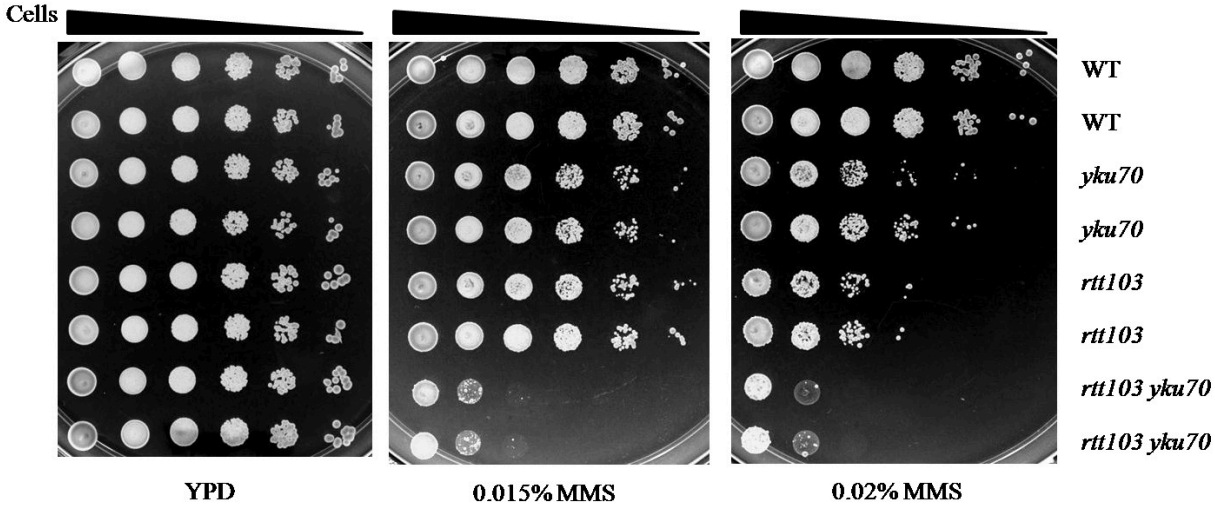


Figure 16: MMS sensitivity of *rtt103*. WT **(**KRY193), *yku70* (KRY172), *rtt103* (KRY284) and *rtt103yku70* (KRY286) strains were grown in YPD broth and 5µl of 10 fold serial dilution was spotted on YPD and YPD + MMS plates in duplicates and incubated at 30°C for 2-3 days.

As seen in figure 16, WT cells grow normally on YPD plate and on plates containing increasing concentrations of MMS (lanes 1 and 2). *yku70* were sensitive to MMS compared to WT at both 0.015% and 0.02% MMS concentration (compare lanes 2 and 3 with lanes 1 and 2). *rtt103* were even more

sensitive than *yku70* cells (compare lanes 5 and 6 with 3 and 4). *rtt103yku70* were more sensitive to MMS than either of the single mutants (compare lanes 7 and 8 with 3, 4 and 5, 6). *rtt103* exacerbates the DNA damage sensitivity of *yku70*. The result of this experiment indicates that *RTT103* is important for maintaining genome stability.

5.2.4.2 Quantification of MMS sensitivity of *rtt103*

MMS sensitivity of *rtt103* was quantified by plating around 200 cells from serially diluted cultures on YPD medium. After 3 days of incubation on YPD and YPD + 0.015% MMS plates at 30°C, the colonies grown on both plates were counted. The fold sensitivity to MMS for WT cells was taken as 1 and the sensitivity of the strains to MMS was calculated by dividing the number of colonies/ml on 0.015% MMS plate for WT by the number of colonies/ml on 0.015% MMS plate for the respective strain at 30°C. Table 7 shows the quantification values of three different experiments. The average value of these three experiments shows that *rtt103* were around 95 fold more sensitive to MMS than WT at 0.015% MMS and *yku70* were around 45 fold more sensitive. *rtt103yku70* were approximately 700 fold more sensitive compared to WT. These data indicate that *rtt103* enhances the *yku70* defect phenotypes in a synergistic manner.

Strain	No of colonies/ml at 30°C	No of colonies/ml at 0.015% MMS	Fold sensitivity compared to WT
WT	180×10^7	65×10^7	1
	198×10^7	78×10^7	1
	189×10^7	67×10^7	1
yku70	205×10^7	1.8×10^7	43.3
	190×10^7	1.5×10^7	44.6
	191×10^7	1.2×10^7	55
rtt103	185×10^7	0.7×10^7	92.8
	189×10^7	0.8×10^7	97.5
	201×10^7	0.8×10^7	97.5
rtt103yku70	180×10^7	0.09×10^7	722
	210×10^7	0.12×10^7	650
	186×10^7	0.12×10^7	744

Table 7: Quantification of MMS sensitivity of *rtt103*. Quantification was done by plating around 200 cells on YPD medium from the MMS experiment (Figure 16). After 3 days of incubation at 30°C on 0.015% MMS plates the colonies grown were counted and sensitivity was quantified.

5.2.4.2 Sensitivity to *Eco*RI endonuclease

Since *rtt103* showed sensitivity to MMS, we also checked the sensitivity of *rtt103* to DNA damage by introducing many DNA DSBs in the genome by *Eco*RI endonuclease. This was done by transforming YCpGal plasmid containing the *Eco*RI endonuclease gene under the control of galactose promoter. This plasmid was transformed into WT, *yku70, rtt103* and *rtt103yku70* strains. The plasmid contains *URA3* selectable marker. After transformation the transformants were selected on SC-URA plates. Colonies which were grown on

these plates after incubation were again restreaked on SC-URA plates. The colonies were then grown overnight in SC-URA broth. The liquid cultures were ten-fold serial diluted and 5µl was spotted on SC-URA (for maintaining the plasmid) and SC-URA GAL plates (for induction of *Eco*RI endonuclease). The result of the experiment is shown in figure 17.

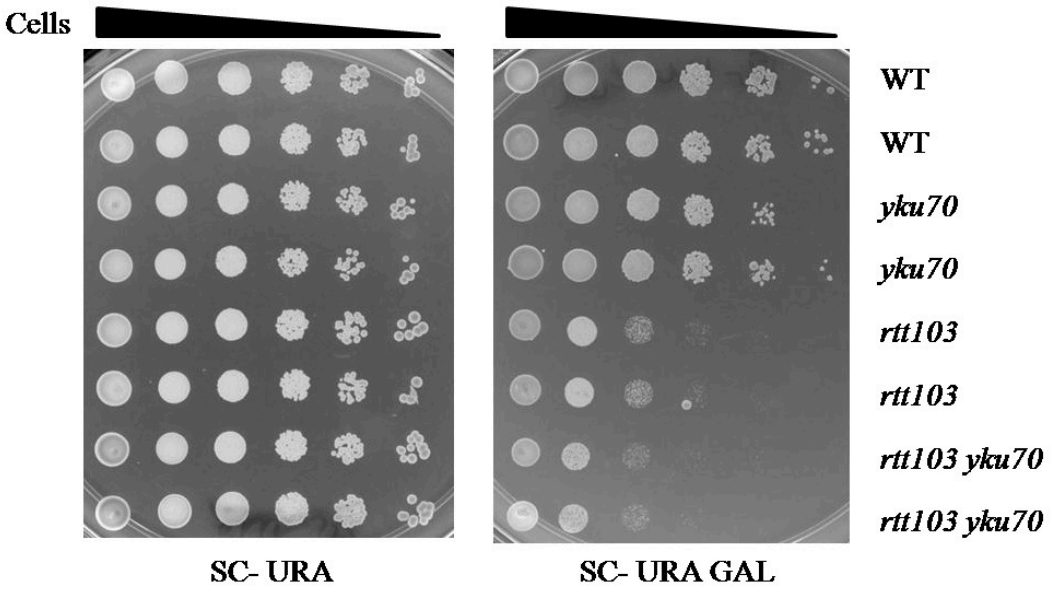


Figure 17: Sensitivity of *rtt103* to *Eco*RI endonuclease: WT (KRY105), *yku70* (KRY171), *rtt103* (KRY230) and *rtt103yku70* (KRY290) strains transformed with YCpGal plasmid were 10 fold serial diluted and spotted on SC-URA and SC-URA GAL plates.

As seen in figure 17, WT cells grow normally on galactose plates (lanes 1 and 2). *yku70* were slightly more sensitive to endonuclease induction compared to WT (compare lanes 3 and 4 with 1 and 2). *rtt103* were more sensitive than WT (compare lanes 5 and 6 with 1 and 2) and *rtt103yku70* were also similar in

sensitivity as *rtt103.* This experiment also shows that *RTT103* has a critical role in responding to DNA damage induced by *Eco*RI.

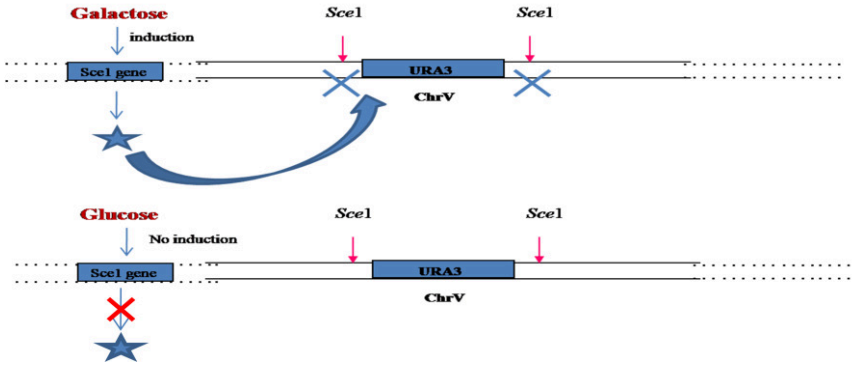
5.2.4.3 Sensitivity to *Sce-I* endonuclease

As MMS and *Eco*RI endonuclease generate multiple DNA breaks all over the genome, and could potentially affect several genome functions, we next tested if *RTT103* is required when single or two breaks are specifically introduced. In order to do this, we used a strain where two sites recognized by *I-SceI* endonuclease have been introduced bracketing *URA3* gene on chromosome V (see schematic in figure 18a) (Marcand et al., 2008). The two sites were in opposite orientation. The sequence encoding the nuclease was inserted at a different locus and placed under the control of galactose inducible promoter.

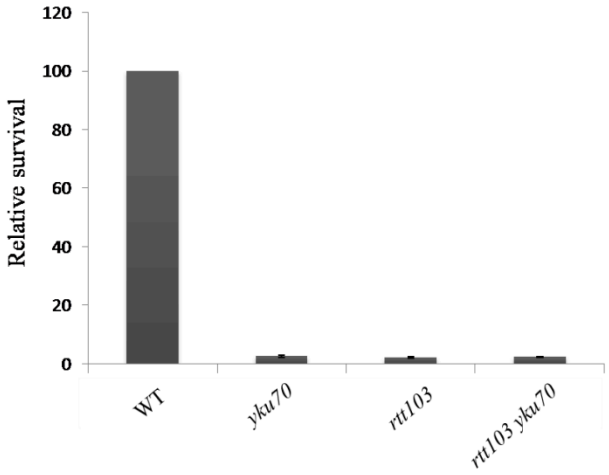
To this end we generated *yku70* and *rtt103* deletions in the strain described above and tested the efficiency of colony formation on galactose plates. When *I-SceI* endonuclease is induced by galactose, breaks were generated on either side of *URA*3 gene. When the cells were grown on glucose medium the endonuclease will not be produced. Wild type cells can repair the break and form colonies or under continuous induction, repair with mutations (usually loss of a couple of nucleotides) and therefore lose the recognition site and become resistant to further digestion by the enzyme (Figure 18b). The strains that cannot repair the DNA break were growth arrested, and were not able to form colonies on galactose plates. We tested wild type, *yku70*, *rtt103* and

yku70rtt103 deletions carrying the *I-SceI* sites and endonuclease for recovery from *I-SceI* cut by plating on plates containing galactose. Numbers of full sized colonies that appeared after 3 days were counted. As shown in figure 18b, *yku70*, *rtt103* and *yku70rtt103* deletions were all severely affected and very few colonies could be recovered. However, wild type cells were able to form colonies as they could repair the breaks at the expected frequency. We noticed that even though *rtt103* deletions did not form many full-sized colonies, there were numerous tiny colonies in this single mutant (Figure 18c). This suggests that *rtt103*, unlike *yku70* cells underwent a few divisions before they succumbed. Based on the data we conclude that *RTT103* is required for repair of specific induced DSBs.

A


Galactose
induction
Sce1 gene
Sce1
Sce1
URA3
ChrV
Glucose
No induction
Sce1 gene
Sce1
Sce1
URA3
ChrV

B


Relative survival
120
100
80
60
40
20
0
WT
yku70
rtt103
rtt103 yku70

C

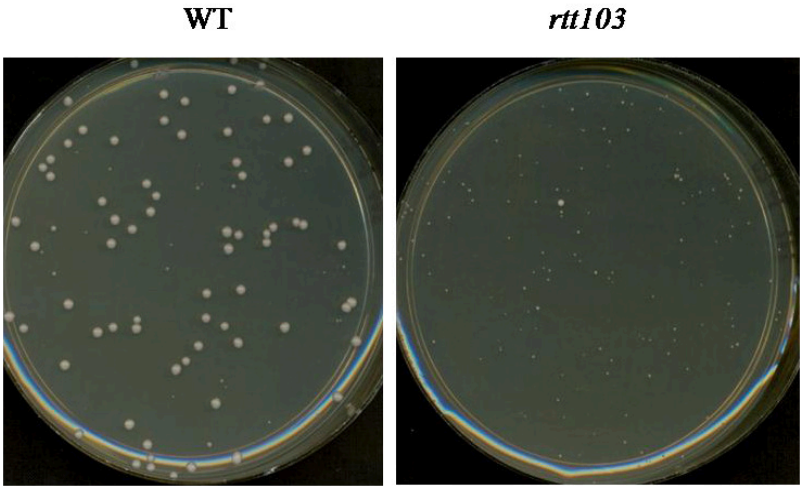

WT
rtt103

Figure 18: Sensitivity of *rtt103* to induced DNA DSBs. (18a) Schematic representation of the *URA3* region showing *I-SceI* site and its mode of action. **(18b)** Strains of WT (KRY304), *yku70* (KRY376), *rtt103* (KRY375) and *rtt103 yku70* (KRY379) with two *Sce*-I sites on either side of the *URA3* gene were induced with galactose to produce DSBs. The relative survival on galactose versus glucose was calculated from three independent cultures for each strain and error bars show SD. **(18c)** Strains of WT (KRY304) and *rtt103* (KRY376) were plated on galactose plates and the tiny colonies that were formed on *rtt103* after 3 days of incubation were shown.

