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School of Biology

Genes required to maintain telomeres in  
the absence of telomerase in  
*Saccharomyces cerevisiae*

*by*

**MOHAMMAD KDAIMES H. ALOTAIBI, MSc**

Thesis submitted to the University of Nottingham for  
the degree of Doctor of Philosophy

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## Abstract

In the absence of telomerase, *Saccharomyces cerevisiae* telomeres erode leading to senescence. Rare cells can survive after this stage as they can elongate their telomeres utilizing homologous recombination. Two different types of survivors can be easily distinguished by Southern blot. Type I survivor cells, elongate the telomere by amplifying Y' elements and require *RAD51*, *RAD54*, *RAD55* and *RAD57* for establishment. Type II survivors elongate their telomere by amplifying TG<sub>1-3</sub> repeats, however, they require the following genes to be established: *RAD50*, *MRE11* and *XRS2*, *RAD59*, *SGS1* and *KU80* in some cases. Both types require the gene *RAD52*.

In this study several candidate genes were deleted individually in diploid type II survivor strains. The main aim of this work was to see if these genes were required for type II telomere maintenance. Most of these genes are not required for type II telomere maintenance at least until ~150 generations after deleting these genes. The exceptions were *KU80* and *RPB9*. *Ku80*Δ strains switched to a new survivor type that is similar to type I and continued for the long term. *RPB9* was required for two independent type II survivor strains to survive, whereas the third type II strain did not require this gene at ~150 generations after deleting the gene. After many generations (~ 350), this strain switched to type I.

At long term propagation (~500 generations) after deletion of the candidate genes, all type II strains displayed telomere shortening until the propagation was stopped. However, *Rad50*Δ strains switched to type I after long term.

Finally, the absence of the candidate genes did not affect the sensitivity of type II survivor strains to temperature. On the other hand, type II survivor strains with some genes deleted displayed sensitivity to UV.

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# Glossary

## Abbreviations:

ALT	Alternative lengthening of telomeres
APBs	ALT-associated PML bodies
BER	Base Excision Repair
BIR	Break induce replication
C	Cytosine
CHEF	Clamped homogenous electric fields
DDR	DNA damage response
DSB	Double strand break
DSBR	Double strand break repair
DNA	Deoxyribonucleic acid
EDTA	Ethylenediaminetetraacetic acid
EtBr	Ethidium bromide
FANCD2	Fanconi anaemia group D2
FEN1	Flap endonuclease 1
G	Guanine
HAATI	Heterochromatin amplification-mediated and telomerase-independent
HR	Homologous recombination
LiAc	Lithium Acetate
LB	Luria Bertani
MA	Maleic acid
MMR	Mismatch repair
MRX	MRE11/RAD50/XRS2 complex
NER	Nucleotide excision repair
NHEJ	Non-homologous end joining
PCR	Polymerase chain reaction
PEG	Polyethylene glycol
PML	Promyelocytic leukaemia
RB	Retinoblastoma
RCR	Rolling-circle replication
RNA	Ribonucleic acid
SDS	Sodium dodecyl sulphate
SGD	Saccharomyces Genome Database
SLS	Scientific Laboratory Supplier
SSA	Single strand annealing
ssDNA	Single strand DNA
T	Thymine
T-loop	Telomere-loop
TBE	Tris-borate-EDTA buffer
TE	Tris-EDTA buffer
TERT	Telomerase reverse transcriptase
UV	Ultra-violet
YEPD	Yeast extract Peptone Dextrose medium

## **Gene and protein nomenclature**

Gene names are written in upper case italics, while proteins names are written in standard upper case followed by p (e.g. *RAD50* is the gene and RAD50p is the protein). Genes knockouts are lower case italics and given the  $\Delta$  suffix (*rad50* $\Delta$ ) or the connector::<sub>2</sub> followed by the knockout cassette used (*rad50*::KANMX).

## **Chapter 1: Introduction**

### ***1.1. Telomere historical background***

Telomeres were discovered in the 1930s. In 1938, Hermann Muller observed a difference between the DNA breaks and the chromosome ends in *Drosophila melanogaster* (reviewed in (Gilson and Segal-Bendirdjian, 2010)). He recognized that chromosome ends are able to protect themselves from end to end fusions. A few years later (in 1941) Barbara McClintock discovered the same role of chromosome end protection in *Zea mays* (McClintock, 1941). Both studies revealed that changes such as rearrangements can occur at internal DNA breaks but not at chromosomes ends. Therefore, they hypothesized a special structure that provides chromosome end protection. H. Muller called this structure a telomere (from the Greek *telos* for end and *meros* for part). At that time, telomeres were known to provide DNA integrity, but it was not known yet what their real structure and how they work (Gilson and Segal-Bendirdjian, 2010). Telomere structure was later known as DNA-protein complexes that cap chromosomes ends providing stability.

In the 1960s, Hayflick described a new view of cellular aging. He discovered that human diploid cells can multiply for a limited time in culture. Cells can only reach a certain number of divisions in vitro, the maximum number of divisions termed the "Hayflick limit" (Shay and Wright, 2000). At the Hayflick limit, cells displayed morphological and biological changes leading to cell cycle arrest. This is called "cell senescence" (Shay and Wright, 2000).

In the 1970s, James Watson and Alexey Olovnikov noted that the DNA replication cannot continue till the end of linear chromosome (reviewed in

(Gilson and Segal-Bendirdjian, 2010). It is known that the DNA polymerases replicate DNA in the 5'-3' direction with an RNA primer required for this mechanism. DNA replication cannot reach the chromosome end, raising a specific problem. J. Watson called this problem as "end replication problem". As result of this problem, telomeres erode with each cell division. This erosion will stop cellular propagation when telomeres are too short (Gilson and Segal-Bendirdjian, 2010).

At the same time Alexey Olovnikov was able to find a link between the end replication problem, described by Watson and the cellular senescence, described by Hayflick. He recognized that the DNA shortening with each cell division may explain why cell propagation can only continue for a certain numbers of doublings. This view can explain the Hayflick limit (Olovnikov, 1973). Thus, telomere shortening in normal human cells can be used as cellular clock that displays how many times cells were replicated (Gilson and Segal-Bendirdjian, 2010).

In the 1978, Elisabeth Blackburn found a specific hexameric repeat at the telomere of the ciliated protozoan, *Tetrahymena thermophila*. This repeat consists of TTGGGG sequence providing telomere protection from degradation (Blackburn and Gall, 1978, Szostak and Blackburn, 1982). Ciliate telomeric DNA present at one end of linear chromosome was found to serve as template for synthesis of a new telomere in budding yeast (Szostak and Blackburn, 1982). Thus, the telomeric repeat was hypothesized to be conserved in eukaryotes and involved in an important mechanism for cell maintenance (Gilson and Segal-Bendirdjian, 2010). Later on, telomeres in human cells were

found to also consist of repeats, but the sequence is TTAGGG (Moyzis et al., 1988).

In 1985, C. Greider and E. Blackburn discovered the enzyme that replicates telomeres, solving the end replication problem (Greider and Blackburn, 1985). This enzyme helps cells to divide without losing telomeres. The enzyme was first called "a telomere terminal transferase". The purification of this enzyme showed that it consisted of an RNA component and a protein. The RNA part contained the CCCCAA sequence that used as template for the TG telomere, whereas the protein component was necessary for the enzymatic function as reverse transcriptase (Greider and Blackburn, 1987, Greider and Blackburn, 1989). More details about human and yeast telomere structure and functions are given in the following sections.

### ***1.2. Introduction to telomeres and their functions***

Telomeres are DNA-protein structures found at the ends of eukaryotic linear chromosomes and are necessary for genome stability (O'Sullivan and Karlseder, 2010). The important functions of this structure are provided by its short unique repetitive sequences that are termed TG<sub>1-3</sub> repeats. This G-rich repeat is heterogeneous in its sequence and size among most eukaryotic species. The TG<sub>1-3</sub> repeat terminus is elongated by the ribonucleoprotein telomerase (Cech and Lingner, 1997, Cech et al., 1997, Lingner and Cech, 1998). There are several proteins that associate with telomeres to enable them to achieve their functions. Telomeres provide crucial functions for chromosome ends that lead to genome stability. Telomeres protect chromosome ends from degradation and end to end fusions allowing the cells



to distinguish telomeres from double strand breaks (DSB), and they have an important role in chromosome segregation. End protection and replication are the most important functions of telomeres (Pampalona et al., 2010).

### **1.2.1. Telomere protection**

Telomere protection is an important property that is required for genome integrity. However, telomere protection can be provided by different factors. First, telomerase is essential for telomere protection. It is activated to elongate short telomeres (Cech et al., 1997, Lingner and Cech, 1998). Therefore, telomerase always keeps telomeres at an optimal length to protect telomeric DNA, subtelomeric DNA and nearby genes from degradation. Second, the single-strand DNA binding proteins, human POT1 and yeast CDC13p have a significant role to protect chromosome ends from degradation and end to end fusion, along with STN1p and TEN1p (Pennock et al., 2001, Garvik et al., 1995, Baumann and Cech, 2001). Third, the KU heterodimer proteins (KU70/80) also have been found as a main factor for telomere protection. They protect telomeres from nucleolytic degradation and end to end fusion (Hsu et al., 2000, Polotnianka et al., 1998, Fisher and Zakian, 2005). However, it has been recently found that EST1p provide a new pathway for telomere protection that differs from CDC13p and KU proteins (Tong et al., 2011).