5.2.5 *RTT103* is not required for plasmid end-joining

As a specific double strand break assay indicated that *RTT103* was required to repair these breaks in vivo, we directly tested if end joining is affected in *rtt103* by plasmid rejoining assay. This assay reports the efficiency of end-joining by NHEJ. When linearized CEN plasmids are transformed into yeast, they are recircularized by end joining (most efficient) or are integrated into the genome (less efficient). However, when end-joining is defective as seen in *yku*70/80 mutants or DNA ligaseIV mutants (Milne et al., 1996), very few transformants are recovered. Equal amount of pRS313 plasmid (containing *HIS3*) was digested with *Eco*RI to completion. The linearized DNA was then transformed into wild type, *yku70*, *rtt103* and *yku70rtt103* deletions by lithium acetate method and the transformants were quantified. Parallel transformations with equal amount of supercoiled version of the same plasmid were performed to enable normalization for minor differences in transformation efficiencies between strains and between experiments. Cells were plated and colonies arising on SC-HIS plates were counted after 2-3 days incubation at 30°C. Since

the linearized plasmid should be recircularized in order to be propagated, the number of transformants obtained with the linear plasmid normalized to the number obtained with the supercoiled plasmid provides us the data about ability of the yeast strain to mediate repair of the DSB produced by the restriction enzyme. The data are represented as fraction of linear plasmid recovered relative to the supercoiled plasmid. As expected in wild type cells a high number of transformants were obtained which indicates repair with high efficiency of approximately 70% and in the *yku70* very few could be recovered as this protein is involved in the repair of the DSBs (Figure 19). In *rtt103* the levels were comparable to wild type suggesting that *RTT103* is not required for end-joining of plasmids.

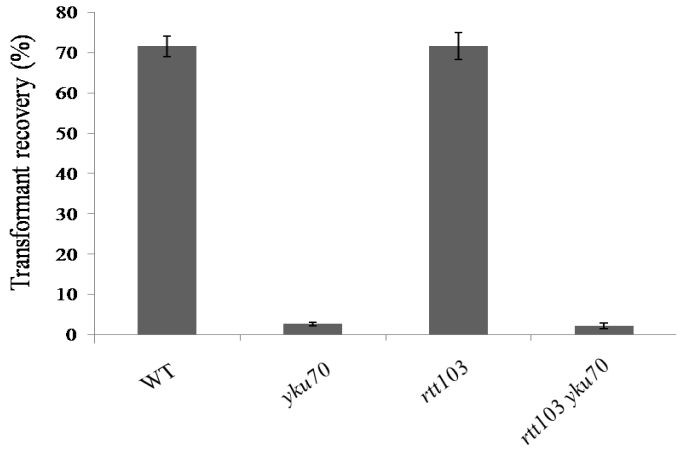


Figure 19: *RTT103* is not required for end-joining of plasmid. WT (KRY105), *yku70* (KRY171), *rtt103* (KRY230) and *rtt103yku70* (KRY290) strains were transformed with supercoiled or linearized pRS313. The transformants were plated on SC-HIS plates in duplicates and incubated at 30°C for 2-3 days. The value plotted was the percentage of linear plasmid recovered relative to supercoiled plasmid for each strain from three independent transformation experiments.

5.2.6 *rtt103* cells are not sensitive to UV damage

We also tested to see if *rtt103* were sensitive to damage by UV irradiation. The strains were grown overnight in YPD broth and the cultures were then serial diluted and spotted on YPD plates. The plates were then exposed to 254nm UV light in a transilluminator. They were wrapped in aluminium foil and incubated at 30°C for 2-3 days. As controls *rad1* was used that is extremely sensitive to UV radiation and *yku70* that is not sensitive to UV. We found that even though *rad1* were sensitive to UV, *rtt103* were not (Figure 20) and were quite indistinguishable from wild type. These results indicate that *RTT103* is required for recovery from MMS or endonuclease digestion, but not from UV induced damage. In sum the data described above show that *RTT103* is required for repair of chromosomal breaks although could be dispensable for UV damage.

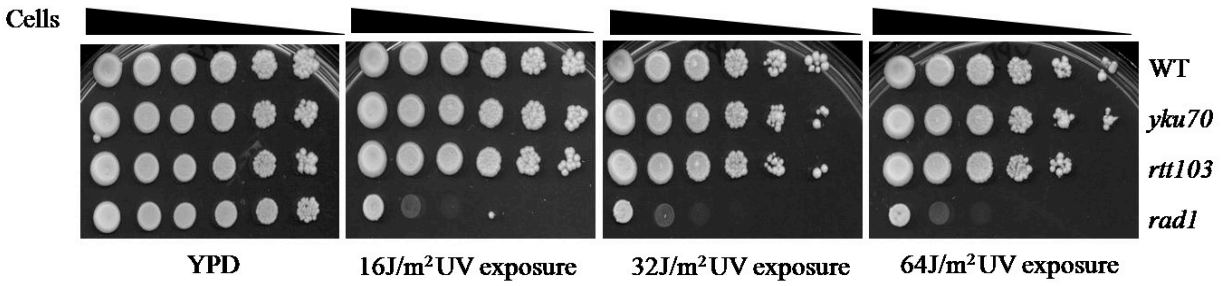


Figure 20: *RTT103* is not required for repairing UV damage. Wild type (KRY105), *yku70 (*KRY171), *rtt103 (*KRY230) and *rad1* (KRY473) strains were grown to mid log phase, 10-fold serially diluted and spotted on YPD plates. They were then exposed to UV radiation and incubated at 30°C for 2 days.

5.2.7 *RTT103* deletion strains are defective in sporulation

As all these experiments indicated that *RTT103* is essential for efficient repair of DNA damage, we tested if the naturally occurring double-strand breaks also require *RTT103*. When yeast cells undergo meiosis, an early step in prophase I, is introduction of several double-stranded DNA breaks by Spo11, many of which initiate recombination events and others that are repaired without recombination. In order to test if *RTT103* has any effect on the repair of these breaks we generated diploid *rtt103 / rtt103* cells and wild type diploid cells. These diploids were then plated on potassium acetate plates to induce sporulation. After 3 days of incubation on YPK plate at room temperature, the cells were picked and the number of spores was counted under the microscope. We obtained undetectable amounts of spores from the *rtt103/ rtt103* diploids (4 out of 800, 0.5%) although the wild types cells sporulated efficiently (35%) on the same plates. To further confirm this, we stained the cells with Dapi. As shown in figure 21, we could clearly see 4 nuclei in many wild type cells but in *rtt103* diploids there were none (upper panel). In order to confirm that the sporulation defect is actually because of absence of Rtt103p, we transformed empty vector and CEN or 2μ *RTT103* into *rtt103* diploids and checked the status of sporulation. In *rtt103* diploids which were transformed with empty vector, there is no detectable sporulation. However, *rtt103* carrying a single or multicpoy *RTT103* on a plasmid could sporulate as efficiently as the wild type cells (lower panel). We note that the genome-wide screen for meiosis and

sporulation reported that *rtt103* sporulated normally (Enyenihi and Saunders, 2003). However, we found that *rtt103 / rtt103* diploids obtained from BY4741 and BY4742 also was severely defective in sporulation (no spores at all) and this defect could be complemented by wild type *RTT103* on a plasmid. It is possible that *rtt103* escaped detection in the genome-wide study. These data indicate that *RTT103* is essential for successful meiosis, probably because it is essential to repair the induced double strand DNA breaks.

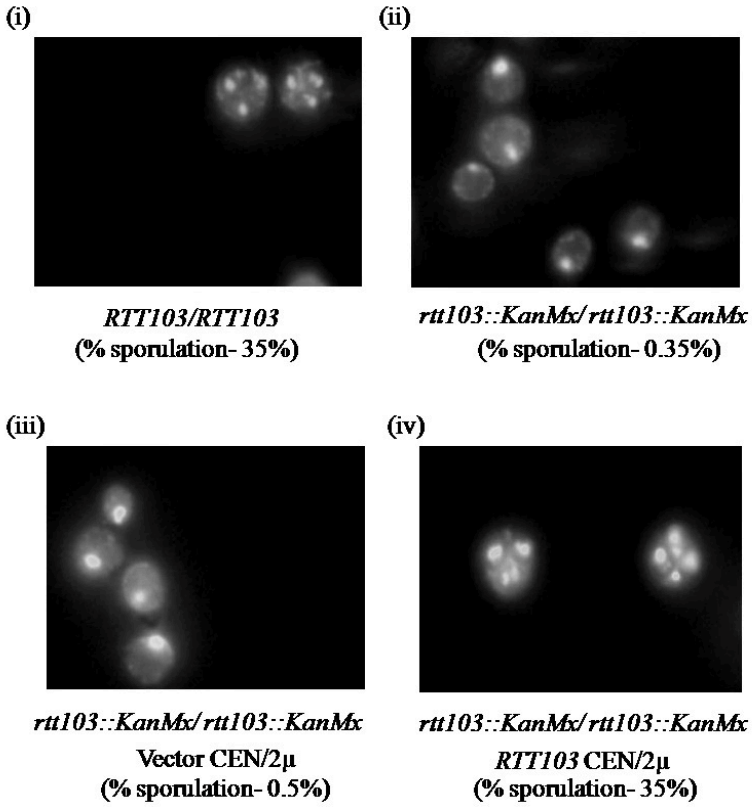


Figure 21: *rtt103* homozygous strains are severely defective in sporulation. WT **(i)** and *rtt103* homozygous diploids **(ii)** were incubated on YPK plates for induction of sporulation and stained with DAPI to visualize nuclei after 4 days. *rtt103* were transformed with either empty vector **(iii)** or with single copy/2μ of *RTT103* **(iv)** and incubated on YPK plate for 4 days. DAPI images from this stage were shown.

5.3 Summary

There is some indirect evidence from various genome-wide studies to suggest that *RTT103* might be involved in damage response and other chromosomal functions as Rtt103 interacts genetically with a plethora of genes involved in chromatin or genome stability (Cherry et al., 1998). For eg., *rtt103* show increased Rad52 foci, a mark of increased spontaneous damage (Alvaro et al., 2007).

In chapter 4 it was shown that *RTT103* overexpression suppressed the ts and MMS sensitive phenotype of *yku70*. This gave us a clue that *RTT103* is involved in DNA repair. So *rtt103* null mutants were generated to understand the function of *RTT103*. *rtt103* cells have telomere length similar to WT and there is no loss in telomere silencing. Though *rtt103* cells did not show any temperature sensitivity, this null mutation exacerbated the ts of *yku70. rtt103* are sensitive to DNA damaging agents like MMS and are sensitive to *Eco*RI endonuclease. The sensitivity of *yku70* to MMS is increased when *rtt103* is mutated. These experiments tell us that *RTT103* and *YKU70* function in parallel pathways for maintaining genome integrity. As in the above experiments the damage is done all over the genome, we have checked if *RTT103* is important when there are induced DSBs. With the *Sce*-1 experiment it was established that *RTT103* is required when there is single DSB in the genome and cells require Rtt103 for the survival. We also showed that naturally occurring DSBs which are produced during sporulation, requires *RTT103*. This requirement may be

because Rtt103p is required for repairing the DSBs and supports the data which was obtained from the DNA damage experiments. In summary our current data show that though Rtt103 is required for repairing the breaks generated *in vivo*, it is not required for the repair of extra chromosomal breaks.

Chapter-6

Mechanism of action of Rtt103

6.1 Introduction

Maintenance of genome integrity is essential for survival of cells and multiple pathways contribute to this process. When there is DNA damage, the signalling pathway will be activated, leading to cell cycle arrest, which is later resumed after the DNA is repaired. This pathway consists of sensors (Rad24, Mec1 etc.,) transducers (Rad9) and effectors (Chk1 and Rad53). Rad53 activates Dun1, which in turn leads to induction of transcription of DNA damage inducible genes.

Recently, evidence has accumulated to indicate the critical role of 3′ end processing in responding to damage. A general response to UV treatment is the reduction of poly A+ mRNA (Ljungman et al., 1999) and 3′ end processing is also affected. CstF-50 is a component of CstF, (cleavage stimulation factor), which is an essential polyadenylation factor. Upon UV induced damage it interacts with BARD1/BRCA1 complex. This work also shows that CstF-50, RNAPolII, BARD1 and BRCA1 associated with the sites of repaired DNA (Mirkin et al., 2008). In bacteria, Rho, which is a transcription termination factor, is also shown to associate with the sites of DNA damage (Washburn and Gottesman, 2011). Sen1 helicase has recently been shown to be important in maintaining genome integrity (Mischo et al., 2011; Skourti-Stathaki K et al., 2011). Mutations in any of the genes encoding THO confer impairment of transcription and a transcription-dependent hyper-recombination phenotype (Aguilera A., 2002; Jimeno et al., 2002). Both Sen1 and THO complex proteins

prevent transcription associated genome instability by restricting the occurrence of RNA:DNA hybrids that might occur during transcription.

Since the mechanism of action of Rtt103 in maintaining genome stability is not yet known, all the possible mechanisms were tested.

6.2 Results

6.2.1 Transcription termination factors show differential response to DNA repair

Rtt103p copurifies with Rat1 and Rai1 proteins and is synthetically lethal with *rai1* (Kim et al., 2004). Rat1, Rai1 and Rtt103 proteins were found to crosslink very strongly at the 3′ends of the genes predicting their involvement in transcription termination. Since Rat1, Rai1 and Rtt103 proteins act in a complex during transcription termination, we wanted to test if the *RAT1* and *RAI1* genes show any DNA damage sensitivity when exposed to DNA damaging agents.

Firstly, we checked the sensitivity of the transcription termination factors to MMS. WT, *rat1-1* and *rai1* strains were taken, grown overnight in YPD. The grown cultures were then 10 fold serial diluted and the spotted on YPD and YPD + MMS plates to check for the damage sensitivity. As shown in figure 22a,

growth of *rtt103* was inhibited by MMS, whereas, growth of *rat1-1* and *rai1* was comparable to the corresponding WT strains.

To further investigate if these factors were required when specific double-strand breaks were introduced, we generated *rai1* and *rtt103* in a strain carrying galactose inducible HO endonuclease that generates a single break in the MAT locus. As shown in figure 22b, although *rtt10*3 are very sensitive to endonuclease induction, *rai1* were not. These results indicate that transcription termination per se is not responsible for sensitivity of *rtt103* to DNA breaks and suggest a unique or atleast a more prominent role for *RTT103* in maintaining genome stability.