### **1.2.2. Telomere replication**

Replication origins located in sub-telomeric region are expected to initiate the telomere replication mechanism (Wellinger et al., 1993). Small RNA primers

initiate the lagging strand synthesis to replicate the G-rich strand. The last RNA primer is removed to leave a gap which cannot be filled. The C-rich strand is replicated by leading strand synthesis. A blunt end is formed as result of this replication. This end is resected by nucleases and helicases generating a TG-overhang, reviewed in (Sampathi and Chai, 2011). Telomeres are mainly maintained by telomerase (Lundblad, 2003). Telomerase consists of two central components (Greider and Blackburn, 1985, Greider and Blackburn, 1987). The catalytic subunit which is a reverse transcriptase, and the RNA template, which varies in size and sequence throughout species. The telomerase RNA component (the template) is complementary to telomeres' TG repeat (Singer and Gottschling, 1994, Feng et al., 1995). Telomerase uses its RNA component to extend telomeres. However, telomeres can be extended in the absence of telomerase (Lundblad and Blackburn, 1993, Sandell and Zakian, 1993, Teng and Zakian, 1999). The telomerase-independent telomere replication relies on the homologous recombination pathway (HR) (Teng and Zakian 1999).

### ***1.3. The structure of telomeres and subtelomeres***

#### **1.3.1. The structure of *S. cerevisiae* telomeric and subtelomeric regions**

Telomeres in *S. cerevisiae* consist of  $350 \pm 50$  bp of heterogeneous TG<sub>1-3</sub> repeats (Wang and Zakian, 1990, Shampay et al., 1984) (Fig. 1.1). RAP1p is a DNA binding protein and is involved in telomere length regulation. The TG<sub>1-3</sub> repeat has RAP1p binding sites every 18 bp (Gilson et al., 1993). RAP1p plays important roles in transcriptional activation and repression, recombination, gene silencing, and telomere structure, and is essential for cell growth (Shore, 1994).

The *S. cerevisiae* subtelomeric region consists of two main components, core-X elements and Y' element (Fig. 1.1). Core-X is present at all yeast chromosomes ends (Louis, 1995). It is approximately 475 bp, and contains an ARS (Autonomously replication sequence), and in most cases contains a binding site for (ARS) binding factor 1 ABF1p (Louis et al., 1994, Pryde et al., 1995). Core-X, along with yKU is thought to play an important role in genome stability by mediating a fold-back structure that represses recombination at yeast telomeres (Marvin et al., 2009a, Marvin et al., 2009b). Moreover, SIR3p and RAP1p bind the X-elements (Zhu and Gustafsson, 2009), therefore, X-elements have a crucial role in silencing adjacent genes. SIR3p and RAP1p at X-elements are thought to contact the same proteins bound to the TG repeats, which leads to the fold-back structure at telomeres (Zhu and Gustafsson, 2009). ABF1p is a site-specific DNA binding protein found at many locations in the yeast genome. It is a multifunctional protein expressed in *S. cerevisiae* (Chasman et al., 1990), it is also involved in gene silencing within subtelomeric regions (Pryde and Louis, 1999). There is a GC-rich sequence in between ARS and the Abf1p binding site (Pryde et al., 1995).

The Y' element component is found adjacent to the TG<sub>1-3</sub> repeat sequence at some yeast telomeres (Louis, 1995). It is highly conserved, and found in 0-4 tandem copies (Chan and Tye, 1983b, Walmsley et al., 1984, Chan and Tye, 1983a). There are two classes of Y' elements, Y'-long (6.7 kb) and short (5.2 kb) (Louis and Haber, 1990b, Louis and Haber, 1992, Chan and Tye, 1983b). Y' elements vary between strains in terms of their copy number and location. 26 to 30 Y's were found in the YP1 strain, falling into both sizes. These Y's

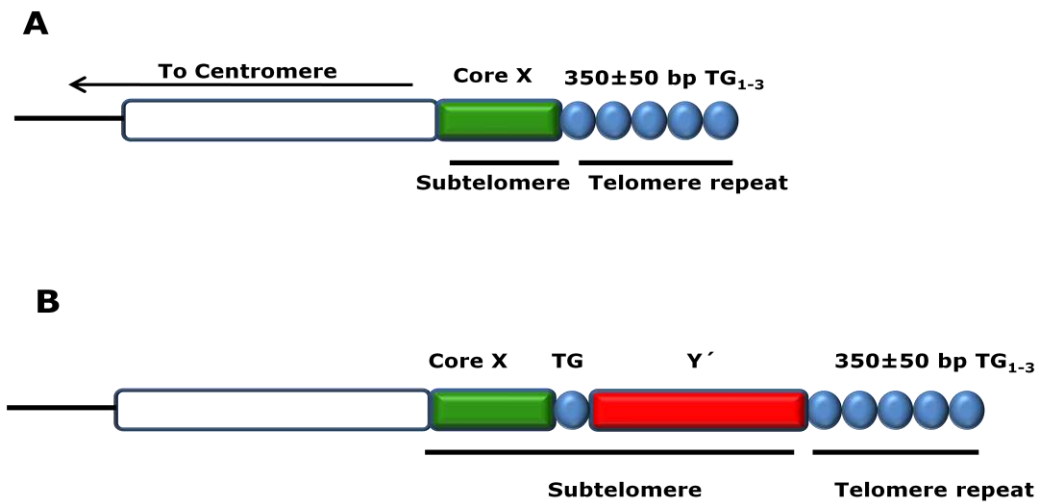
were found at 19 out of 32 telomeres, concentrated in the large chromosomes. Whereas the Y55 strain only has 14 to 16 Y's in both sizes (Louis and Haber, 1990b, Walmsley et al., 1984). The presence of Y' element tandem arrays can be found in long or short size, but not a combination of both sizes (Louis and Haber, 1990b). They are separated by short sequences (50 -100 bp) of TG<sub>1-3</sub> repeats (Walmsley et al., 1984, Louis et al., 1994). Y' elements are thought to have originated as mobile elements (Louis and Haber, 1992). Mobile elements have been found to play important role in telomere maintenance in some eukaryotes. For instance, *Drosophila* uses retrotransposons to lengthen its telomeres (Pardue and DeBaryshe, 2008). Y' elements can move from one telomere to others by recombination generating tandem copies. These elements can be lost from the chromosome ends as a result of recombination between the TG repeats or X elements (Louis and Haber, 1990a). However, the subtelomeric regions in *S. cerevisiae* display very low levels of reciprocal recombination compared to the adjacent regions, which exhibit high recombination rates (Barton et al., 2008).

Several small elements called STR-D, C, B and A are located between core X and the telomere, or Y' elements if present (Louis et al., 1994). Variable short TG sequences have also been found between core X and Y' element (Walmsley et al., 1984, Louis et al., 1994). Core X and Y' elements are assembled in nucleosomes, whereas, the TG<sub>1-3</sub> repeat are assembled in non-nucleosomal chromatin structure termed the telosome (Wright et al., 1992).

### **1.3.2. The structure of human telomeric and subtelomeric region**

Telomeres in human cells consist of a repeat sequence (TTAGGG)<sub>n</sub> at an average of 5 kb to 15 kb in somatic cells (Moyzis et al., 1988). This repeat sequence has been found to be greater than 20 kb in germline tissues down to 2 kb in senescing cells (Levy et al., 1992). Telomeres in humans end with a single strand TG repeat that is heterogeneous in size (~35 to ~600 bp) (Sfeir et al., 2005). This single strand is involved in the formation of a T-loop structure (Griffith et al., 1999). Complex regions of segmental duplicated DNA tracts that are termed subtelomeric repeat DNA, are found adjacent to the human terminal repeat. The duplicated tracts have a high sequence similarity (90% to > 99.5%), and they vary in segment length (1 kb to >200 kb), reviewed in (Riethman et al., 2005).

Several proteins play crucial roles in human telomere biology. They consist of six subunits called shelterin, and they involve TRF1p, TRF2p, TIN1p, RAP1p, TPP1p, and POT1p. Three of the shelterin subunits, TRF1p, TRF2p, and POT1p directly connect to the telomere repeat and they are interconnected by the other subunits, TIN2p, TPP1p, and RAP1p. TRF1p and TRF2p, both bind to the sequence, 5'-TAGGGTT-3' in duplex DNA (Court et al., 2005, Hanaoka et al., 2005). The most conserved component of shelterin, POT1p has two strong binding sites specifically to a single-strand, 5'-(T) TAGGGTTAG-3' at 3' end and internal locations (Lei et al., 2004, Loayza et al., 2004). However, shelterin proteins are involved in many aspects of human telomeres. For example they have a significant role in forming T-loop structures and repressing HR. However, non-shelterin proteins such as the MRE11- complex



**Figure 1.1. Telomere and subtelomere structure of *Saccharomyces cerevisiae***

**A-** Telomere has only core X. **B-** telomere that has core X and Y' element. Two types of telomere are displayed, some with only core X and some with core X and Y' element. Y' element can be found in 0-4 copies at telomere. Telomeres end with TG repeats. Core X and Y' elements and Y' elements themselves are separated by short TG repeats.

(MRE11, RAD50, and NBS1) are also involved in human telomere function and structure (Assenmacher and Hopfner, 2004).

### **1.3.3. Telomeric and subtelomeric in other organisms**

Some organisms have different telomere structures than the usual TG repeat that is present at yeast and human telomeres. *Drosophila melanogaster* has a special telomere structure that includes tandem arrays of retrotransposable elements, HeT-A, TART and TAHRE. Telomeres are elongated by transposition of these three unusual structures (Pardue and DeBaryshe, 2008). In the plant *Alliaceae* and some related species, cells display a highly repetitive satellite and or rDNA sequences (Pich et al., 1996), also the mosquito *Anopheles gambiae* exhibits similar repetitive satellite structures (Biessmann et al., 1996). These telomere patterns are thought to be replicated by recombination.

## **1.4. Mechanisms of chromosome end protection**

### **1.4.1. Single stranded overhangs at the chromosome end**

The telomeric DNA consists of TG repeat heterogeneous structure. The G-rich strand runs in the 5' to 3' direction towards the chromosome end. While, the complementary C-rich strand run in the opposite direction (5' - 3'). The G-rich strand is longer than the C-rich forming a 3' end single strand. Two different DNA synthesis mechanisms contribute to generate the telomeric single strand. Lagging strand synthesis occurs on the G-rich strand and ends with a gap which cannot be repaired. This gap results from the removal of RNA primer fragment from replication, therefore a single strand is generated.

Leading strand synthesis by telomerase generates a blunt end DNA structure that is acted upon by a nuclease to form the 3' end (Chai et al., 2006, Jacob et al., 2003, Makarov et al., 1997). Single strand lengths vary among species. The *S. cerevisiae* single strand is 12 – 14 bp long (Larrivee et al., 2004). Human cells have a single strand with an average of 35 to 600 bp (Sfeir et al., 2005). The single strand is a substrate for telomerase to elongate telomeres (Cech et al., 1997, Lingner and Cech, 1998).

The 3' end single strand is capable of forming secondary structures that are thought to play an important role in telomere biology. The most likely role of this structure is protection of the chromosome ends (de Lange, 2002). The T-loop is a common secondary structure in higher eukaryotes that forms by invasion of the single strand into the telomere repeat from the same chromosome (Griffith et al., 1999). It has been found in humans, mice, chickens, plants, ciliates and Trypanosomes (Cesare et al., 2003, Griffith et al., 1999, Murti and Prescott, 1999, Nikitina and Woodcock, 2004). Folding back or T-loop structures are mediated by protein-protein interactions that are proposed to occur in *S. cerevisiae* (de Bruin et al., 2000, de Bruin et al., 2001, Pryde and Louis, 1999). T-loop formation is mediated by human TRF2 protein (Stansel et al., 2001, Amiard et al., 2007).

#### **1.4.2. Telomere associated proteins in *S. cerevisiae***

Many proteins are directly or indirectly associated with telomeres in *S. cerevisiae*. CDC13p is a single strand binding protein (Bourns et al., 1998, Lin and Zakian, 1996). CDC13p assembles with STN1p and TEN1p to form the



CST (Cdc13/ Stn1/Tin1) complex that provides telomere protection (Grandin et al., 2001, Grandin et al., 1997). Moreover, CDC13p interacts with the catalytic subunit of DNA polymerase, Pol1 and the telomerase RNA-associated protein, EST1. This interaction helps telomerase to replicate telomeres (Evans and Lundblad, 1999, Qi and Zakian, 2000). RAP1p is a DNA binding protein that binds to the telomere at every 18 bp (Gilson et al., 1993). It is also negatively regulates telomere length by the interaction with RIF1p and RIF2p (Wotton and Shore, 1997, Marcand et al., 1997). KU (ku70/ ku80) proteins play important role to lengthen telomeres (Stellwagen et al., 2003, Zappulla et al., 2010). The DNA DSB repair MRX-complex (MRE11p, RAD50p, and XRS2p) is also involved in telomere biology (Assenmacher and Hopfner, 2004).

#### **1.4.3. Telomere associated proteins in human**

A number of proteins are associated with telomeres in human cells. These proteins form a shelterin complex which consists of TRF1p, TRF2p, POT1p, TIN2p, TPP1p, and RAP1p (de Lange, 2005). POT1p is a single stranded DNA binding protein that binds to the 3' overhang in human cells (Lei et al., 2004, Loayza et al., 2004). TRF2p is proposed to play an important role in T-loop formation (Griffith et al., 1999, Stansel et al., 2001). It has been recently found that Rap1p inhibits non-homologous end joining (NHEJ) mechanism, leading to genome stability (Sarthly et al., 2009). The DSB repair proteins (MRE11p, RAD50p, NRS2p) also play an important role in telomere maintenance (Lamarche et al., 2010). The KU heterodimer proteins enhance TRF2p chromatin association, therefore affecting telomere protection (Fink et al., 2010).

## ***1.5. Telomere maintenance by telomerase***

### **1.5.1 The holoenzyme telomerase**

Telomerase is the enzyme that replicates telomere repeats. It is a ribonucleoprotein enzyme and consists of two core components (Greider and Blackburn, 1987, Greider and Blackburn, 1985). Telomerase components are the highly conserved catalytic subunit, reverse transcriptase and the RNA template. The catalytic subunit in human cells is TERT (telomerase reverse transcriptase) (Meyerson et al., 1997, Nakamura et al., 1997) and in *S. cerevisiae* cells is EST2p (ever short telomeres 2) (Lendvay et al., 1996, Lingner et al., 1997). The RNA template varies among species regarding its size and template sequence. In humans, the RNA template contains 11bp (5'-CUAACCCUAAC-3') which is complementary to the human telomere repeat (TTAGGG) (Feng et al., 1995). In *S. cerevisiae*, the *TLC1* gene encodes the RNA template that contains 5'-CACCACACCCACACAC-3' which is complementary to yeast telomere repeat TG<sub>1-3</sub> (Singer and Gottschling, 1994).

### **1.5.2. Telomerase accessory proteins**

There are a number of proteins that are associated with telomerase. Some of these proteins are conserved among species.

#### **1.5.2.1. Telomerase associated proteins in *S. cerevisiae***

The two main components of telomerase are the catalytic reverse transcriptase (EST2p) (Lingner et al., 1997) and the RNA template (Singer and Gottschling, 1994). Two more regulatory (EST1p and EST3p) proteins are essential for functional telomerase (Hughes et al., 2000, Lundblad and

Szostak, 1989, Lendvay et al., 1996). Absence of any of these components leads to telomere shorting and death for most cells (Singer and Gottschling, 1994, Lendvay et al., 1996). EST1p interacts with CDC13p to recruit EST2p to telomeres (Evans and Lundblad, 1999). However, CDC13p binds to the telomere single strand DNA, contributing to both telomere protection and replication (Pennock et al., 2001). The association between EST2p and EST3p requires EST1p (Osterhage et al., 2006). KU proteins bind to the telomerase RNA enabling telomerase to act on telomeres (Stellwagen et al., 2003). Moreover, KU proteins bind to telomerase RNA at multiple sites leading to telomere lengthening (Zappulla et al., 2010).

#### **1.5.2.2. Telomerase associated proteins in human**

Some proteins that are associated with human telomerase have been found. These proteins are homologous to yeast EST1p. Three of these proteins are, EST1Ap, EST1Bp and EST1Cp (Reichenbach et al., 2003, Snow et al., 2003). Both, EST1Ap and EST1Bp are associated with telomerase activity in human cells. They are involved in telomere protection and lengthening. However, EST1Cp has not been found associated with telomerase (Redon et al., 2007). The human KU proteins interact directly with the RNA telomerase component, hTR (Ting et al., 2005). However, KU proteins also have been found associated with the human telomerase subunit, hTERT (Chai et al., 2002).

#### **1.5.3. Telomere length homeostasis**

Telomere length is regulated by a number of proteins that are present at chromosomes ends in humans and yeast. The balance of different mechanisms

that lengthen and shorten telomeres, keep their length at a certain equilibrium average. Telomeres length is reduced with each cell division as a result of telomere replication and nuclease activities. At short telomeres, telomerase is activated to extend the TG repeat (Cech et al., 1997, Lingner and Cech, 1998). A number of proteins are involved in telomere lengthening, by mediating telomerase access to the telomeres. At the same time some proteins prevent telomere lengthening. For example the absence of RIF1p/RIF2p proteins increases telomere length (Wotton and Shore, 1997). Therefore, it is obvious that telomere length is regulated by the balance between telomerase extendable and non-extendable states (Teixeira et al., 2004). However telomeres can be extended in the absence of telomerase (Le et al., 1999, Teng and Zakian, 1999).

#### **1.5.3.1. Regulation of telomere length in *S. cerevisiae***

Many proteins are involved in telomere length regulation in *S. cerevisiae*. The usual telomere length is  $350 \pm 50$  bp of TG repeat. Some proteins contribute to telomere elongation and some contribute to their shortening, keeping telomere length at the certain average. These proteins are involved in different categories such as DNA repair (YKU70p, YKU80p, RAD50p, MRE11p, and XRS2p), DNA replication (ELG1p, PIF1p and POL32p), telomere protection (CDC13p, STN1p, and TEN1p), telomere heterochromatin (RAP1p, RIF1p, RIF2p, SIR3p, and SIR4p), RNA metabolism (UPF1p, UPF2p and UPF3p) (Askree et al., 2004, Blackburn, 2001), and checkpoints (MEC1p and TEL1p) (Ritchie et al., 1999). However, telomere length is affected by mutations of 272 nonessential genes that are involved in many cellular mechanisms (Askree et al., 2004, Gatbonton et al., 2006).

### **1.5.3.2. Regulation of telomere length in humans**

Telomere length in human cells is maintained at 5 – 15 kb. Many proteins play a crucial role to keep telomeres at that length. These proteins affect telomere protection and telomerase function. Six proteins that are called the shelterin complex provide protection to human telomeres. They consist of TRF1p, TRF2p, TIN2p, RAP1p, TPP1p, and POT1p. TRF1p and TRF2p directly bind the double-stranded telomere repeats. TRF2p has an important role in T-loop formation (Stansel et al., 2001). POT1p is a single strand binding protein that binds the overhang and suppresses the DNA repair activities. In addition, these proteins protect telomeres from HR and NHEJ (Sarthy et al., 2009, de Lange, 2005). However, the POT1p-TPP1p complex, negatively and positively regulate telomerase access to telomere (Wang et al., 2007, Xin et al., 2007). Therefore they are involved in lengthening and shortening telomeres.