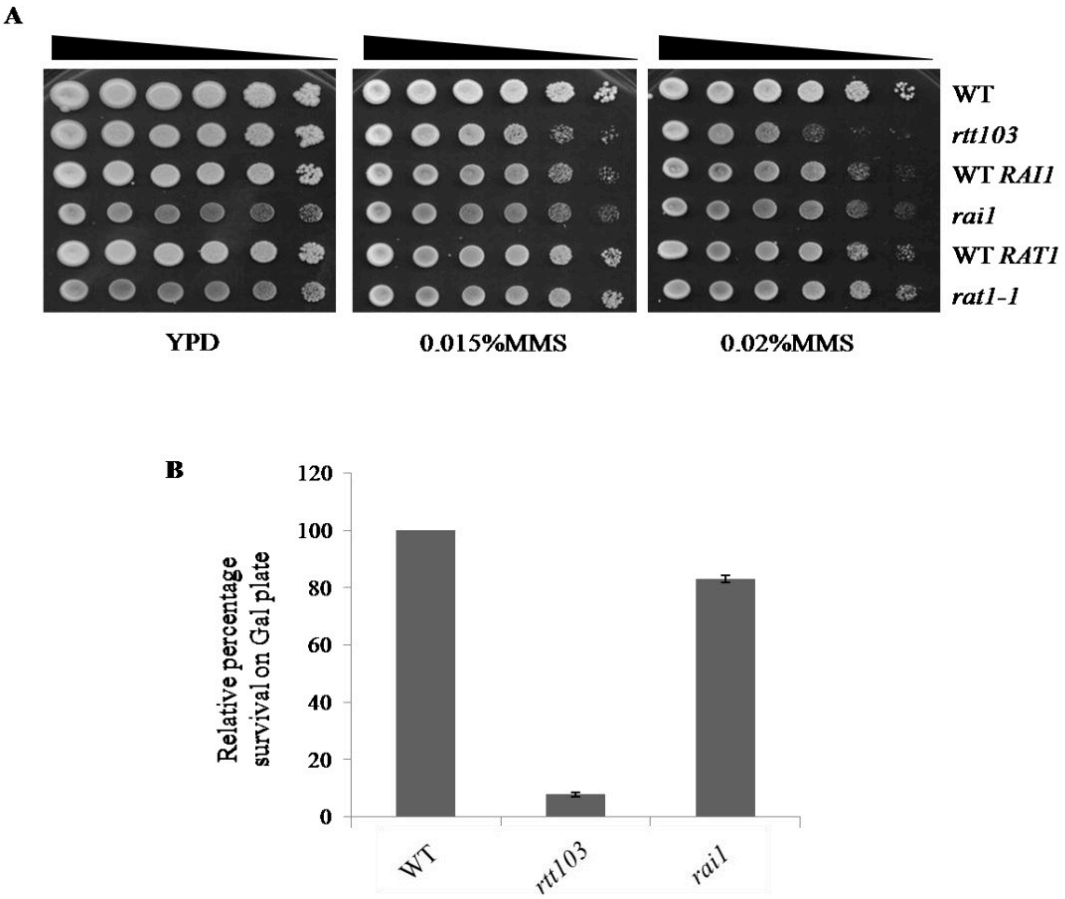


Figure 22: *rat1-1* and *rai1* mutants are not sensitive to MMS and HO endonuclease (22a) WT (KRY105), *rtt103* (KRY230) WT *RAI1* (KRY631), *rai1* (KRY632), WT *RAT1* (KRY633) and *rat1-1* (KRY634) strains were grown to mid-log phase. 5µl of 10-fold serial dilutions of yeast cultures were plated on YPD plates containing MMS and incubated at 30°C for 2-3 days. **(22b)** WT (KRY622), *rtt103* (KRY646), *rai1* (KRY624), were induced with galactose for HO endonuclease to produce DSBs. The percentage survival on galactose compared to WT was calculated from three independent experiments and error bars denote SD.

6.2.2 Epistasis analysis with genes involved in DNA damage signalling

Rtt103 is required for repairing chromosomal breaks and exacerbates the sensitivity of *yku70/80* mutants to genotoxic agents. To further investigate this,

we tested if *rtt103* affected the loss of other repair and DNA damage signalling genes in a similar manner. To this end we generated double mutants of *rtt103* with some of the genes involved in signalling pathway. We have done MMS sensitivity assay for checking this. *rtt103rad9*, *rtt103rad24*, *rtt103mec1* and *rtt103tel1* showed similar sensitivity to MMS as *rad9* (figure 23 (i), *rad24* (figure 23 (ii), *mec1* and *tel1* (figure 23 (iii) respectively. So *RTT103* is not epistatic to the genes in the signalling pathway.

We also tested if *RTT103* is epistatic to homologous recombination genes. We have chosen *RAD51* and *RAD52* and checked for the sensitivity of the double mutants. As seen in figure 23 (iv) and (v), *rtt103* does not exacerbate the sensitivity of *rad51* or *rad52* to DNA damage agents.

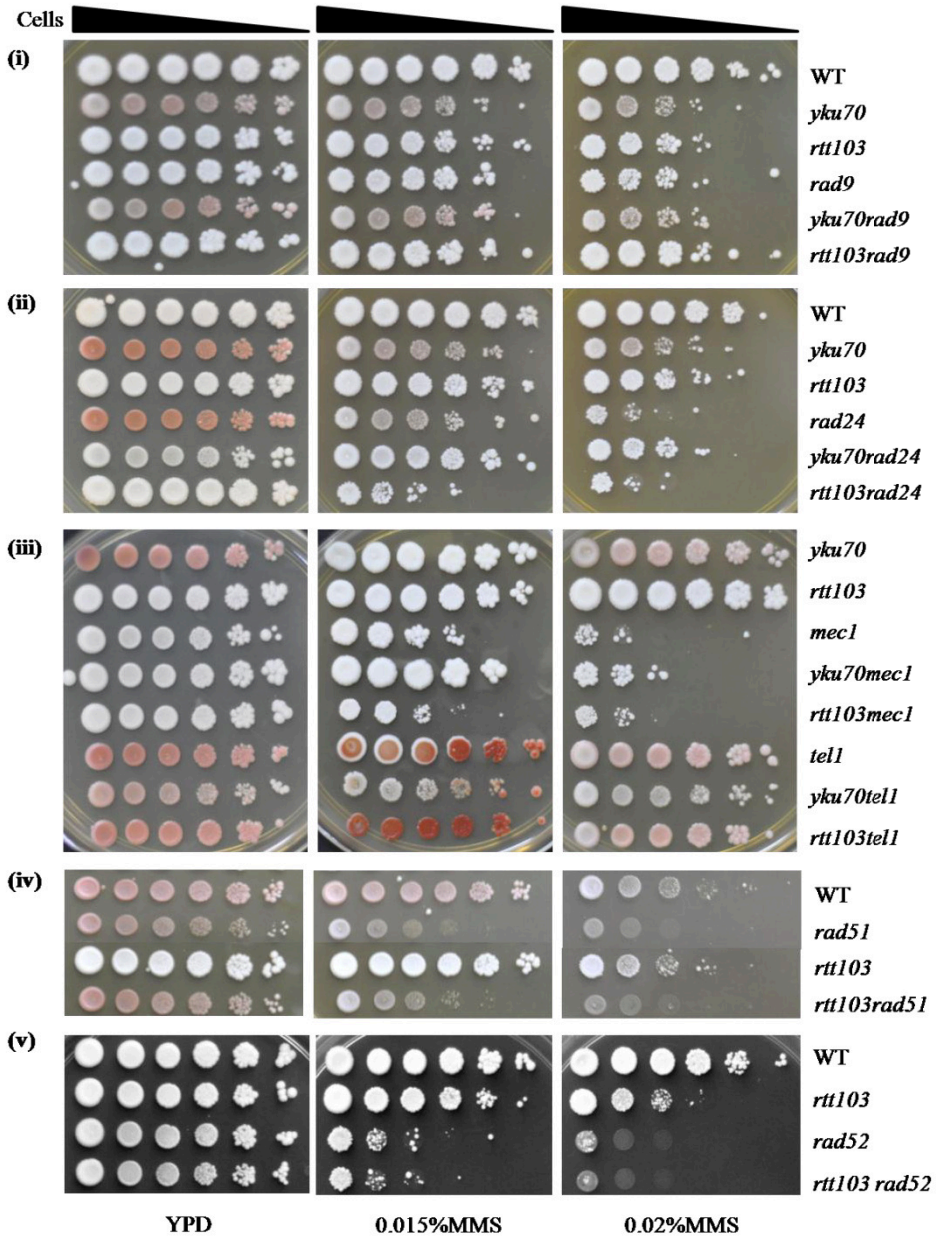


Figure 23: Epistasis analysis: WT (KRY105), *yku70* (KRY172), *rtt103* (KRY230), *rad9* (KRY365), *yku70rad9* (KRY369), *rtt103rad9* (KRY371), *rad24* (KRY366), *yku70rad24* (KRY367), *rtt103rad24* (KRY372), *mec1* (KRY443), *yku70mec1* (KRY444), *rtt103mec1* (KRY445), *tel1* (KRY440), *yku70tel1* (KRY441), *rtt103tel1* (KRY442), *rad52* (KRY477), *rtt103rad52* (KRY676), WT (KRY2), *rad51* (KRY617), *rtt103* (KRY231) and *rtt103rad51* (KRY681) strains were grown overnight, 10 fold serial diluted and spotted on YPD and YPD + MMS plates and incubated at 30°C for 2-3 days.

6.2.3 DNA damage signalling is intact in *rtt103*

A key difference between the plasmid end-joining assay and the chromosome break assays is the nature of response to damage: when there is a chromosome break, cells arrest their division cycle and signal the presence of the break to the repair proteins. This signal transduction cascade culminates in the recruitment of proteins that execute the repair and when the repair is complete, the signal is turned off. But in case of plasmids, as the break is not on the chromosome but on an extra-chromosomal element, there is no cell cycle arrest or activation of the signalling cascade. Because *rtt103* were sensitive to chromosomal breaks but not plasmid breaks, we reasoned that the end-joining process per se is not affected but the signal transduction cascade could be affected in *rtt103*. In order to test this, we checked the phosphorylation of Rad53, the final effector kinase in the pathway. Rad53 phophorylation activates the phosphorylation of Dun1 protein by Rad53 kinase; Dun1 activation leads to transcriptional upregulation of a set of repair specific genes. Rad53 is phosphorylated in response to DNA damage primarily by Mec1 and this is facilitated through interactions between Rad9 and Rad53.

WT, *yku70* and *rtt103* were transformed with plasmid encoding *RAD53-9xMYC* with *LEU2* marker (Pellicioli et al., 1999). The transformants were selected on SC-LEU plates and restreaked on SC-LEU plates. They were then picked up and inoculated in SC-LEU broth, grown overnight and then 0.03% MMS was added to one half of each culture to introduce DNA damage and

incubated for 2 hours. The other half was incubated for the same time without adding MMS. Total protein was then extracted from the cultures. Western blots were performed using anti-myc antibody. As shown in figure 24a, the untreated cells show a sharp band at ~110kDa. Upon treatment with MMS, we see an upward shift in the Rad53 band and it also turns fuzzy. This indicates that it gets phosphorylated upon DNA damage. This shift in molecular weight is same in all three strains, showing that the damage signalling cascade is active in *rtt103*. The same blots were further probed with antibodies to Sir2 protein to establish that the fuzzy bands are not due to abnormal separation of proteins.

As a control experiment we tested the phosphorylation of Rad53 in a mutant deficient in signalling, *mec1*. As shown in figure 24b, no phosphorylation of Rad53 could be detected (no fuzzy band), as *MEC1* is required for phosphorylating Rad53.

After the DNA is repaired the Rad53 should get dephosphorylated. So we checked if this is happening in *rtt103*. The strains were treated with MMS for 2 hours and then the MMS was washed in ice cold 10% sodium thiosulphate 2-3 times. The washed cultures were then incubated for 4 and 6 hours for checking the dephosphorylation. As shown in figure 24c, Rad53 got dephosphorylated in WT and *rtt103*. This experiment shows that the signalling of the damaged DNA is intact in *rtt103.*

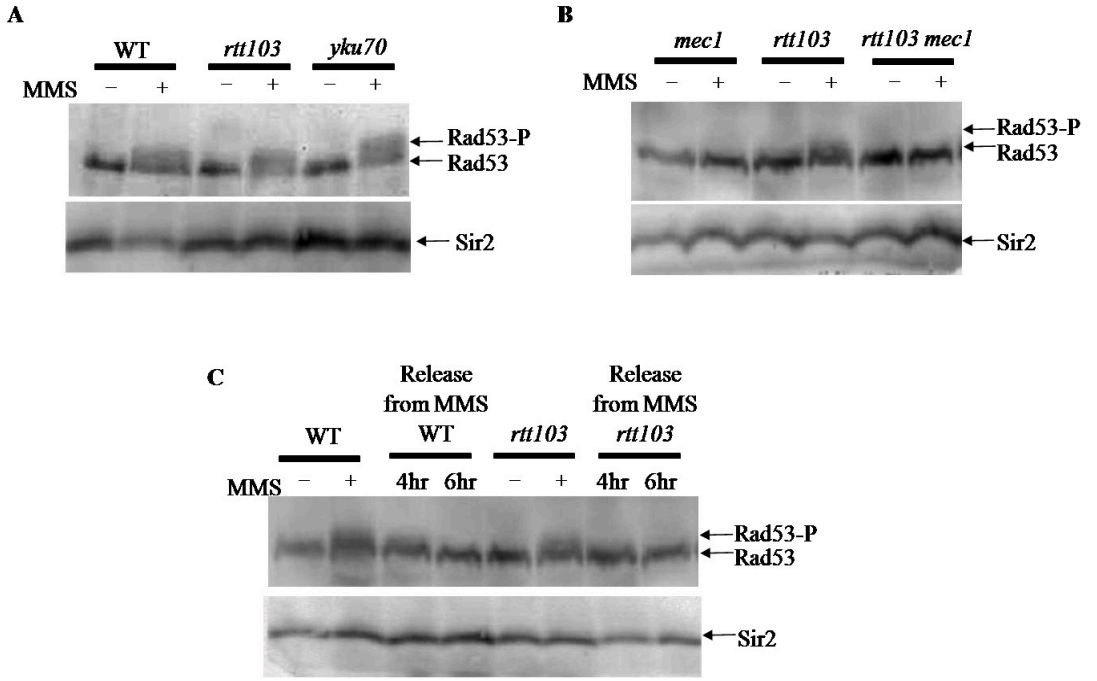


Figure 24: *rtt103* cells show normal Rad53 phosphorylation and dephosphorylation: (24a) WT (KRY105), *yku70* (KRY171), *rtt103* (KRY230) cells containing *RAD53-Myc* was treated with MMS for 2hrs and anti-Myc western blots were performed. **(24b)** *rtt103* (KRY230), *mec1* (KRY443) and *rtt103mec1* (KRY445) were treated with MMS as described above. Rad53 phosphorylation could not be detected in *mec1* mutants. Same blot was probed with Sir2 to confirm normal separation of proteins in the gel. **(24c)** Treated cells (KRY105 and KRY230) were released from MMS and western blots were performed after 4 or 6 hours after release. The disappearance of the slow moving band indicates dephosphorylation. The same blots were probed with Sir2 antibody for loading control.

The downstream effect of this signalling is the transcriptional upregulation of the DNA damage signature genes, namely, *DUN1*, *PLM2*, *RAD54*, *RNR2* and *RAD51*. Their expression is substantially induced in response to both MMS and ionizing radiation (Gasch et al., 2001). To test if *rtt103* had any defects in the induction of transcription, RNA levels of *DUN1*, *PLM2*, *RAD54*, *RNR2* and *RAD51* genes in WT and *rtt103* with and without the

DNA damage were tested by Northern blots. WT and *rtt103* were grown overnight in YPD and then the cultures were divided into two. To one half of the cultures 0.03% MMS was added and incubated for 3hrs. The other half was incubated for the same time without MMS. RNA was extracted from the cultures. 10µg of total RNA was loaded onto formaldehyde agarose gels. The RNA was then transferred onto nylon membrane and then probed with the respective DNA probes. We found, as expected, these genes were induced upon DNA damage. As seen in figure 25(i), there was no difference in the levels of RNA induced between wild type and *rtt103*. Figure 25(ii) shows RNA loading control. This indicates that the *rtt103* is unlikely to affect the levels of DDR genes. However, it is still possible other genes are affected by *rtt103* as the genome-wide transcriptional studies indicate transcriptional induction of several genes. These data further confirm that the DNA damage is sensed and the core downstream response is activated in an *rtt103*.

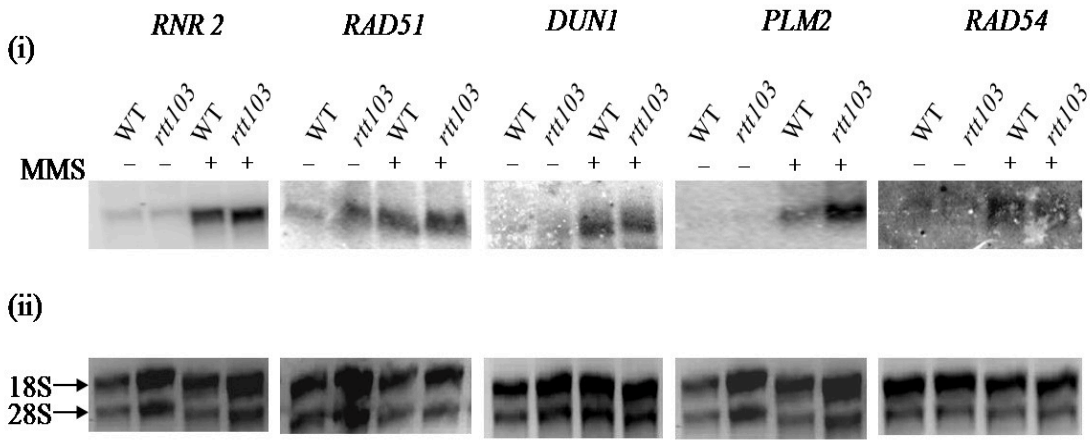


Figure 25: Expression of several DNA damage responsive genes is similar in WT and *rtt103*: RNA was isolated from WT (KRY105) and *rtt103* (KRY230) and northern blots were done to check the expression of *RNR2, RAD51, DUN1, PLM2* and *RAD54*. *rtt103* show similar pattern of upregulation of *RNR2, RAD51, DUN1, PLM2* and *RAD54* as seen in WT cells. Upper panels (i) show the northern blots probed with *RNR2, RAD51, DUN1, PLM2* and *RAD54* while the lower panel (ii) shows the corresponding agarose gel stained with ethidium bromide as loading control.

6.2.4 *rtt103* do not enhance chromosomal recombination events

Connections between transcription and genome instability have been well established by studying mutants in the multiple processes that ultimately lead to the accumulation of mature RNA in the cytoplasm (Aguilera, 2002). For example, components of the THO complex and Sen1 restrict the occurrence of RNA:DNA hybrids that might occur during transcription. A hallmark of THO complex mutants and *sen1-1* is the increased chromosomal instability by promoting recombination between direct repeats. We tested if *rtt103* also showed similar hyper-recombination phenotypes in the assay described previously (Santos-Rosa and Aguilera, 1995).

rtt103, *yku70* and *hpr1* deletions were generated in a strain carrying *URA3* and *ADE2* flanked by a defective *leu2-k* repeats (Figure 26a) and recombination was tested in wild type and the null mutants (Figure 26b). If recombination occurs between the *leu2* repeats, *URA3* and *ADE2* genes will be lost. The strains were grown overnight in YPD and the cultures were 10 fold serial diluted and spotted on SC and SC-FOA plates. Depending on the growth

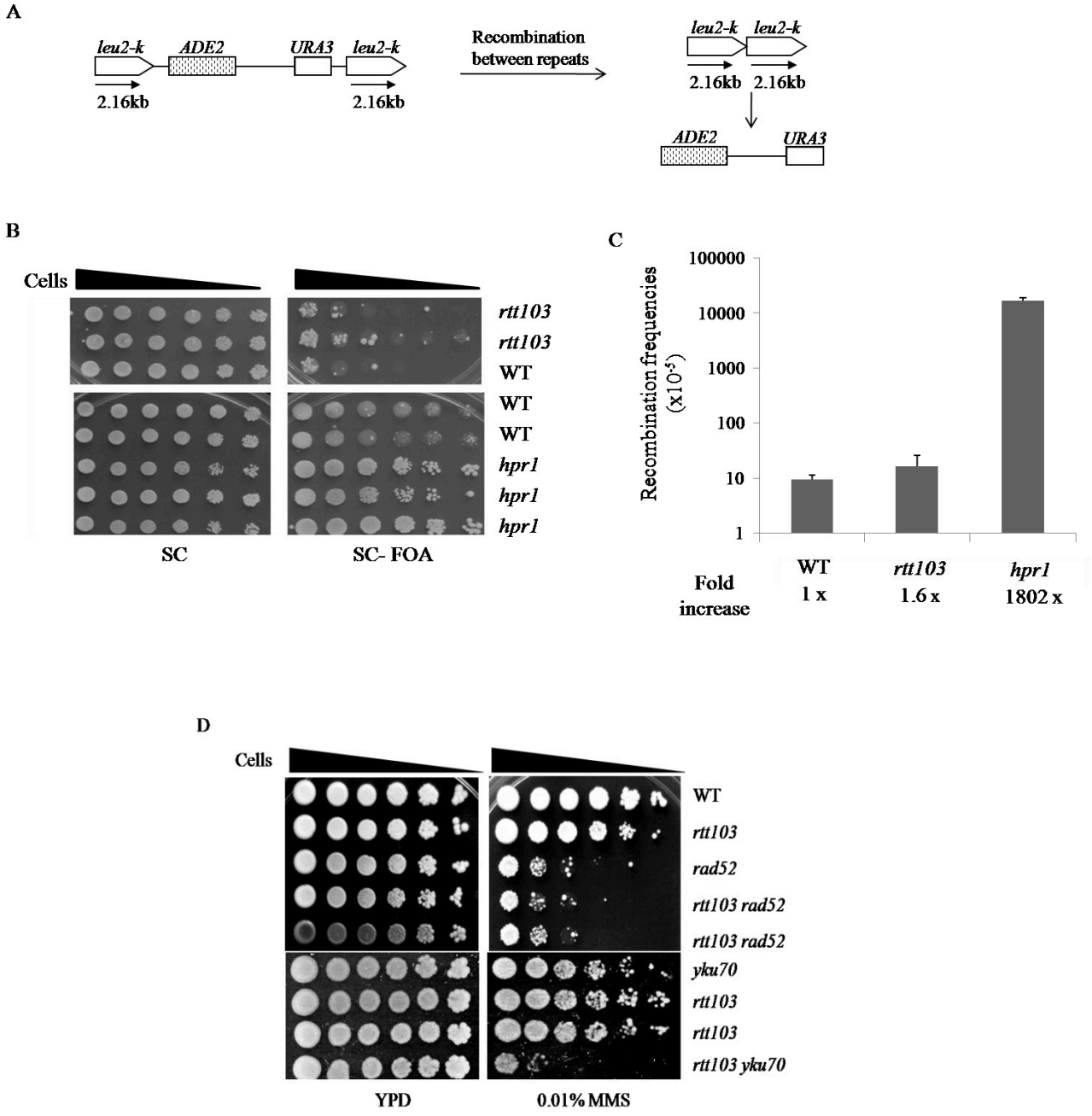
on SC-FOA plates the loss of *URA3* was analyzed. Growth on FOA plate indicates the loss of *URA3* (hyper-recombination) and no growth indicates no recombination. As seen in figure 26b and as expected, wild type cells had low levels of recombination and *hpr1* which was taken as a positive control had high levels of recombination. However, *rtt103* had recombination frequencies indistinguishable from wild type cells.

The serial dilutions were then plated on SC and SC-FOA plates for calculating the frequency of recombination. Number of colonies on FOA plate per 1,00,000 colonies on SC plate were counted and graph was plotted (Figure 26c). *rtt103* has similar recombination frequency as WT and *hpr1* cells had more than thousand fold increase in recombination. These results show that *rtt103* do not stimulate hyper-recombination between repeats.

As it has been demonstrated that *sen1-1* mutants show increased hyper-recombination, we conclude that Rtt103 is unlikely to function in the same pathway as Sen1 to prevent genome stability. Additionally, *sen1-1* shows synthetic genetic interactions with genes involved in the homologous repair pathway and none with genes involved in NHEJ. However, *rtt103* appears to have the opposite effect, by showing synthetic phenotype of increased sensitivity to DNA damage with *yku70* as described in the chapter 4. We also tested the *rtt103rad52* double mutants for damage sensitivity and found that it does not show increased sensitivity (Figure 26d).

We further tested the hyper-recombination phenotype by using a plasmid LLac which has *TRP* marker (Figure 26e). This plasmid has *leu2* alleles flanking the LacZ gene under the control of *LEU2* promoter. *LEU2* is normally not functional in this plasmid. If recombination occurs between the *leu2* repeats, LacZ gene will be lost, the repeats will come together and *LEU2* will be functional. This plasmid was transformed into WT, *rtt103* and *hpr1* and the transformants were selected on SC-TRP plates. The colonies were restreaked on SC-TRP plate. From this individual colonies were picked, serial diluted and then equal number of cells were plated on SC-TRP (total number of colonies) and SC-LEU (number of recombinants) plates. Recombination frequency per 10,000 cells was calculated. As shown in figure 26f, the frequency of recombination of *hpr1* is approximately 57 fold above the WT levels and that of *rtt103* is approximately 8 fold high which is not significant compared to *hpr1*. This data also indicates that recombination occurs at slightly higher than wild type levels in *rtt103.*

Taken together these data indicate that although both *rtt103* and *sen1* affect genome stability, their mechanisms might be distinct.


A
leu2-k
ADE2
URA3
leu2-k
2.16kb
2.16kb
Recombination between repeats
leu2-k
leu2-k
2.16kb
2.16kb
ADE2
URA3
B
Cells
rtt103
rtt103
WT
WT
WT
hpr1
hpr1
hpr1
SC
SC- FOA
C
Recombination frequencies (x10-5)
100000
10000
1000
100
10
1
WT
rtt103
hpr1
Fold increase
1 x
1.6 x
1802 x
D
Cells
WT
rtt103
rad52
rtt103 rad52
rtt103 rad52
yku70
rtt103
rtt103
rtt103 yku70
YPD
0.01% MMS

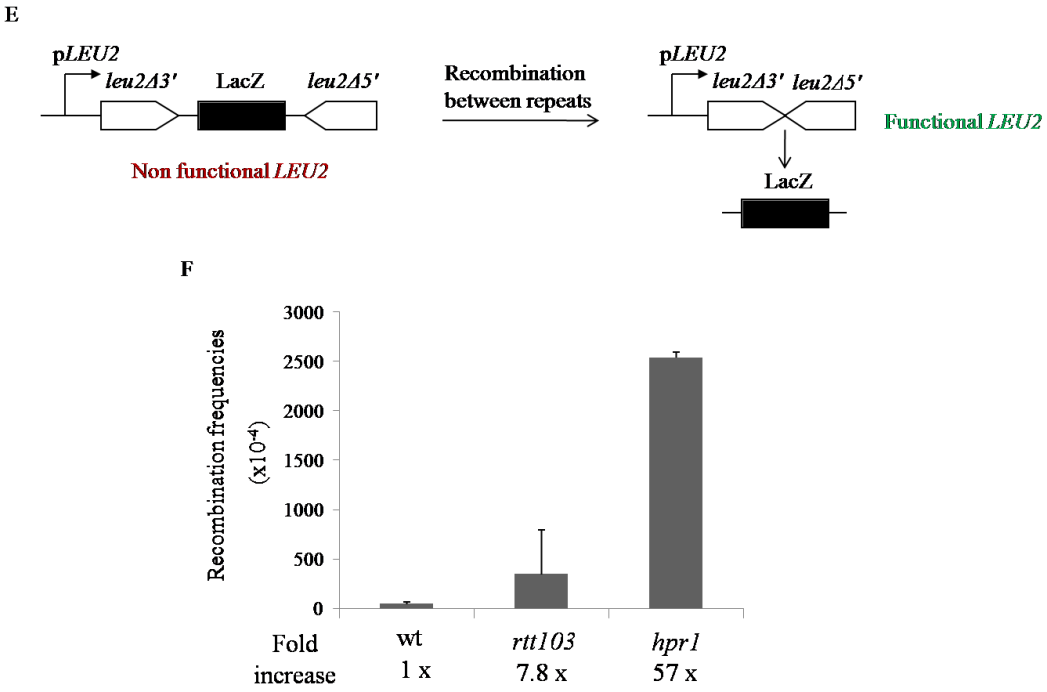


Figure26: *rtt103* mutants do not stimulate hyper-recombination between repeats (26a) Schematic representation of chromosomal region containing *leu2-k* repeats flanking the *ADE2* and *URA3* genes. **(26b)** WT (KRY615), *rtt103* (KRY650), and *hpr1* (KRY652) strains containing the *ADE2*, *URA3* genes flanked by *leu2-k* repeats were serial diluted spotted on SC plates (viability) and SC+FOA plates (recombinants). **(26c)** The recombination frequency per 1,00,000 cells was plotted and was determined for three independent segregants. Error bars denote SD. **(26d)** WT and strains carrying deletions of *rtt103* (KRY230), *rad52* (KRY477), *rtt103rad52* (KRY674) and *rtt103yku70* (KRY290) were tested for sensitivity to MMS. *rtt103* does not affect the sensitivity of *rad52* to DNA damage induced by MMS. *rtt103* exacerbates the sensitivity of *yku70* to MMS. **(26e)** Schematic representation of plasmid construct, containing *leu2* alleles flanking the LacZ gene. **(26f)** WT (KRY105), *rtt103* (KRY230) and *hpr1* (KRY626) mutants were transformed with LLac plasmid. Transformants were grown for 3–4 days on SC-TRP plates at 30°C. Quantification of recombinants formed from four colonies of four different transformants is presented.

6.2.5 Rtt103 associates with sites of DNA damage

The results shown above indicated that Rtt103 is critical for DNA repair but did not follow the same pathways as Sen1, the other transcription

termination factor shown to affect genome stability. Since there were reports that CstF in mammals and Rho in bacteria which are transcription termination factors play role in genome stability by binding the damaged DNA at the cut site, we tested if Rtt103 also functions by binding to damaged DNA. Chromatin immunoprecipitation (ChIP) experiments were done to confirm this.

Rtt103 was first tagged with 13xMYC (Appendix 2.3). To confirm that the addition of the tag did not make the protein non-functional, we tested the sensitivity to MMS. WT, *yku70*, *rtt103* and *RTT103* 13xMYC strains were taken, grown overnight in YPD and the cultures were serial diluted. They were then spotted on YPD and YPD + MMS plates. As shown in figure 27a, WT cells grew normally on MMS plates. *yku70, rtt103* and *rtt103yku70* are sensitive to MMS as shown in chapter 4 and 5. If tagging of the protein makes it non-functional, *RTT103* Myc strains should be sensitive to MMS. But as shown in figure 27a the tagged strain grew normally on MMS plates, similar to WT indicating that the protein is functional.

We tested the localization of Rtt103. Immunolocalization was performed (Pasupala N et al., 2012). The cells were then stained with DAPI for staining the DNA, Nsp1 for staining the nuclear pore complex and Myc for Rtt103 protein. As seen in figure 27b, Rtt103 was extensively localized to the nucleus.