Non-shelterin proteins are also involved in telomere length regulation. For instance, the Mre11- complex proteins play an important role in telomere length maintenance (Assenmacher and Hopfner, 2004). Moreover, KU proteins are important for telomere protection and maintenance (Wang et al., 2009).

### ***1.6. Telomeres in the absence of telomerase***

In the absence of telomerase, telomeres erode with each cell division. This degradation is due to the incomplete replication of telomeres that is known as the end replication problem. In *S. cerevisiae*, telomeres lose 3-5 bp with each cell division (Lundblad and Szostak, 1989). In humans, telomeres lose 50-150 bp with each cell division (Blasco et al., 1997, Harley et al., 1990).

### **1.6.1. The end replication problem**

Two different mechanisms collaborate to synthesize the DNA. One strand, called the leading strand, is synthesised continuously in the direction of fork movement. The leading strand is synthesised by polymerase  $\delta$ . This synthesis leads to generating a blunt end (Fig.1.2). The blunt end must be acted upon by a nuclease to generate a 3' overhanging end. The other strand, called the lagging strand, is synthesised in the opposite direction to fork replication movement. RNA fragments prime short discontinuous segments of DNA that are called Okazaki fragments. The RNA fragments are  $\sim 10$  nt long, and the Okazaki DNA segments are  $\sim 125$  nt long in *S. cerevisiae* (Bielinsky and Gerbi, 1999). The lagging strand is synthesised by DNA polymerase  $\alpha$ . After the lagging strand has been completed, the RNA fragments must be removed and the DNA segments must be ligated. Finally, the gaps between the synthesised DNA are filled by DNA polymerase. The gap at the 5' end cannot be filled (Fig.1.2). This leads to telomere shortening with each cell division (reviewed in (Smogorzewska and de Lange, 2004, Waga and Stillman, 1998, Bambara et al., 1997)).

### **1.6.2. Telomeres in the absence of telomerase in *S. cerevisiae***

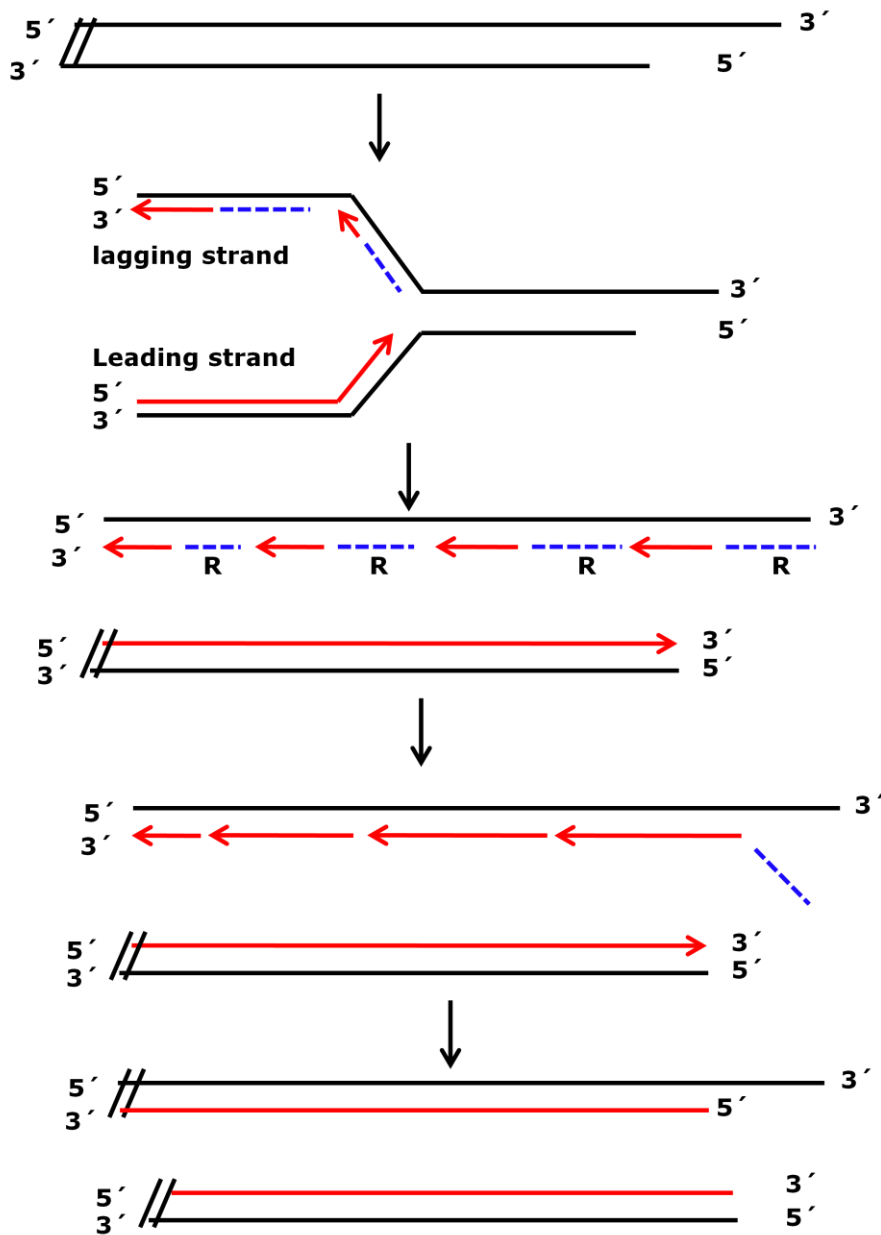
The absence of some genes that are involved in telomerase activity or telomere capping can affect chromosome end maintenance, leading to telomere erosion over time. These genes include telomerase components, *EST2* and *TLC1* (Lendvay et al., 1996, Singer and Gottschling, 1994), telomerase accessory factors (*EST1*, *EST3* and *CDC13*) (Lendvay et al., 1996, Lundblad and Szostak, 1989) and Checkpoints pathways, when double mutation of *MEC1* and *TEL1* cause senescence (Ritchie et al., 1999). Also,

telomere capping functions affect telomere length and double mutation of YKU70 and MRE11 causes erosion (Maringele and Lydall, 2002, Ritchie and Petes, 2000). Moreover, the absence of the *KU70* gene results in telomere shortening and temperature sensitivity (Manolis et al., 2001).

Telomerase dysfunction leads to telomere erosion with each cell division. Cells display normal growth for many generations after telomerase disruption, but at ~60 - 80 generations most cells die (Lundblad and Blackburn, 1993, Teng and Zakian, 1999). At this point cells enter senescence, which is defined as period of minimum growth rate (Fig. 1.3). During and post-senescence, the mutation rate (Hackett et al., 2001) and genome instability are increased. Most cells arrest at senescence due to telomere shortening and DNA damage response mechanisms activation (AS and Greider, 2003). A small population of cells are able to pass this stage using recombination pathways to maintain telomere function and generate survivors (Fig. 1.3).

### **1.6.3. Telomerase and human cells**

Telomerase activity is strongly repressed in human somatic cells. However, telomerase is active in some normal tissues such as ovaries, and testis (Hsiao et al., 1997) reviewed in (Autexier and Lue, 2006). Telomerase is highly expressed in most (90%) cancer cells, leading to telomere elongation (Bryan et al., 1997). However, in the absence of telomerase, telomeres degrade at the average 50-150 bp with each cell division (Allsopp et al., 1992, Harley et al., 1990).



**Figure 1.2. The end replication problem**

The leading strand is synthesised in the direction of fork movement to replicate the C-rich strand. Whereas, the lagging strand is synthesised in the opposite direction to fork movement to replicate the G-rich strand. RNA fragments are used to initiate lagging and leading strand synthesis. The removal of the distal RNA fragment leads to telomere shortening with each cell division. One telomere of a chromosome is shown. Red lines indicate new DNA strands.



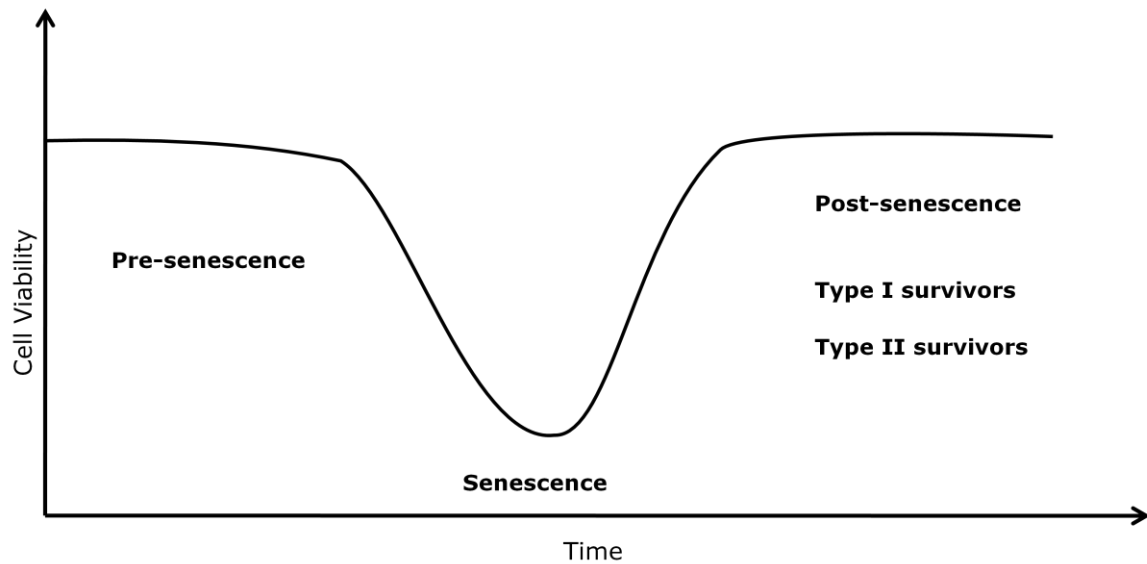
Cultured normal human somatic cells have a short lifespan, the cells stop growing after several divisions (Hayflick and Moorhead, 1961). The cell division arrest is termed cellular senescence. At this stage, cells can suffer from massive chromosomal abnormalities. Some cells without telomerase can bypass the senescence stage, but then die later at crisis, where genome instability is too great to be viable. At crisis some cells can reactivate telomerase and continue dividing for long time, and some can elongate their telomeres by a recombination pathway (Bryan et al., 1997, Bryan et al., 1995).