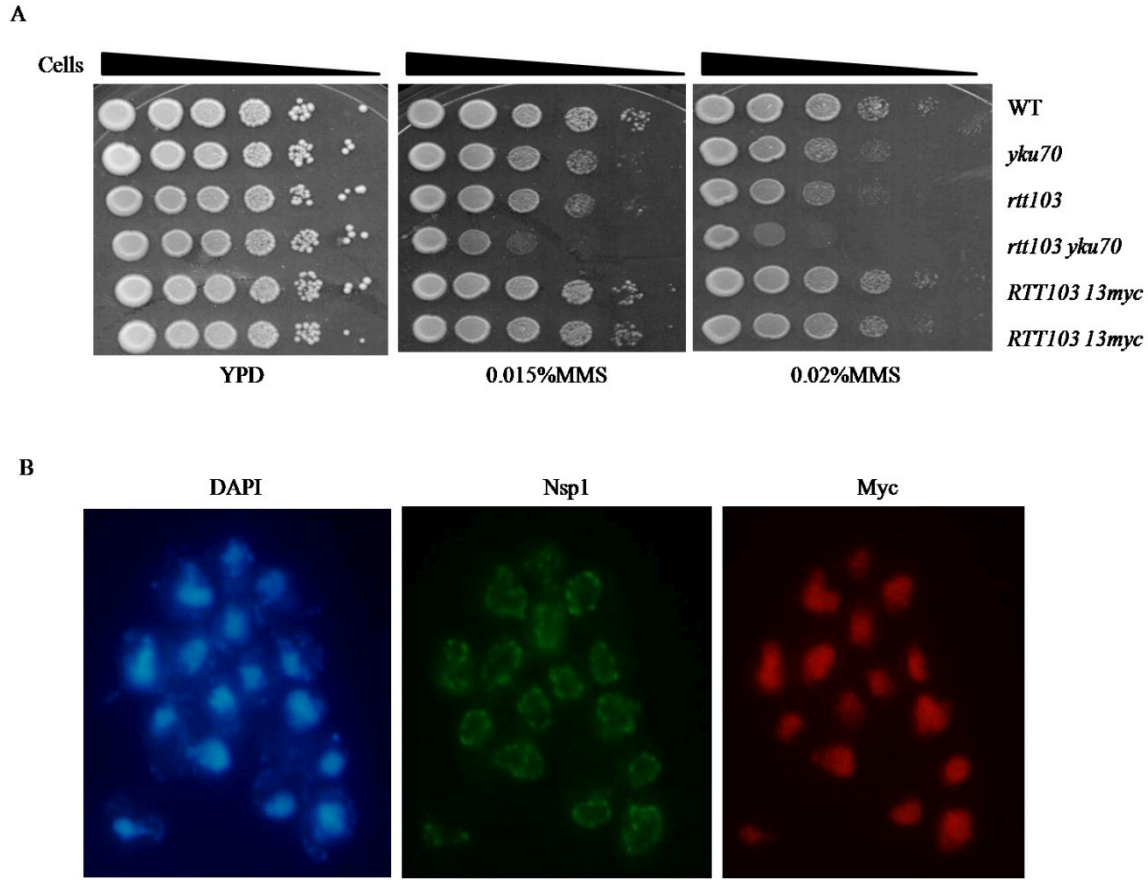


Figure 27: Localization of Rtt103p. (27a) WT (KRY105), *yku70* (KRY171), *rtt103* (KRY230), *rtt103yku70* (KRY290) and *RTT103* 13MYC (KRY184) strains were spotted on YPD containing various concentrations of MMS. 5µl of 10 fold serial dilutions of the strains were spotted and incubated at 30°C for 2 days. *rtt103* and *yku70* are sensitive to MMS where as *RTT103* 13Myc is not MMS sensitive indicating that the C-terminal tagging did not affect the function of *rtt103.* **(27b)** The Rtt103 Myc strain is stained with anti-rabbit myc antibody. The nucleus is marked by both DAPI staining and antibody to a nuclear pore complex protein, Nsp1.

We then introduced this tag in the I-*Sce*I strain, and performed ChIP experiments to test the binding of Rtt103 to break sites. Before performing ChIP experiments we first ascertained the time point at which there was significant

cut by *Sce*I. The Rtt103Myc strain was grown overnight in 2% raffinose media and then divided into four cultures. One was incubated without adding galactose. To the other 3 halves galactose was added to induce the *Sce*I endonuclease after washing raffinose and incubated for 1hour, 3hours and overnight. DNA was then extracted by zymolyase method from all the cultures. DNA was digested with *Bgl*II (Figure 28a) and southern blot was done (Figure 28b) to check the cutting of *Sce*I.

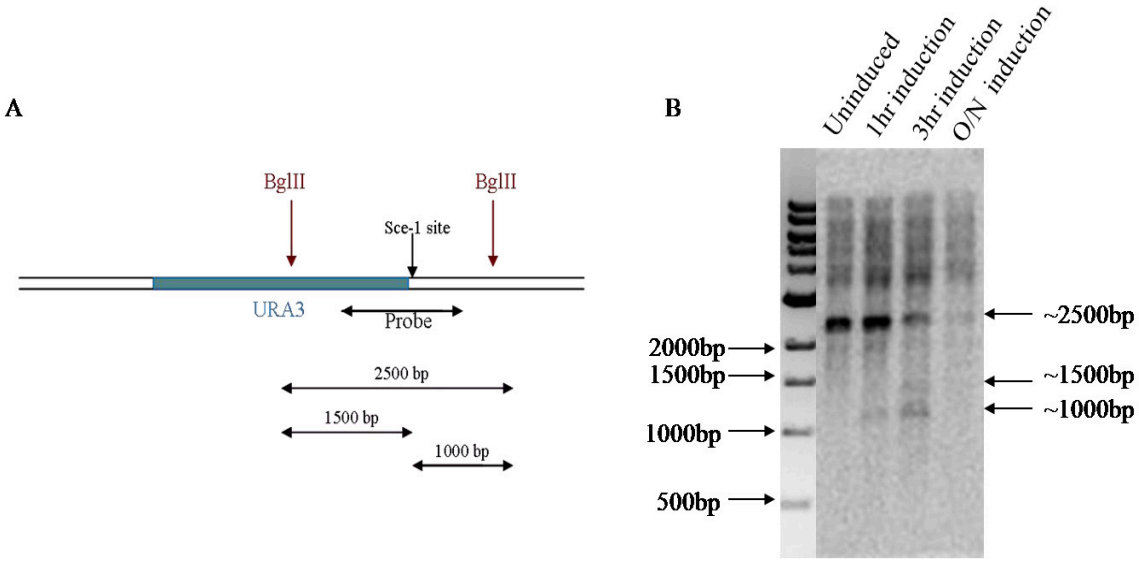


Figure 28: Induction of I-*Sce*1 cut by galactose. **(28a)** Schematic representation of the *URA3* region showing I-*Sce*I site, the region used as probe and the products that were produced after the I-*Sce*I cut. **(28b)** Southern blot showing the I-*Sce*I cut. The genomic DNA was isolated from the uninduced, 1hr, 3hr and overnight induced strain (KRY448) with galactose. The *Bgl*II digested DNA was run on agarose gel, transferred on to membrane and then probed. Uninduced gives a product of ~2500bp and the I-*Sce*I cut (induced) gives an uncut product of ~2500bp and cut products of ~1500 and 1000bp.

As seen in figure 28b the *Sce*I site was digested completely after 3hrs of induction in galactose media which gave 1500 and 1000bp bands. The undigested (uninduced) sample gave only one band of 2500bp. So we have chosen 3hr time point for doing the ChIP experiments.

We first performed the ChIP experiment with Yku80 13xMYC strain (Appendix 2.4), as it was already known that Yku proteins bind to the damaged DNA. I-*Sce*I enzyme was induced for three hours and then cross-linked with formaldehyde and immunoprecipitated using anti-myc antibodies. PCR primers were chosen at the site of damage, 0.2kb, 0.5 and 1kb away from the cut site (Figure 29a). *SPS2* on chromosome IV was chosen as negative control. As shown in figure 29b, there was no significant association of Yku80 with any of the regions around the cut site when the endonuclease was not induced (blue bars). However, upon induction, the association of Yku80 went up only in the vicinity of the cut site (red bars). *SPS2* region did not show any increased association upon endonuclease induction, suggesting that Yku80 protein binding is specific to regions of damage.

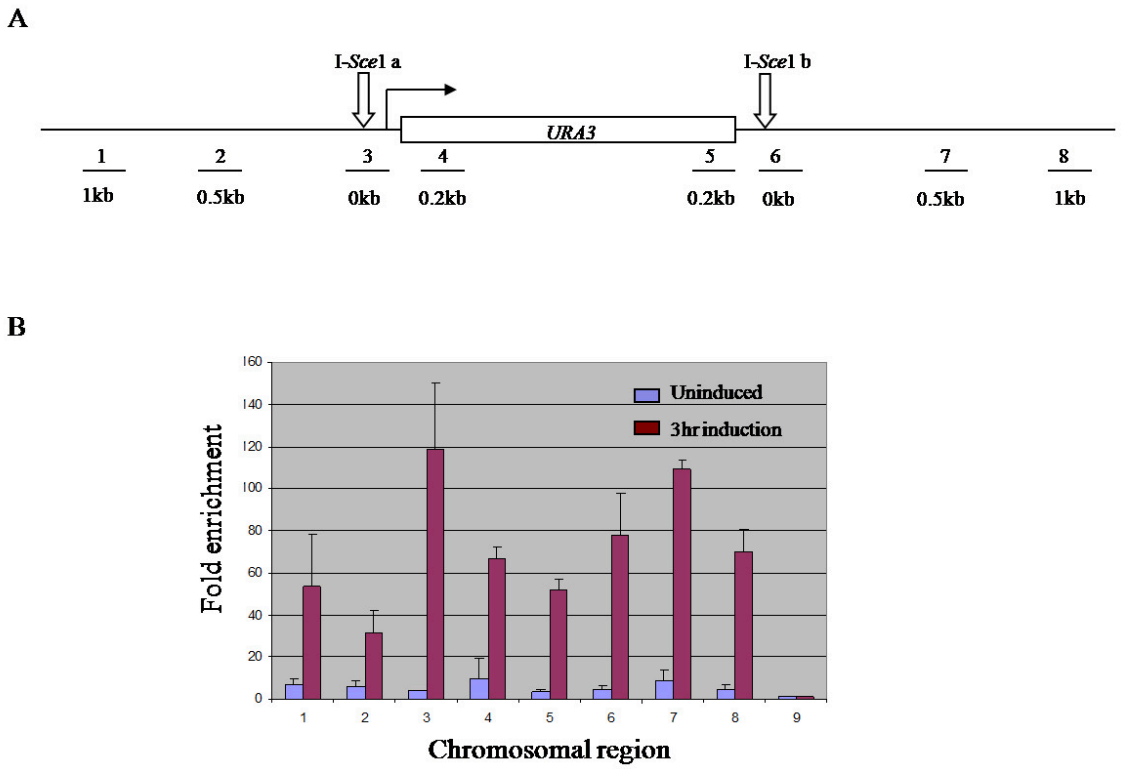


Figure 29: Binding of Yku80p to the site of DNA damage. (29a) Schematic diagram of region in and around *URA3* with two flanking I-*Sce*1 sites. Bars (1-8) represent the regions up to 1kb away from Sce1 sites, at which Rtt103p binding is checked. **(29b)** ChIP experiment to show Yku80p binding at the site of damage. Yku80 13Myc strain (KRY447) was grown in galactose medium to induce the expression of I-*Sce*I enzyme for three hours and then cross-linked with formaldehyde and immunoprecipitated using anti-myc antibody. RT PCR was done to check the fold induction of Yku80p at the sites indicated. The numbers on the x-axis indicate the binding site of primers which is shown in the schematic diagram. The number on the y-axis indicate the fold induction of Yku80p compared to the internal *SPS2* gene (9) which is a house keeping gene.

As the control experiment established that the cut and subsequent physiological response of recruiting Yku80 was normal, we proceeded to test the binding of *RTT103* Myc. I-*Sce*I enzyme was induced for three hours and then cross-linked with formaldehyde and immunoprecipitated using anti-myc

antibodies. PCR primers were chosen at the site of damage, 0.2kb, 0.5, 1, 2, and 3kb away from the cut site (Figure 30a). *SPS2* on chromosome IV and a telomere proximal site on chromosome VI were chosen as negative controls. Additionally, 3′ ends of two genes, *PMA1* and *ADH1*, where Rtt103 was shown to be enriched were also tested. As shown in figure 30b, except for 3′ ends of *PMA1* and *ADH1* where Rtt103 was expected to be enriched, there was no significant association of Rtt103 with any of the other regions tested when the endonuclease was not induced (blue bars). However, upon induction, the association of Rtt103 went up more than five-fold only in the vicinity of the cut site (red bars). *SPS2*, telomere regions, regions further away from the cut site, *PMA1* and *ADH1* did not show any increased association upon endonuclease induction, suggesting that Rtt103 protein binding is specific to regions of damage. These data strongly indicate that Rtt103 functions through association at the site of damaged DNA.

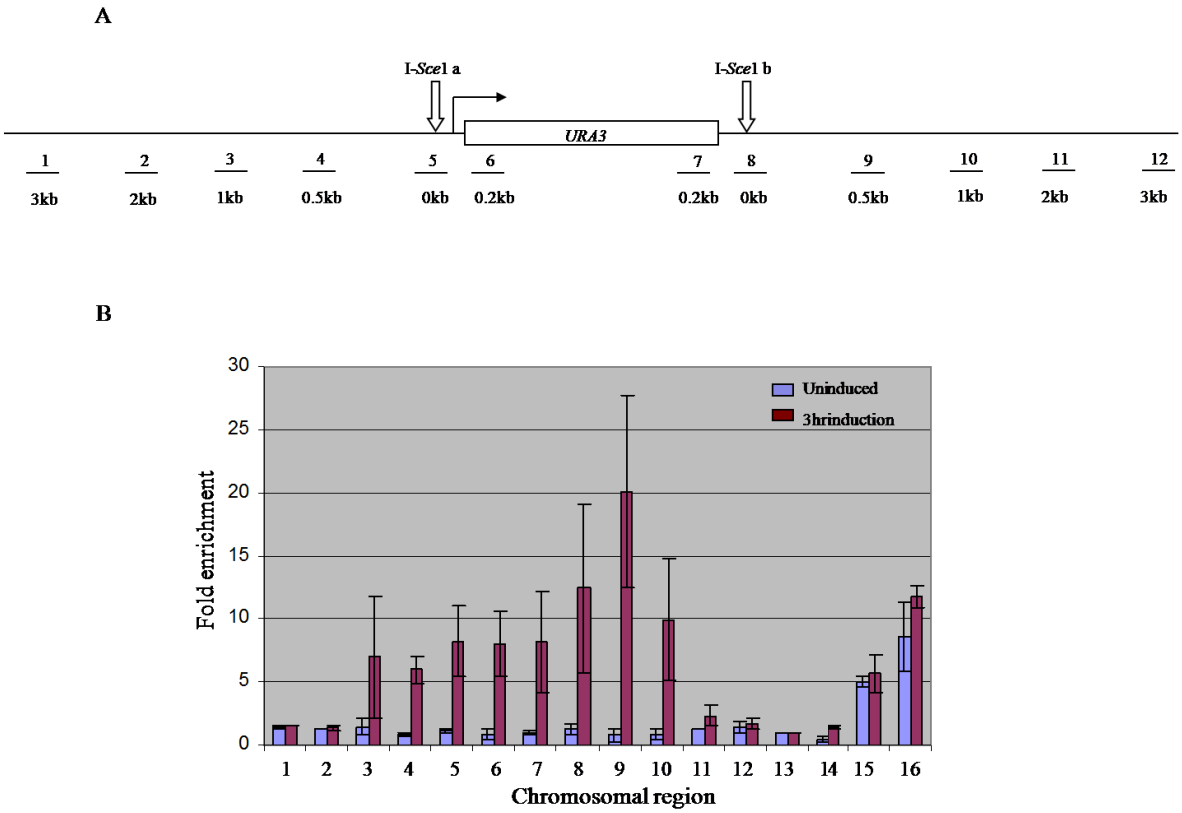


Figure 30: Binding of Rtt103p to the site of DNA damage. (30a) Schematic diagram of region in and around *URA3* with two flanking I-*Sce*I sites. Bars (1-12) represent the regions up to 3kb away from I-*SceI* sites, at which Rtt103p binding is checked. **(30b)** ChIP experiment to show Rtt103p binding at the site of damage. Rtt103Myc strain (KRY448) was grown in galactose medium for three hours and then cross-linked with formaldehyde and immuoprecipitation with anti-myc antibody. X-axis indicates the loci tested (see schematic) and y-axis shows the fold change of Rtt103p binding compared to *SPS2* internal control; blue bars represent Rtt103p association just prior to galactose induction (no cut) and red bars represent association 3 hours after induction. *SPS2* (13) and 10kb from the telomere VI R (14) are negative controls. *PMA1* 3′ region (15) and *ADH1* 3′ region (16) where Rtt103 is reported to crosslink heavily are used as positive controls.