Tumour repressor proteins play critical roles in cell cycle arrest at senescence. Inactivation of these proteins, p53 and retinoblastoma (Rb) allow cells to overcome senescence (Chai et al., 2005).

A link has been found between telomere length and human age. Some somatic cells such as fibroblasts and leukocytes from old people display short telomeres compared to the same type of cell from young people (Harley et al., 1990, Hastie et al., 1990).

### ***1.7. Telomerase-independent telomere maintenance***

Some organisms are able to maintain their telomeres without telomerase using different mechanisms. Telomeres in *Drosophila melanogaster* are maintained by transposition of retrotransposon elements (reviewed in



**Figure 1.3. The cell viability of *S. cerevisiae* cells in the absence of telomerase**

Cell viability decreases at pre-senescence due to telomeres erosion. At senescence most cells die due to telomere shortening. Rare cells can bypass senescence and generate survivors. These cells use recombination to maintain their telomeres.

(Villasante et al., 2008)). Some other organisms are thought to maintain their telomeres by recombination such as *Anopheles gambiae* (Roth et al., 1997), *Allium cepa* (Pich and Schubert, 1998), and *Chironomus* (Saiga and Edstrom, 1985). Moreover, human and *S. cerevisiae* cells are able to elongate their telomeres in the absence of telomerase, using homologous recombination pathways.

### **1.7.1. Telomerase-independent telomere maintenance in *S. cerevisiae***

*S. cerevisiae* cells are able to maintain their telomeres in the absence of telomerase. There are at least two types of post-senescence cells that are termed type I and type II survivors. It is easy to distinguish between these two survivors using a *XhoI* restriction enzyme and Southern blot analysis probing with a Y'-TG<sub>1-3</sub> probe. In type I survivors, cells amplify Y' elements to maintain their telomeres (Fig. 1.4). Whereas, in type II survivors, cells amplify TG repeats to provide telomere function (Fig. 1.4) (Chen et al., 2001, Lundblad and Blackburn, 1993, Teng and Zakian, 1999, Le et al., 1999).

#### **1.7.1.1. Type I post-senescent survivors**

Type I survivors cells display amplification of Y' elements followed by short TG<sub>1-3</sub> (Teng and Zakian, 1999). Type I survivors cells are hypothesized to use telomere-telomere recombination to elongate their telomeres. First, in the absence of telomerase, telomeres erode with each cell division until the Y' element is at the terminus. Second, recombination is initiated within the Y' elements. If recombination occurs in the internal Y' element on a donor chromosome that has a number of Y' elements, then the amplification of Y's

will be seen (Fig. 1.5-A). Some chromosomes have only X elements. Therefore, if the resection reaches X element then recombination is likely to occur with other X element. Many chromosomes have both X elements and Y' elements. Thus it is possible that all chromosomes can pick up Y's (Chen et al., 2001). It is easy to differentiate between two classes of Y' element in type I survivor cells using Southern blot analysis. These classes termed Y' long and Y' short elements with 6.7 kb and 5.2kb sizes respectively (Lundblad and Blackburn, 1993). Type I survivors cells grow slower than wild-type and they display fluctuating growth rates with re-senescence during growth. Type I survivors can change to type II survivors in some cases (Teng and Zakian, 1999).

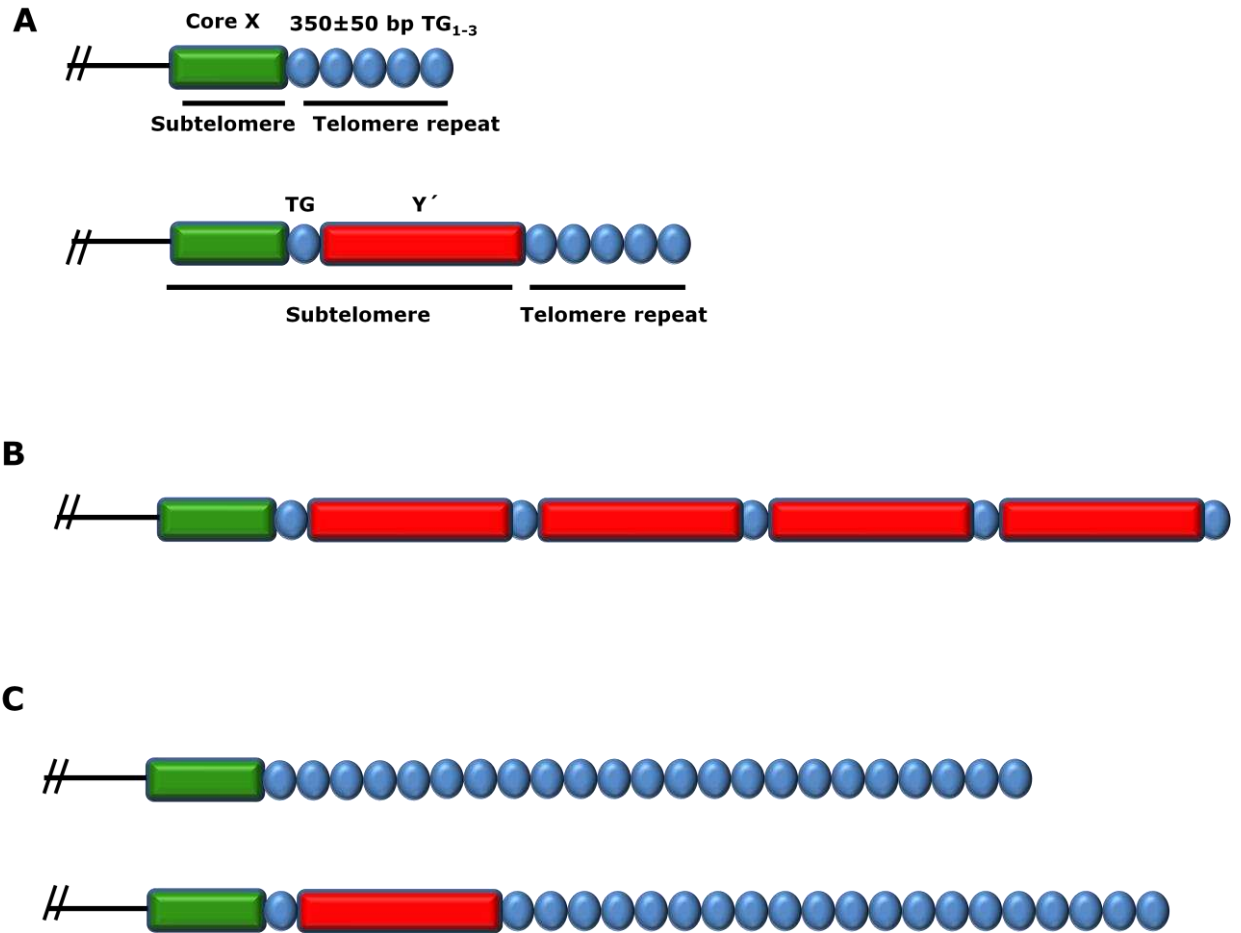
#### **1.7.1.2. Type II post-senescent survivors**

Type II survivors cells display long TG repeat amplification that can be up to 12 kb long or more. Cells initiate recombination within the TG repeats. The 3' single strand can invade into a TG repeat of other telomeres and copy them until the end. Telomere recombination can occur by an intrachromosomal pathway. The single strand can be looped back and invade the TG repeat from the same telomere (Chen et al., 2001). However, modest amplification of Y' elements can be seen in some cases of type II survivor strains (Teng and Zakian, 1999). Type II survivor cells display a dynamic telomere length with gradual shortening and then further elongation. Cells exhibit similar growth to wild-type strains. They grow faster than type I survivor strains (Chen et al., 2001).