6.3 Summary

Rtt103 functions in DNA damage response (Chapters 3-5). To understand the mechanism underlying the action of Rtt103 various experiments were done. Rtt103 associates with other transcription termination factors Rat1 and Rai1 and helps in transcription termination. To know if Rtt103 functions in the damage response through this pathway, DNA damage assays were done in *rat1-1* and *rai1.* But *rat1-1* and *rai1* cells were not sensitive to DNA damage indicating that Rtt103 has unique role in protecting the genome, which is different from Rat1 and Rai1. Signalling was also normal in *rtt103* as shown by the epistasis, Rad53 phosphorylation, dephosphorylation and northern blot experiments. We have also shown that Rtt103 does not function by resolving RNA:DNA hybrids, unlike Sen1 and THO complex proteins.

Since transcription factors like CstF and Rho were recently shown to function in damage response by binding to DNA near the damage site, we hypothesized that Rtt103 might also function by binding to the damaged DNA. Our ChIP experiments confirm this, and have shown that Rtt103 directly binds to the damaged DNA and protects the genome.

Chapter-7

Discussion

Telomeres are specialized ends of the chromosomes consisting of simple sequence repeats and proteins that bind to these sequences. Telomeres protect chromosome ends from fusion and prevent the natural chromosome ends from being recognized as double strand DNA breaks by the DNA repair apparatus. The proteins involved in double-strand break repair, Yku70/80p, are localized to the telomeres and are critical for protection of chromosome ends. In an effort to understand the mechanisms through which this heterodimer protects chromosome ends, we initiated a genetic screen to isolate suppressors of *yku70* mutants. In the process we have discovered an unanticipated role for transcription termination factor, Rtt103, in maintaining genome integrity.

7.1 *RTT103* overexpression suppresses Yku70 defect phenotypes

Ku was first identified as a protein that is recognized by certain human autoimmune sera and is a relatively abundant nuclear protein (Francoeur et al., 1986; Mimori et al., 1981; Mimori and Hardin, 1986; Yaneva et al., 1985). It binds with high selectivity to DNA DSBs and other discontinuities in the genome (de Vries et al., 1989; Falzon et al., 1993; Paillard and Strauss, 1991). Ku proteins are expressed in all eukaryotic cells suggesting that they have a fundamental role in general DNA metabolism. It binds to DNA ends in a sequence independent manner (Milne et al., 1996; Siede et al., 1996).

yku70 null strains exhibit temperature sensitive growth (Feldmann and Winnacker, 1993). *yku70* cells can form colonies at 37°C on solid medium, but these cells do not grow when replicated on fresh medium at 37°C (Barnes and Rio, 1997). Yku70 deficient cells have anomalous DNA content. Majority of unbudded cells have 2N DNA content and majority of large budded cells have 4N DNA content. Although we know that Yku has multiple roles at the telomeres, molecular means of its multiple functions is not completely understood.

We used the temperature sensitive phenotype of Yku70 as a tool to understand its function. We set up a multi-copy suppressor screen to identify the interacting partners of Yku that are able to suppress the temperature sensitivity. *yku70*Δ was transformed with a genomic library in a high copy number plasmid and plated at 35°C and incubated for 2 days. *yku70* form small colonies at this temperature. Any large colonies that were formed indicate that the ts of *yku70* might be suppressed. From this screen we identified *RTT103* as one of the suppressors of Yku70 temperature sensitive phenotype. *RTT103* was initially identified as a gene that when mutated increases the Ty1 transposition. Later it was shown to be involved in transcription termination along with its interacting proteins Rat1 and Rai1. As Yku70 is a protein that is involved in NHEJ pathway of DNA repair, we tested if *RTT103* suppresses the DNA repair phenotype of Yku and we found that *RTT103* overexpression suppresses this

phenotype. Thus *RTT103* was isolated as a gene that when overexpressed suppresses the Yku70 defect phenotypes.

7.2 *RTT103* is involved in maintaining genome stability

Rtt103 is an abundant nuclear protein, however no clear function has been demonstrated. It contains a carboxy-terminal interacting domain (CID) or RPR. This domain interacts with the conserved C-terminal domain of RNA polymerase II and proteins are recruited via this interaction to the actively transcribed chromatin (Lunde et al., 2010). Since *RTT103* overexpression suppresses the ts and DNA repair phenotype of Yku, we have constructed an *RTT103* deletion strain to check if it has any role in DNA repair.

rtt103 cells did not show any ts phenotype, but the ts phenotype of *yku70* was enhanced when *rtt103* was mutated. So *rtt103* exacerbates the ts phenotype of *yku70*. *rtt103* cells do not show TPE and their telomere length was similar to WT. To check if *RTT103* is directly involved in DNA repair, MMS treatment was done to *rtt103* cells and it was observed that these cells were sensitive to this DNA damaging agent. When *rtt103* was mutated in a *yku70* strain, these *yku70rtt103* double deletions were more sensitive than either of the single deletion strains. This indicates that Rtt103 and Yku70 function in two different pathways to protect the genome from instability. In order to confirm that this gene is involved in maintaining genome stability, we have done DNA damage to *rtt103* cells by additional methods like using *Eco*RI endonuclease or *Sce*I

endonuclease. These experiments also established the involvement of Rtt103 in DNA repair. As all these experiments clearly indicated that *RTT103* is essential for efficient repair of DNA damage, we asked if the naturally occurring double-strand breaks also require *RTT103*. Sporulation of *rtt103* homozygous strain was done to check this and we found that this strain is defective of sporulation. So *RTT103* may be involved in repairing the DSBs, as the artificially induced DSBs produced by different agents could not be repaired in the absence of *RTT103.* For the first time we have reported the involvement of *RTT103* in DNA repair.

7.3 Mechanism of action of *RTT103*

Rtt103p copurifies with Rat1 and Rai1 and were found to crosslink very strongly at the 3′ ends of the genes predicting their involvement in transcription termination. Since Rat1, Rai1 and Rtt103 proteins are believed to act in a complex during transcription termination, we tested if the loss of *RAT1* and *RAI1* genes leads to any DNA damage sensitivity like *rtt103* when exposed to DNA damaging agents. *rtt103*Δ cells, as described above, were sensitive to MMS, whereas, *rat1-1* and *rai1* mutants were not. To further investigate if these factors were required when specific double-strand breaks were introduced, we generated *rai1* and *rtt103* in a strain carrying galactose inducible HO endonuclease. Although *rtt10*3 were very sensitive to endonuclease induction, *rai1*Δ cells were not. UV sensitivity was also checked for these mutants and was observed that *rat1-1* and *rai1* mutants were not sensitive to UV damage. In the genome wide screens where *RTT103* was obtained as a factor involved in

maintaining genome stability, *RAT1* and *RAI1* were not obtained which indicate that transcription termination per se is not responsible for sensitivity of *rtt103* to DNA breaks and suggest a unique or atleast a more prominent role for *RTT103* in maintaining genome stability.

There is some indirect evidence from various genome-wide studies to suggest that *RTT103* might be involved in damage response and other chromosomal functions as Rtt103 interacts genetically with a plethora of genes involved in chromatin or genome stability (Cherry et al., 1998). For eg., *rtt103*Δ show increased Rad52 foci, a mark of increased spontaneous damage (Alvaro et al., 2007). These could be due to spontaneous breaks in the unperturbed cells. *rtt103* is synthetically sick with *dna2, orc2-1* and *orc5-1*. In all these networks neither *rat1* nor *rai1* appear. Given that *RAT1* is essential and may have been missed in these screens, if termination defects were the primary reason for picking up *rtt103*, *rai1* is likely to have appeared in a few of these screens. This suggests that *RTT103* may have an additional role in protecting genome integrity.

Because *rtt103* were sensitive to chromosomal breaks but not plasmid breaks, we tested if signalling could be affected in *rtt103*. In order to test this, we checked the phosphorylation of Rad53, the final effector kinase in the signalling pathway. WT and *rtt103*Δ cells were treated with MMS and it was observed that upon treatment Rad53 got phosphorylated in *rtt103* similar to WT. This phosphorylation was also seen in I-*Sce*I and *Eco*RI induced damage.

This experiment shows that the signalling of the damaged DNA is intact in *rtt103*. We checked the dephosphorylation in *rtt103* and the results showed that it is similar to WT. The downstream effect of this signalling is the transcriptional upregulation of the DNA damage signature genes (*DUN1*, *RAD54*, *RNR2*, *RAD51, PLM2* etc*)*. Their expression is substantially induced in response to both MMS and ionizing radiation (Gasch *et al.*, 2001). To test if *rtt103* had any defects in the induction of transcription, RNA levels of *RNR2, RAD51*, *RAD54, DUN1* and *PLM2* genes in WT and *rtt103* with and without the DNA damage, were tested by Northern blots . There was no difference in the levels of RNA induced between wild type and *rtt103*. This indicates that the *RTT10*3 deletion is unlikely to affect the levels of DDR genes. However, it is still possible that expression of genes other than these, are affected by *rtt103,* as the genome-wide transcriptional studies indicate transcriptional induction of several genes upon DNA damage. Nevertheless, these data confirm that the DNA damage is sensed and the core downstream response is activated in *rtt103*.

Maintenance of genome integrity is essential for survival of cells and multiple pathways contribute to this process. There are reports showing link between transcription associated processes and genome stability. For example, mutations in the components of the THO complex lead to transcription associated hyper-recombination phenotype (Jimeno *et al.*, 2002). Recently, this has been shown for a transcription termination factor in yeast and humans, Sen1 (Mischo *et al.*, 2011; Skourti-Stathaki *et al.*, 2011). In THO complex mutants

and *sen1-1* mutants there is increased chromosomal recombination induced by transcription when transcription occurs between direct repeats. Since *RTT103* is also a transcription associated factor, we hypothesized that due to improper processing of transcripts, R loop induced genome instability might be elevated in *rtt103*, and therefore tested the hyper-recombination phenotype. *rtt103* cells did not show hyper-recombination phenotype in two different assays. So we conclude that Rtt103 is unlikely to function in the same pathway as Sen1 to prevent genome instability. Another intriguing link between RNA processing and genome stability was found when the human pre-RNA processing complex was purified, KU70/80 and DNAPK were consistently copurified (Shi et al., 2009).

There are reports showing direct binding of the transcription associated factors at the sites of DNA damage. In bacteria it was shown that Rho, which is a transcription termination factor, binds to the DNA at the site of DNA damage and clears the RNAPolII from the site, so that the DNA is free for the repair proteins to bind and repair the DNA (Washburn and Gottesman, 2011). In mammalian cells CstF recruits BARD1/BRCA1 complex when there is DNA damage and promotes the ubiquitination and degradation of RNA PolII which is bound to the DNA at the site of damage. Alternately, this complex recruits the transcription coupled repair proteins which repair the DNA, and later RNApolII resumes its function (Mirkin *et al.*, 2008). So we hypothesized that Rtt103 associate with the DNA when there is damage. For checking the binding

of Rtt103, we have done chromatin immunoprecipitation (ChIP) experiments. There was no significant association of Rtt103 with any of the regions tested when the endonuclease was not induced. However, upon induction, the association of Rtt103 went up more than five-fold only in the vicinity of the cut site. *SPS2* and telomere regions did not show any increased association upon endonuclease induction, suggesting that Rtt103 protein binding is specific to regions of damage. These data strongly indicate that Rtt103 functions through association at the site of damaged DNA.

7.4 A model for the possible molecular function of Rtt103 at DNA damage sites

Rtt103 has been isolated with transcription termination complexes and our studies show that it is essential for DNA damage response. We suggest a hitherto unidentified role for this protein in genome stability. Although there is evidence of interaction between DNA repair and transcription, dynamics of RNA PolII is not completely understood. When there is DNA damage the RNA PolII transcription is arrested and it is cleared from the site so that the repair machinery can access and repair the damaged DNA. Recent studies have shown that RNAPI and RNAPII are affected when there are DSBs which leads to transient repression of transcription (Kim et al., 2007; Kruhlak et al., 2007; Shanbhag et al., 2010). Another recent study has shown that the transcription arrest is not due to the DSB per se but it is due to the DNAPK. If the activity of the kinase is lost, then the transcription is resumed which can lead to

production of mutated transcripts (Pankotai et al., 2012). Based on our observations we hypothesize that termination factor Rtt103 become associated with DNA damage sites to disengage RNA polymerase from the damaged sites in order to prevent synthesis of aberrant transcripts and to make the DNA available for repair by the repair machinery.

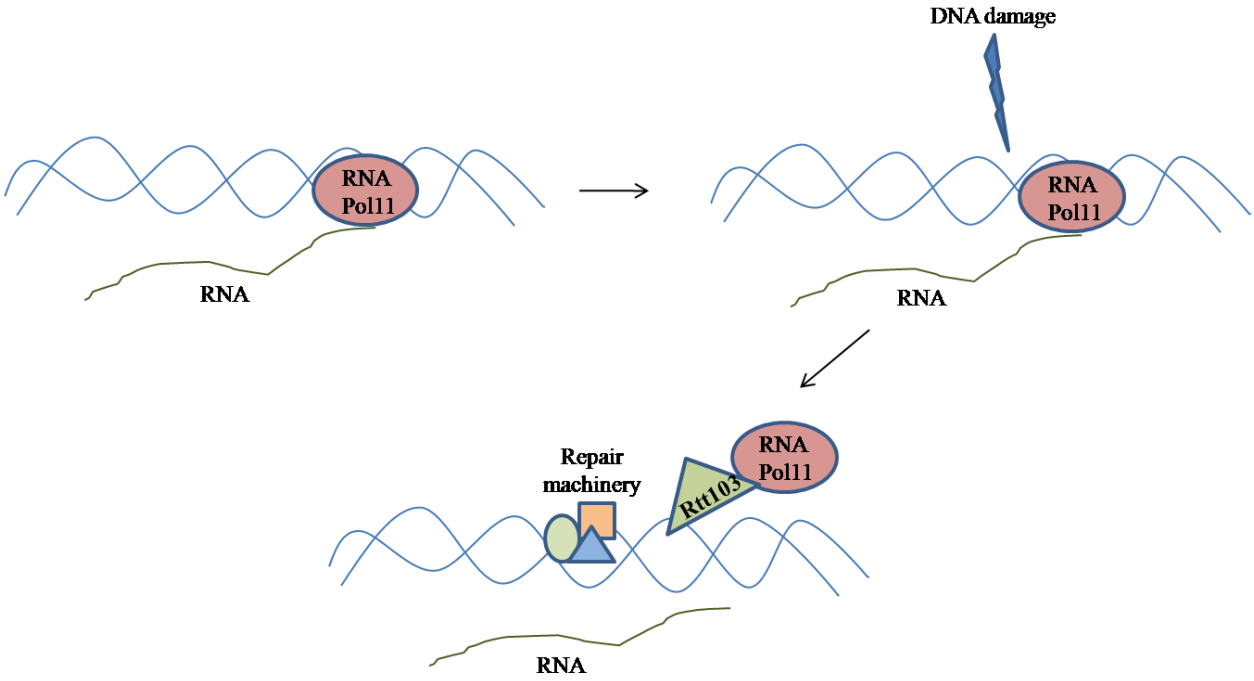


Figure 29: A possible mode of action for RTT103 at the site of damage

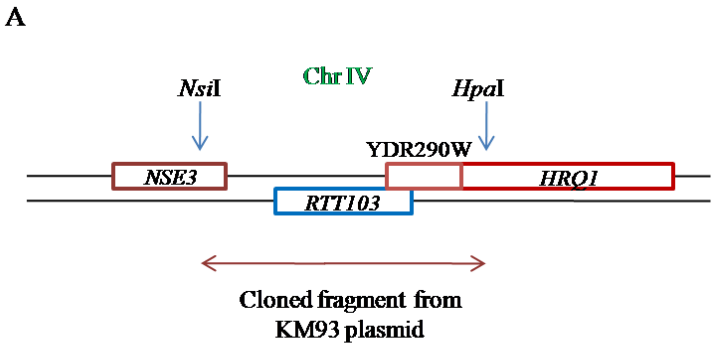
This model predicts that Rtt103 associates with RNA polymerase II upon DNA damage and clears the site. This can be tested by a detailed ChIP analysis by measuring RNApol II association with the DSB. Additionally, as the association is proposed to happen via the CID domain, this can be tested by making mutants of Rtt103 and further characterization of direct interactions between RNA polymerase II and Rtt103.

In summary, our studies have identified a novel factor that is essential for protection of the genome and suggest an important role for transcription termination factor(s) in response to DNA damage.

Appendix

1.1 Construction of *RTT103* clone in Yeplac181 vector (CKM233)

RTT103 gene was subcloned into Yeplac181, 2µ vector carrying *LEU2* and ampicillin resistance markers. KM93 genomic library plasmid was digested with *Hpa*I and *Nsi*I restriction enzymes and DNA fragment of size 2,500bp containing full length *RTT103* gene along with its promoter region was ligated into *Pst*I and *Sma*I digested Yeplac181 (CKM6) vector listed in Table 2. The resulting plasmid was confirmed by digesting with *Eco*RI restriction enzyme. Yeplac181 vector backbone contains one *Eco*RI site at poly cloning region and insert contains two *Eco*RI sites. Therefore, upon digestion, the clones give three fragments of size 6273, 1538 and 458bp as seen in figure A1.b.



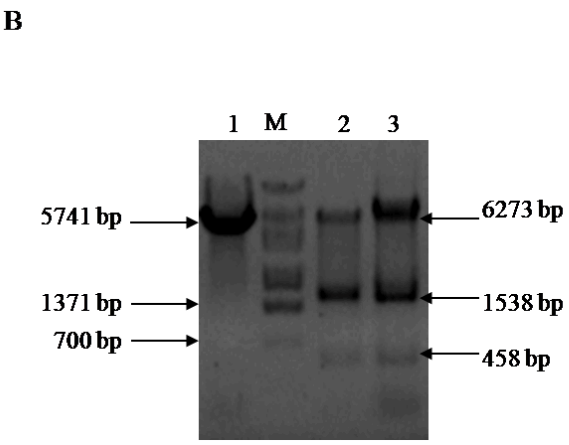


Figure A1: Construction of *RTT103* clone in Yeplac181 vector. (A) Schematic representation of digested fragment from KM93. (B) Confirmation of *RTT103* clone by digesting with *Eco*RI enzyme that releases three fragments of size 6.2kb, 1.5kb and 458bp. Lane 1 is *Eco*RI cut CKM6 and lanes 2 and 3 are *Eco*RI cut CKM 233 plasmid.

1.2 Construction of *RTT103* clone in YCplac22 vector (CKM261)

RTT103 gene was sub cloned in multi copy YCplac22 vector. CKM233 plasmid was digested with *Kpn*I & *Sph*I and 2.5 kb DNA fragment containing *RTT103* gene (1.2 kb) and its promoter region was cloned into *Kpn*I & *Sph*I digested YCplac22 vector. The resulted plasmid (CKM261) was confirmed by digesting with *Kpn*I & *Sph*I restriction enzymes. Therefore upon digestion with *Kpn*I & *Sph*I enzymes, the clone gives two fragments of size 4821bp and 2551bp as seen in figure A2.

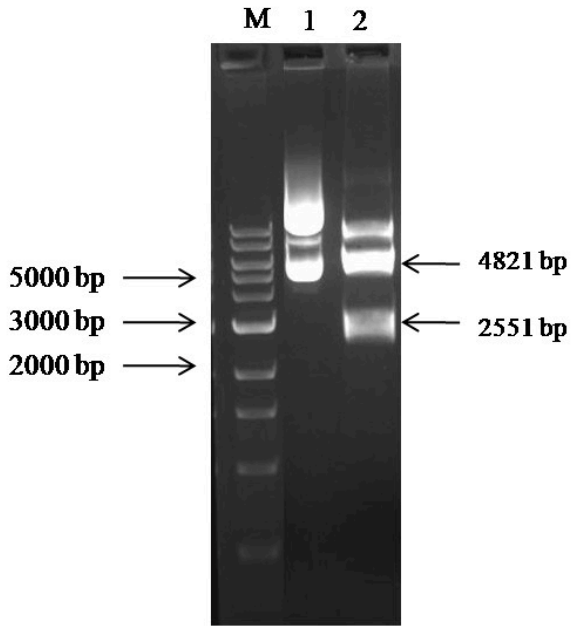


Figure A2: Confirmation of *RTT103* clone in YCplac22 vector (CKM261). Confirmation of *RTT103* clone by digesting with *Kpn*I & *Sph*I enzymes that releases two fragments of size 4.8kb (YCplac22 vector backbone) and 2.5kb (insert containing *RTT103* gene with promoter). Lane 1 represents uncut CKM1 and lane 2 represents *Kpn*I & *Sph*I digested CKM261.

2.1 Construction of *yku70::KanMx*

PCR amplification of DNA fragment for *YKU70* gene deletion. *yku70* null mutant was constructed by knocking off complete gene with *KanMx* marker by using PCR based homologous recombination method (Longtine et al., 1998). The forward primer for deleting *YKU70* gene was designed by selecting 47 bp just upstream of start codon and reverse primer by taking 50bp sequences downstream of stop codon. The sequences of the primers used are listed in table 3. Plasmid E335 (listed in table 2) was used as template for amplifying *KanMx* marker. The figure A3.B shows the DNA fragment of size 1559bp amplified by PCR (knock-out module). This DNA was transformed into competent yeast cells by LiAc method. It gets integrated into the genome by homologous recombination. The transformants were selected on YPD plate containing 200µg of G418 drug. Then single colonies were picked up and the genomic DNA was extracted by phenol:chloroform:isoamylalcohol (25:24:1) method.

Screening PCR for *yku70* null mutant. Screening PCR was done for *yku70* null mutant by using the forward primer (around 19bp) that gets annealed within the deletion module and the reverse primer (20nt) that anneals

with the unaltered downstream region of the *YKU70* gene. The sequences of the primers are given in table 3. The diagnostic PCR product of around 387bp in figure A3.C shows that *YKU70* gene has been replaced by *KanMx* marker by homologous recombination.

Southern confirmation for *yku70* null mutant. Genomic southern was done for further confirmation of *yku70* null mutant. Genomic DNA from yeast strains that showed positive for *YKU70* knockout in screening PCR was isolated by zymolyase method. This genomic DNA was subjected to 6hrs restriction digestion with *Kpn*I enzyme. The digested genomic DNA was run on 0.8% agarose gel and transferred to nylon membrane. The bands were detected by southern hybridization of the blot with alpha p-32 radiolabelled *KanMx* probe. . This was expected to yield 3.0kb and 2.0kb fragments in *yku70* null mutant and WT strain will not give any band. *KanMx* probe was made by digesting E335 plasmid (CKM67) with *Bam*HI and *Eco*RI enzymes. The DNA fragment of size 1500bp having KanMx gene was taken as template and radiolabelled using BRIT random radiolabelling kit. The blot was washed and exposed to autoradiogram. Figure A3.D shows the presence of 3.0kb and 2.2kb bands confirming that the particular yeast strain is *yku70* null mutant.

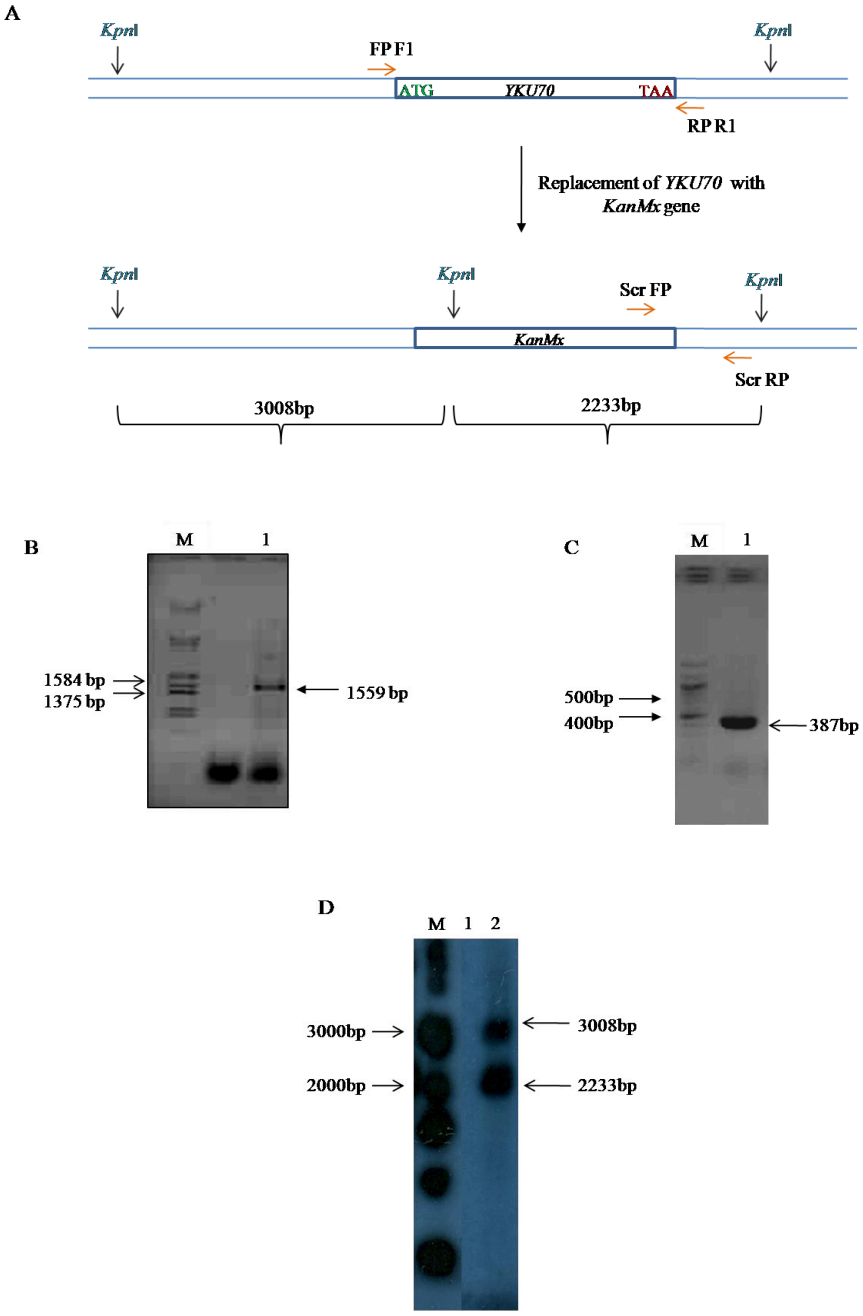
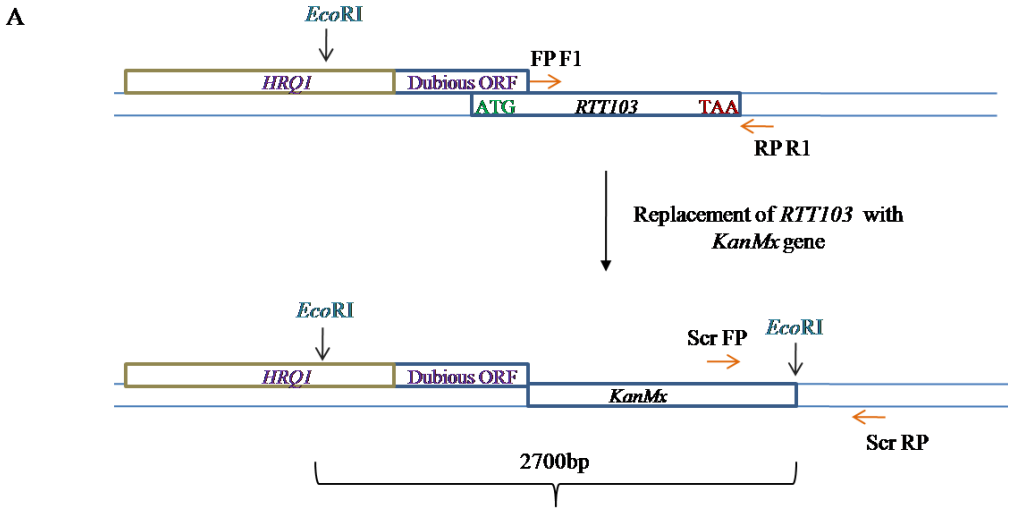


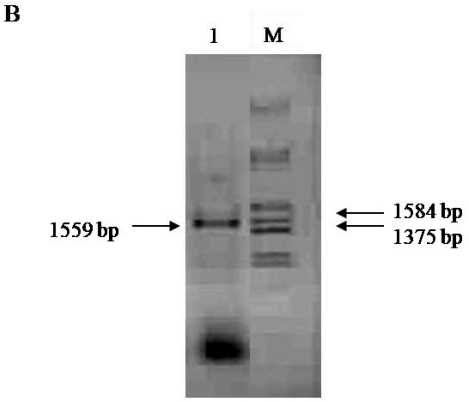
Figure A3: Construction of *yku70::KanMx* null mutant. (A) Schematic representation of knock out. (B) Lane 1 represents PCR product of size 1.5 kb used for replacing *YKU70* gene with *KanMx* marker. (C) Lane 1 reprsesents screening PCR product of size 387bp which shows that *YKU70* gene has been replaced with *KanMx* marker. (D) Genomic southern confirmation for *yku70::KanMx* null mutants. Lane 1 represents *Nco*I digested WT that gives no band and lane 2 represents *Kpn*I digested *yku70* which gives 3.0kb and 2.2kb bands.