### **1.7.1.3. Genetic components needed for survival in the absence of telomerase**

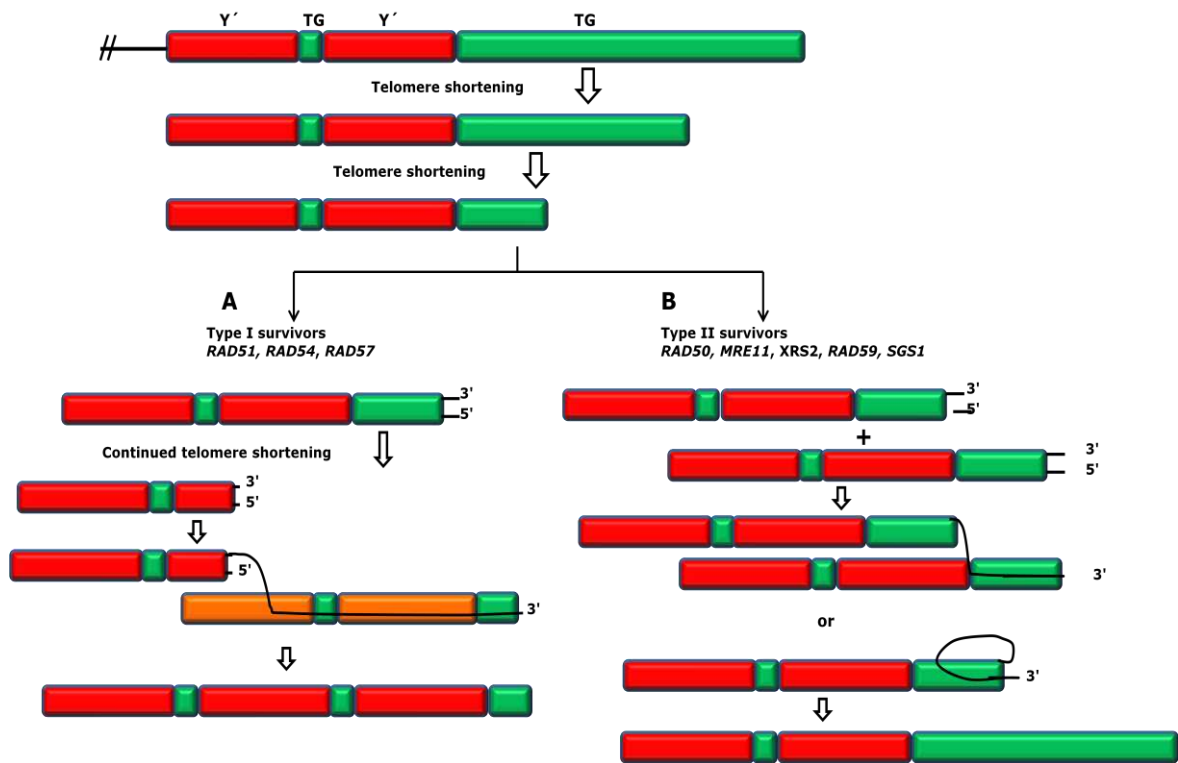
The central recombination gene *RAD52* is essential for both type I and type II survivor establishment. Therefore, none of these survivors can be seen in the absence of *RAD52* (Lundblad and Blackburn, 1993). However, some types of survivors that differ from both type I and type II are established in the absence of both telomerase and *RAD52* (Maringele and Lydall, 2004b, Grandin and Charbonneau, 2009, Lebel et al., 2009). *RAD52* is a central gene for all types of homologous recombination that includes spontaneous and DSB recombination. Thus, absence of *RAD52* leads to defects in gene conversion, single strand annealing (SSA), break induce replication (BIR), double strand break repair (DSBR) and meiosis, reviewed in (Symington, 2002, Krogh and Symington, 2004). The key function of RAD52p is binding to single strand DNA to mediate annealing to the complementary DNA sequences (Mortensen et al., 1996). The Type I pathway require the *RAD51*, *RAD54*, *RAD55* and *RAD57* genes (Teng et al., 2000, Le et al., 1999, Chen et al., 2001). Type II survivors require *RAD50*, *MRE11*, *XRS2*, *RAD59* and *SGS1* (Huang et al., 2001, Le et al., 1999, Chen et al., 2001, Johnson et al., 2001). No survivor strains can be generated in a *rad50 rad51 tlc1* triple mutant. Thus, *RAD50* and *RAD51* are in two different pathways that generate survivors (Le et al., 1999).

RAD51p has an important function in HR. It binds to ssDNA to form a filament searching for a homologous DNA sequence. RAD52p helps RAD51p bind to the ssDNA. RAD51p binding is mediated by other proteins RAD54p, RAD55p, RAD57p (reviewed in(Krogh and Symington, 2004)). Both RAD55p and



**Figure 1.4. Telomere structures in wild-type and post-senescent survivors**

**A-** wild-type telomeres. Core X only and core X-Y' element telomeres shown. **B-** Type I survivors. They amplify Y' element to replicate their telomere. Telomeres in type I survivors ended with short TG repeat. **C-** Type II survivors. They replicate TG repeats to elongate their telomeres. Y' element can found in type II survivors.



**Figure 1.5. Mechanisms of post-senescence survival in *S. cerevisiae***

Type I and type II survivor cells are generated by two different recombination pathways. A telomere containing two copies of Y' elements that separated by short TG repeat and terminate with long TG repeat is represented at the top. In the absence of telomerase, telomeres erode with each cell division leading to cell senescence. Two different types of survivor cells can be generated after senescence stage. A- Type I survivors generated by the amplification of Y' elements. These survivors require *RAD51*, *RAD54*, and *RAD57* genes. Telomere shortening continues until it reaches a Y' element. Single strand of one telomere (red) invades into an Y' element of the donor telomere (orange) and use it as template for telomere replication. B- type II survivor cells generated by the amplification of TG repeat, cells require *RAD50* and *RAD59*. Telomere recombination occurs between TG repeat in one telomere and the TG repeat in another telomere. Single strand of TG repeat is expected to fold back and invade into the TG double strand to use it as template to elongate telomeres. Adapted from (Chen et al., 2001).

RAD57p form a heterodimer that functions as a mediator for RAD51p in the strand exchange. RAD54p is involved in chromatin remodelling and helps RAD51p to bind to ssDNA forming a filament structure (Krogh and Symington, 2004).

The type II survivor pathway requires *RAD52*, *RAD50*, *MRE11*, *XRS2*, *RAD59*, and *SGS1* (Le et al., 1999, Chen et al., 2001, Huang et al., 2001, Johnson et al., 2001). The MRX-complex (*MRE11*, *RAD50* and *XRS2*) performs an important role in telomere maintenance. It is involved in exonuclease activity that generates single stranded DNA at chromosomes ends (reviewed in (Krogh and Symington, 2004)). RAD59p binds to single strand DNA, and then anneals to complementary DNA (Petukhova et al., 1999, Davis and Symington, 2001). *SGS1* is a RecQ helicase that is involved in genome stability and is required to generate type II survivors (Huang et al., 2001, Johnson et al., 2001).

*RAD51*-dependent and *RAD51*-independent repair pathways require different length of homology for strand invasion. The *RAD51*-dependent repair pathway requires approximately 100 bp of homology for strand invasion (Ira and Haber, 2002). This is consistent with the high identity between Y' elements being able to form long homologous strands. On the other hand, the *RAD51*-independent repair pathway that requires *RAD50* only needs about 30 bp of homology for strand invasion (Ira and Haber, 2002). It is known that the telomere TG repeat is heterogeneous. This means less chance for long homology between TG strands. The *RAD50* dependent pathway has been found to be efficient with short homology length of strands (Ira and Haber, 2002).



The Y' elements encode a helicase called Y'-Help1 (Y'-helicase protein 1). The Y' elements are not expressed in most cases, or can be expressed at very low levels (Louis and Haber, 1992). However, they are expressed in meiosis and in the absence of telomerase (Louis, 1995, Yamada et al., 1998). Therefore, it is proposed that Y' helicase may be involved in type I survivor generation (Yamada et al., 1998).

There are additional proteins that are involved in the type II survivor pathway. The cell cycle checkpoint proteins Tel1p and Mec1p are required to establish type II survivors (Tsai et al., 2002). Moreover, the mitotic cyclin CLP2p has been found to be essential for type II survivors, since all *tlc1Δ clb2Δ* cells generate type I survivor cells (Tsai et al., 2002). Type II survivors also require *DEF1*, as the double mutation of *def1Δ est2Δ* or *def1Δ est3Δ* only generates type I survivor strains (Chen et al., 2005). The RIF proteins, known to negatively regulate telomere length, were found to inhibit type II recombination (Teng et al., 2000). The DNA Topoisomerase III (*TOP3*) that is involved in telomere stability and regulation of mitotic recombination interacts with *SGS1* to be required for type II survivor establishment (Tsai et al., 2006). The *Exo1* exonuclease has been found to play a significant role in generating both type I and type II survivors (Bertuch and Lundblad, 2004). This role can be explained by its activity in generating single strand DNA at telomeres.

The replication machinery is important in survivor generation. All three polymerases in *S. cerevisiae*, *POL α* (*CDC17*), *POL δ* (*CDC2*), and *POL ε*

(*POL2*) affect telomere-telomere recombination (Tsai et al., 2002). In the absence of POL  $\delta$  only type II survivors can be seen. Both type I and type II survivors were generated with the disruption of POL  $\alpha$  or POL  $\epsilon$ , but type I was more affected (Tsai et al., 2002). Therefore, it is clear that the DNA replication machinery is important for survival pathways.

#### **1.7.1.4. *RAD52*-independent survivors**

It has been found that three types of *S. cerevisiae* survivors can be generated in the absence of both telomerase and *RAD52*. These survivors differ from *RAD52*-dependent type I and type II survivors, and also they differ from each other. First, cells can bypass senescence and generate survivors in the absence of telomerase, *RAD52* and *EXO1* nuclease (Maringele and Lydall, 2004b). In these survivors, short DNA palindromes induce formation of large DNA palindromes preventing the loss of essential genes. These survivors are termed PAL-survivors (Maringele and Lydall, 2004b). Second, cells can generate survivors in the absence of telomerase and *RAD52* when they have long telomeres (Lebel et al., 2009). The polymerase  $\delta$  subunit *POL32* was not essential for these survivors. These survivor strains displayed a type II-like telomere pattern (Lebel et al., 2009). Third, strains can bypass senescence to generate survivors in the absence of both telomerase and *RAD52* (Grandin and Charbonneau, 2009). Long telomeres were present during senescence, thus this type of survivors was termed ILT for inherited long telomere. ILT survivor strains require *RAD50* and *MRE11* but not *RAD51* and *RAD59* (Grandin and Charbonneau, 2009). Therefore, they use a pathway that clearly different from both type I and type II survivors pathways.

### **1.7.2. Telomerase-independent telomere maintenance in human cells**

The majority of immortalised human cancer cells reactivate telomerase to maintain their telomeres (Kim et al., 1994). The rest of cancer cells utilize an alternative pathway that is termed alternative lengthening of telomeres (ALT) to maintain their telomeres (Bryan et al., 1997, Dunham et al., 2000). ALT mechanisms occur in common sarcomas such as breast carcinomas (Subhawong et al., 2009). Moreover, an ALT phenotype is widespread in glioblastoma multiforme, the common type of brain tumour in adults (Hakin-Smith et al., 2003). It has been recently found that ALT mechanisms are used in carcinomas coming from bladder, cervix, endometrium, esophagus, gallbladder, kidney, liver, and lung (Heaphy et al., 2011). ALT cells display very long heterogeneous telomeres that can be more than 50 kb (Bryan et al., 1995). ALT cells display a telomere pattern that is analogous to yeast type II survivors. ALT cells analogous to yeast type I also can be found (Fasching et al., 2005). The average telomere length in human telomerase-positive cancer cells is normally 10 kb, reviewed in (Nabetani and Ishikawa, 2010). Telomeres length in ALT cells and telomerase-positive cells can be differentiated by Southern hybridisation analysis or by fluorescent *in situ* hybridisation (FISH) using telomere-specific probes (Nabetani and Ishikawa, 2010).

The ALT cells can be characterised by the presence of duplex TTAGGG repeat and single-strand of G-rich, and the presence of some telomere-associated proteins. Moreover, telomeres in ALT cells are able to form t-loops (Cesare and Griffith, 2004). ALT cells are also characterised by nuclear bodies that contain promyelocytic leukaemia (PML) proteins, thus these bodies are called ALT-associated PML bodies (APBs)(Yeager et al., 1999). APBs have other

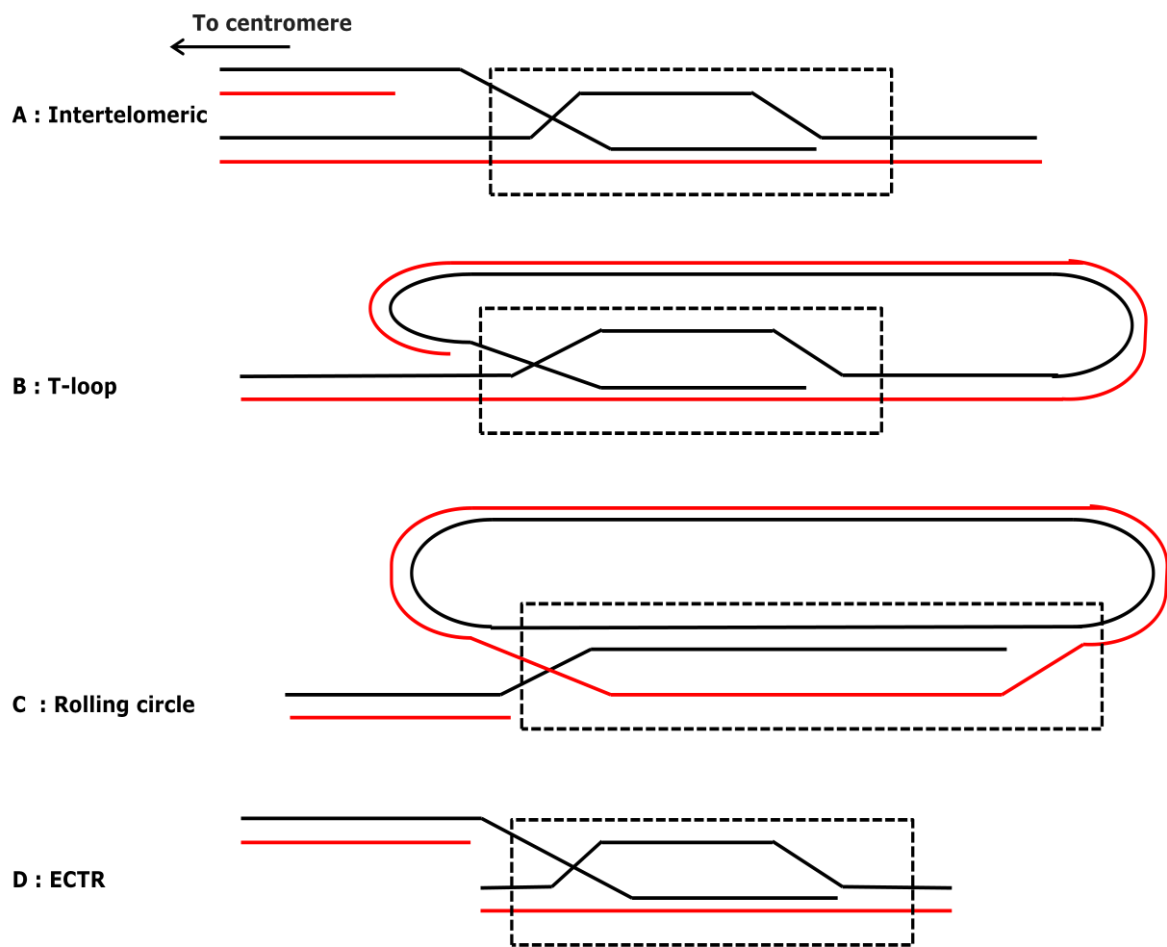
proteins including RAD50p, MRE11p, NBS1p, RAD51p, TRF1p, TRF2p, WRNp (reviewed in (Nabetani and Ishikawa, 2010)). It has been found that ALT cells have circular C-strands (Henson et al., 2009).

The genetic requirements for ALT pathways still need investigation. It is clear that some genes are required for ALT mechanisms. The MRN (*MRE11*, *RAD50* and *NBS1*) complex is essential for telomere length maintenance in ALT cells (Royle et al., 2009). Moreover, a DNA structure-specific recombination endonuclease, *MUS81* is involved in ALT cells survival pathway (Zeng et al., 2009). Additional genes are thought to be required for ALT mechanisms. These genes are flap endonuclease 1 (*FEN1*) (Saharia and Stewart, 2009), and fanconi anaemia group D2 (*FANCD2*) (Fan et al., 2009). Telomeres in human ALT cells are elongated based on recombination mechanisms (Dunham et al., 2000, Henson et al., 2002, Cesare and Reddel, 2010, Nabetani and Ishikawa, 2010). These mechanisms are suggested to involve break-induced replication (BIR). A telomere-telomere recombination pathway is thought to be the important mechanism to elongate telomeres in ALT cells. According to this pathway, the single strand of one telomere invades into the homologous sequence of another telomere. The donor telomere is used as a template, leading to telomere elongation (Fig. 1.6 A). Moreover, T-loops have been found associated with ALT cells and are hypothesized to function in telomere elongation (Cesare and Griffith, 2004). In this mechanism, the telomere folds back and the overhang invades into the telomeric double strand DNA and uses it as template to elongate telomeres (Fig 1.6 B). However, T-loop structure can lead to telomere shortening in some cases through a t-circle (Nabetani and Ishikawa, 2010). Circular DNA molecules are clearly seen in ALT cells

using electron microscopy (Cesare and Griffith, 2004). These molecules are thought to be formed from telomeric DNA (t-circle). Therefore, rolling-circle replication (RCR) is hypothesized to play an important role in telomere elongation in ALT cells (Nabetani and Ishikawa, 2010, Cesare and Reddel, 2010). Based on this mechanism, the telomeric single strand invades into t-circle and uses it as template, leading to telomere elongation (Fig 1.6 C). Furthermore, DNA molecules thought to be linear extrachromosomal telomeric repeats (ECTR) are found in ALT cells (Ogino et al., 1998). Thus it was hypothesized that, the telomeric overhang invades into this molecule and uses it as a template to duplicate telomere length (Fig 1.6 D) (Henson et al., 2002, Cesare and Reddel, 2010, Nabetani and Ishikawa, 2010).

### **1.7.3. Telomerase-independent telomere maintenance in other organisms**

In addition to humans and *S. cerevisiae*, other organisms are able to maintain their telomere length in the absence of telomerase. In addition to *S. cerevisiae*, *Kluyveromyces lactis* and *Schizosaccharomyces Pombe* cells can bypass senescence and generate survivors. *K. lactis* strains are proposed to generate survivors by rolling circle events (McEachern and Blackburn, 1996, Natarajan and McEachern, 2002). These survivors are strongly dependent on *RAD52*. The *K. lactis* survivor strains displayed telomeric-circle (t-circles) double strand DNA at ~100 bp to 3.0 kb length, and G-rich single strands (Basenko et al., 2009, Groff-Vindman et al., 2005).



**Figure 1.6. Mechanisms to elongate telomeres in ALT cells**

Mechanisms that elongate telomeres in ALT cells are based on homologous recombination. Four different pathways are thought to be involved in telomere elongation. **A-** Telomeric recombination occurs between two telomeres. Single strand of one telomere (red line) invades and copies the homologous sequence of another telomere (black line). **B-** T-loop contributes to telomere elongation in ALT cells (see text). **C-** Telomere invades in t-circle DNA and use RCR mechanism to elongate itself. **D-** Linear extrachromosomal telomeric repeats (ECTR) can be used to elongate telomeres in ALT cells. Adapted from (Henson et al., 2002).

In *S. Pombe*, cells can bypass senescence and generate survivors in the absence of telomerase (Nakamura et al., 1998). Most survivor strains circularize all chromosomes and the rest maintain their telomeres by recombination. Survivors that have linear chromosomes were found (Subramanian et al., 2008). It has also found that *S. Pombe* can generate survivors by amplifying and rearranging heterochromatic sequences (Jain et al., 2010). These survivors are termed (HAATI) for heterochromatin amplification-mediated and telomerase-independent.