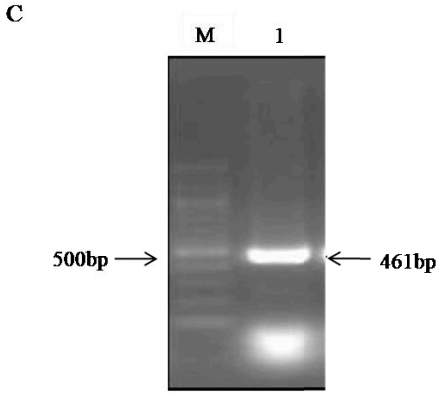
2.2 Construction of *rtt103::KanMx* null mutant

rtt103 knockout with *KanMx* marker was done by following the same method used for knocking out *yku70* gene (Appendix 2.1). The sequences of the primers used for deleting *RTT103* gene are given in table 3. Plasmid E335 listed in Table 2 was used as template to amplify *KanMx* marker for replacing *RTT103* gene. The figure A4.B shows the PCR product of *KanMx* (knock out module of 1559bp). This DNA was transformed into yeast strain. The strain transformed with *KanMx* marker was selected on YPD plate containing 200µg of G418 drug. These transformants were subjected to screening PCR. The sequences of the primers used for screening PCR are given in Table 3. The diagnostic screening PCR product of size 461bp in figure A4.C shows that *RTT103* gene has been replaced with *KanMx* marker.

These strains were further confirmed by genomic southern by hybridizing with *KanMx* probe. The genomic DNA from these strains was digested with *Eco*RI enzyme. WT strain gives no band and *rtt103::KanMx* null mutant gives band at 2.7kb. *KanMx* probe was made by digesting E335 plasmid (CKM67) with *Bam*HI and *Eco*RI enzymes. The DNA fragment of size 1500bp having KanMx gene was taken as template and radiolabelled using BRIT random radiolabelling kit. The result of the autoradiogram of southern blot in figure A4.D shows no band in WT strain and 2.7kb band in *rtt103::KanMx* null mutant which confirms that these yeast strains are *rtt103* null mutants.


A
EcoRI
FP F1
HRQ1
Dubious ORF
ATG
RTT103
TAA
RP R1
Replacement of RTT103 with KanMx gene
EcoRI
Scr FP
EcoRI
HRQ1
Dubious ORF
KanMx
Scr RP
2700bp


B
1
M
1559 bp
1584 bp
1375 bp


C
M
1
500bp
461bp

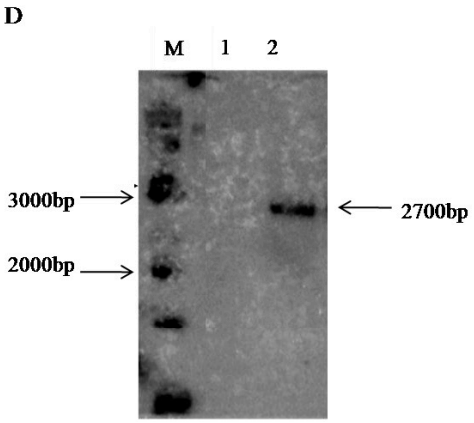

D
M
1
2
3000bp
2000bp
2700bp

Figure A4: Construction of *rtt103::KanMx* null mutant. (A) Schematic representation of knock out. (B) Lane 1 represents PCR product of size 1.5kp used for replacing *RTT103* gene with *KanMx* marker. (C) Lane 1 represents screening PCR product of size 461bp shows that *RTT103* gene has been replaced with *KanMx* marker. (D) Genomic southern confirmation of *rtt1031::KanMx* mutant. Lane 1 represents *Eco*RI digested WT that gives no band and lane 2 represents *Eco*RI digested *rtt103* that gives 2.7kb band.

2.3 Construction of Rtt103-13x-myc strain

Rtt103p was tagged with 13x myc epitope at its C-terminus by PCR based homologous recombination method (Longtine et al., 1998). The forward primer was designed by taking sequence just upstream of the stop codon and in frame so that it does not disrupt the reading frame of the myc epitope and the selectable marker was *KanMx* which would be inserted downstream of the gene. The sequences of the primers are given in Table 3. Plasmid E358 (listed in table 2) was used as template for PCR to amplify 13x myc-*KanMx* DNA fragment. Figure A5.B shows the PCR product of 13x myc-*KanMx* DNA fragment of size 2300 bp which is transformed into yeast strain by following high efficiency LiAC transformation protocol. The transformants were selected on YPD medium containing 200µg G418 drug. The colonies that grew on G418 medium were subjected to screening PCR. The sequences of the primers used for screening PCR are given in Table 3. The diagnostic screening PCR product of 461bp in figure A5.C shows that Rtt103p is tagged with myc epitope at its C-terminus.

These strains were further confirmed by genomic southern by hybridizing with *KanMx* probe. The genomic DNA from these strains was digested with *Bgl*II restriction enzyme by incubating at 37°C for 6-8hrs. WT strain gives no band, whereas Rtt103-13xmyc-*KanMx* strain gives band of size 2.0 kb with *Bgl*II enzyme. A DNA fragment of size 1500bp having KanMx gene resulting from digestion of E335 plasmid was taken as template and radiolabelled using BRIT random radiolabelling kit. The result of the autoradiogram of southern blot in figure A5.D shows no band in WT strain and 2kb band for Rtt103 13xmyc confirming that the yeast strain is tagged with 13myc epitope at C-terminus of Rtt103p.

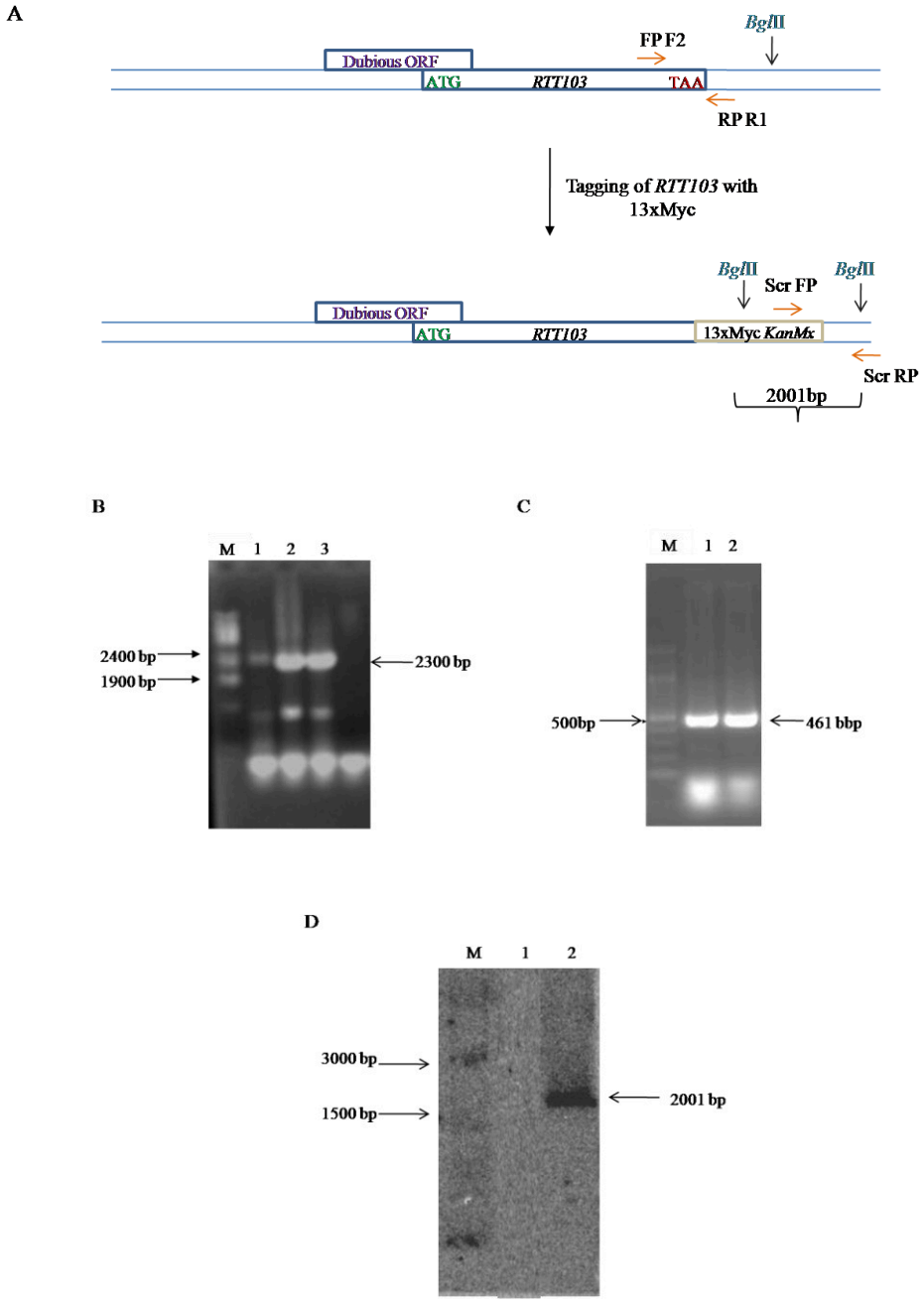


Figure A5: Construction of Rtt103-13xmyc-*KanMx*. (A) Schematic representation of tagging. (B) Lanes 1, 2 and 3 represents 2.3kb PCR product used for tagging 13x myc at C-terminus of *RTT103* gene. (C) Lanes 1 and 2 represent screening PCR product of size 461bp shows that *RTT103* gene has been tagged with 13x myc-*KanMx* at its C-terminus. (D) Genomic southern confirmation of Rtt103-13xmyc-*KanMx* strain. Lane 1 represents *Bgl*II digested WT strain that gives no band and lane 2 represents *Bgl*II digested Rtt103 13xmyc that gives 2.0kb band

2.4 Construction of Yku80-13x-myc strain

Yku80p was tagged with 13x myc epitope at its C-terminus by PCR based homologous recombination method (Longtine et al., 1998). The forward primer was designed by taking sequence just upstream of the stop codon and in frame so that it does not disrupt the reading frame of the myc epitope and selectable marker used was *KanMx*. The sequences of the primers are given in table 3. Plasmid E358 (listed in table 2) was used as template for PCR to amplify 13x myc-*KanMx* DNA fragment. Figure A6.B shows the PCR product of 13x myc-*KanMx* DNA fragment of size 2300 bp which is transformed into yeast strain by following high efficiency LiAC transformation protocol. The transformants were selected on YPD medium containing 200µg G418 drug. The colonies that grew on G418 medium were subjected to screening PCR. The sequences of the primers used for screening PCR are given in table 3. The diagnostic screening PCR product of 465bp in figure A6.C shows that Yku80p is tagged with myc epitope at its C-terminus.

These strains were further confirmed by genomic southern by hybridizing with *KanMx* probe. The genomic DNA from these strains was digested with *Kpn*I restriction enzyme by incubating at 37°C for 6-8hrs. A DNA fragment of size 1500bp having KanMx gene resulting from digestion of E335 plasmid was taken as template and radiolabelled using BRIT random radiolabelling kit. The result of the autoradiogram of southern blot in figure

A6.D shows no band in WT strain and 3.2 kb band for Yku80 13xmyc confirming that the yeast strain is tagged with 13myc epitope at C-terminus of Yku80p.

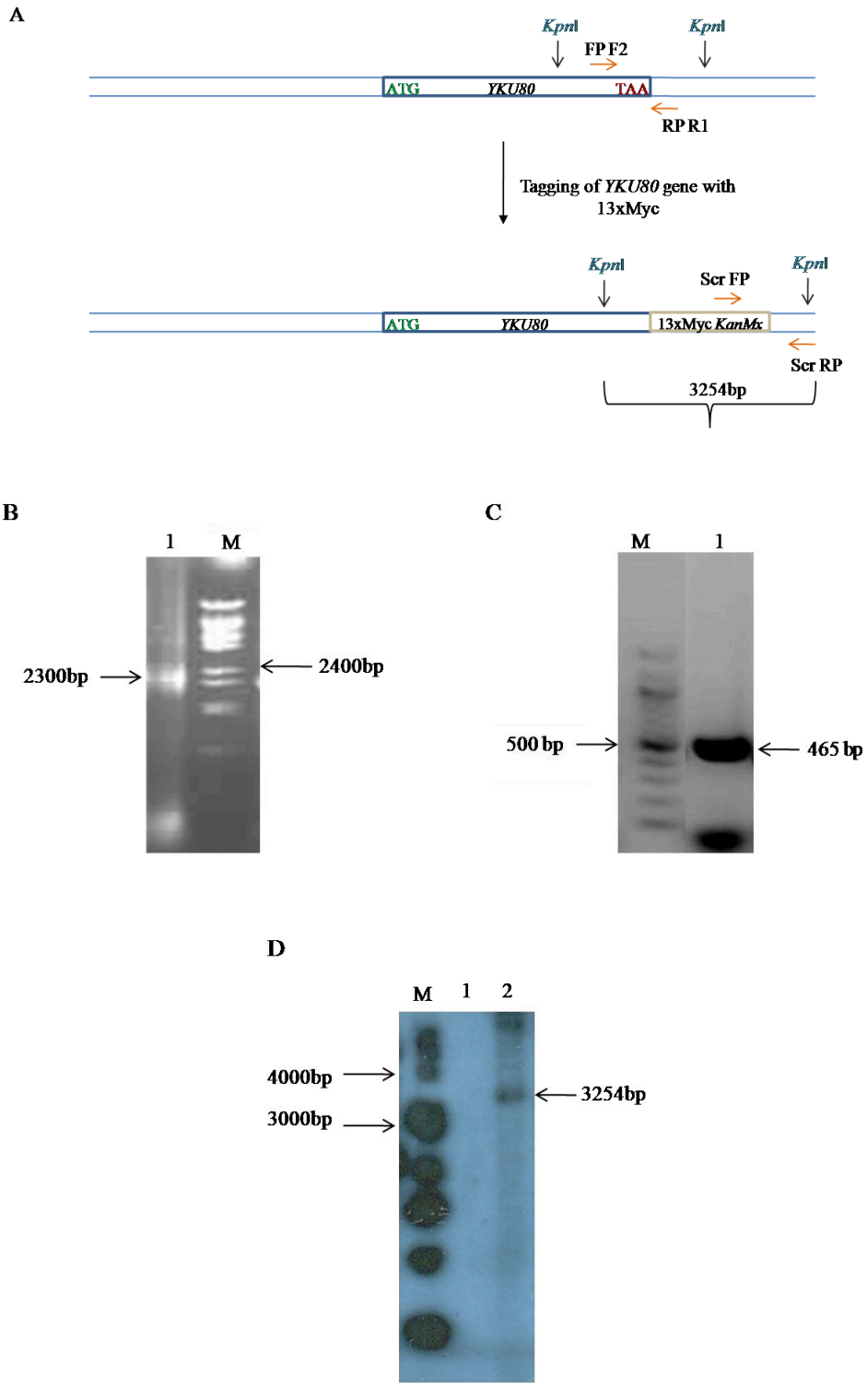


Figure A6: Construction of Yku80-13xmyc-*KanMx*. (A) Schematic representation of tagging. (B)Lane 1 represents 2.3kb PCR product used for tagging 13x myc at C-terminus of *YKU80* gene. (C) Lane 1 represents screening PCR product of size 465bp shows that *YKU80* gene has been tagged with 13x myc-*KanMx* at its C-terminus. (D) Genomic southern confirmation of Yku80-13xmyc-*KanMx* strain. Lane 1 represents *Kpn*I digested WT strain that gives no band and lane 2 represents *Kpn*I digested Yku80 13xmyc that gives 3.2kb.

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