### ***1.8. Mechanisms of post-senescent survival in S. cerevisiae***

In the absence of telomerase, *S. cerevisiae* cells were able to bypass senescence and survive utilizing recombination pathways. Different types of survivors were generated depending on different pathways. Each pathway requires certain genes as discussed above. A number of mechanisms are thought to be involved to generate these survivors as explained below.

#### **1.8.1. Mechanisms by which break-induced replication can generate post-senescent survivors**

Break-induced replication (BIR) and recombination that generate *RAD52*-dependent survivors are similar mechanisms (Signon et al., 2001, Le et al., 1999). After a DSB has occurred at a telomere, strand invasion can occur and initiate a replication fork, which can copy the sequence of the donor strand. BIR is suggested to be involved in telomere replication (Hackett et al., 2001). Two pathways of BIR were found, one is *RAD51*-dependent and the other is *RAD50*-dependent (Davis and Symington, 2004). Some genes are required for each of these BIR pathways which are consistent with genes involved in both

RAD52-dependent survivors. Hence, it suggested that RAD51-dependent BIR is used to generate type I survivors and RAD50-dependent BIR is used to generate type II survivors.

### **1.8.2. Mechanisms by which extra chromosomal circles can generate post-senescent survivors**

Circular DNA molecules were found in all eukaryotic cells (Gaubatz, 1990). The amount of these circular DNA molecules is lower in normal human cells compared to cancer cell lines (Schmidt et al., 2009). The extra-chromosomal DNA molecules were found in ALT cell as double strand telomeric circles (Cesare and Griffith, 2004, Wang et al., 2004). Telomerase-independent cancer cell lines that maintain their telomeres by ALT pathway can use the circle DNA to elongate telomeres (Muntoni et al., 2009). These circular DNA molecules were used as template to elongate telomeres by rolling replication mechanism.

In yeast, circles of Y' elements and TG repeat can be used to generate yeast survivor strains in the absence of telomerase. Circular DNA molecules that are composed of Y' and telomeric repeats have been found in *S. cerevisiae* (Horowitz and Haber, 1985). These circular molecules can be used as template for telomere-telomere recombination. The replication events are proposed to be a rolling circle replication mechanism (Lin et al., 2005). The eroded telomeres can invade a TG repeat circle to generate type II survivors or invade Y' elements ring to generate type I survivors. Both type I and type II *S. cerevisiae* strains display high frequency of TG repeat and Y' elements



circles during or immediately after the formation of survivors (Lin et al., 2005).

DNA circles have been proposed to generate survivors in other yeast species. In *Kluyveromyces lactis*, circle DNA is proposed to establish survivors (Natarajan and McEachern, 2002). Circle molecules that are composed of a G-rich telomeric repeat were found as single and double strand DNA in *K. lactis* (Basenko et al., 2009). This finding supports the generation as survivors by recombination in the absence of telomerase.

### **1.8.3. Mechanisms by which T-loops can generate post-senescent survivors**

T-loop structures are formed by the looping and invasion of telomeric single strand DNA into the telomeric or subtelomeric double strand DNA. T-loops are a common DNA secondary structure in higher eukaryotes (Griffith et al., 1999, Murti and Prescott, 1999, Cesare et al., 2003, Nikitina and Woodcock, 2004). It is proposed to occur in *S. cerevisiae* (de Bruin et al., 2000, de Bruin et al., 2001) but has not been seen. T-loops are proposed to play significant roles at telomeres, for example they can protect telomeres against degradation and they can repress HR. T-loops also can regulate access of telomerase to the telomere. They can be involved in DNA replication leading to telomere elongation in the absence of telomerase. T-loops could mediate the amplification of Y' elements to generate type I survivors and could mediate the amplification of telomere repeat to generate type II survivors. T-loop structures have been found in *K. lactis* yeast (Cesare et al., 2008), and it is

proposed to be involved in telomere recombination. Hence, T-loops may initiate the formation of survivors in strains that lack telomerase. *K. lactis* is related to *S. cerevisiae*. This finding supports the generation of *S. cerevisiae* survivors by the T-loop pathway.

### ***1.9. Aim of this study***

It is well known that *S. cerevisiae* cells are able to generate post-senescent survivors in the absence of telomerase. Two types (type I and type II) of these survivors require *RAD52* for their establishment. Each one of these survivors requires certain genes for its establishment.

The main aim of this study was to test the candidate genes to determine if they were required for type II survivor telomere maintenance. Thus, the candidate genes were deleted individually in established type II survivor strains. Cells with deleted genes were propagated for ~150 generations, and then their telomere structures were examined to see if they continued as type II survivors. Strains that require a certain gene to maintain as type II survivors are expected to die, change to type I or maybe a new type of survivor. Also this study aimed to test the genetic requirements for type II survivors after many generations (~500). Thus, two independent strains for each gene were propagated ~500 generations and the telomere structures were examined. Moreover, one aim of this study was to test type II strains sensitivity to DNA damage reagents (temperature and UV) at ~150 generations after deleting the candidate genes.

## Chapter 2: Materials and Methods

### 2.1. Materials

#### 2.1.1. General laboratory chemicals

General laboratory chemicals were obtained from Sigma, unless otherwise stated.

#### 2.1.2. DNA -marker

Bacteriophage  $\lambda$ DNA digested with *Bst*EII was obtained from New England Biolabs, and was used at a concentration of 100 ng/ $\mu$ l, typically 500 ng were loaded per lane of an agarose gel.

#### 2.1.3. Enzymes

**Table 2.1 Enzymes used with concentration and supplier**

Enzyme	Concentration	Supplier
Taq DNA polymerase	1 unit/50 $\mu$ l reaction	Invitrogen
RNase A	10 mg/ ml	Sigma
Zymolyase	10 mg/ ml	Seikagaku Biobusiness
Proteinase K	10 mg/ ml	Roche
<i>Xho</i> I enzyme	1 $\mu$ l/ 25 $\mu$ l reaction	New England Biolabs

#### **2.1.4. Plasmids**

The plasmid pEL30 contains Y' and TG sequence from *S. cerevisiae* in a pGEM3ZF vector (Watt et al., 1996). It was used in this study as a probe in Southern blots to detect type I and II survivors (Fig 2.1).

#### **2.1.5. Oligonucleotides**

All oligonucleotides were obtained from Invitrogen. Primers were designed using the *Saccharomyces* Genome Database (SGD); their sequences are shown in table 2.2.

#### **2.1.6. Yeast strains**

All *S. cerevisiae* strains used were Y55 strain derivatives (Liti and Louis, 2003), and are listed in table 2.3.

#### **2.1.7. *S. cerevisiae* growth media**

*S. cerevisiae* was grown in Yeast extract Peptone Dextrose medium (YEPD) which consist of 1% w/v yeast extract, 2% w/v bacto peptone, 2% w/v Dextrose-D-Glucose adjusted to pH 6.5. After autoclaving, 10 ml/l of a 5% adenine hemisulphate solution (dissolved in 0.05M HCL, filter sterilised and stored at room temperature) was added. For solid media, 2.5% w/v of bacto-agar was added to the above medium prior to autoclaving. All media were autoclaved at 120°C and 15 psi for 35 minutes. Yeast cells were stored at minus 80°C in 25% v/v glycerol in dH<sub>2</sub>O. The YEPD media components were obtained from scientific laboratory supplier (SLS).

**Table 2.2: primers used in this study**

All primers obtained from (Invitrogen). Four primers were designed for each gene. Forward (Fwd) and reversed (Rev) primers are used to amplify the deleted target gene. Two more short primers (A1 and A4) were used with the cassette primers to make sure if transformation is successfully done as described in chapter 2.

<b>Gene</b>	<b>Description</b>	<b>Sequences</b>
<i>RAD50</i>	Fwd	5'- GAACGACGGAAAGCAGGCatgAGCGCTATCTATAAATTATCT ATTCcgtacgctgcaggtcgac-3'
<i>RAD50</i>	Rev	5'- TATCCCTTCGTAGATATTATGGGGTCTTTTCAATAAGTGACTC TGatcgatgaattcgagctcg-3'
<i>RAD50</i>	A1	5'- GCCTTCACCTCGTTTGTCTTC -3'
<i>RAD50</i>	A4	5'- CAAAGGTGCTTACGTGCTTGC-3'
<i>MRE11</i>	Fwd	5'- TGCCTCGGGGGACTCACTGTTGTGTCCTATGGATATACTTCA TGCGACTGGTCTAATAAACGTACGCTGCAGGTCGAC-3'
<i>MRE11</i>	Rev	5'- TTCGTTGCAGTTCGCACTCGTTTGGGTTTGCTCGTTGGCTTA CTGCTTCCGCTTGACTAATCGATGAATTCGAGCTCG-3'
<i>MRE11</i>	A1	5'- GGACTATCCTGATCCAGACA-3'
<i>MRE11</i>	A4	5'- TTCTTAGCAAGGAGACTTCC-3'
<i>XRS2</i>	Fwd	5'- TATCCCGCTAATCTGATGATTAGCGATTACCCAAAGAGCGA GGACAACAGCATAACGGGAcgtacgctgcaggtcgac-3'
<i>XRS2</i>	Rev	5'- TATTCGATCCAAATCTTTCCATCTCCGTCAAAGCAAACATATC TTCGTTTTCTTCTGTGCatcgatgaattcgagctcg-3'
<i>XRS2</i>	A1	5'- GGACTATCCTGATCCAGACA -3'
<i>XRS2</i>	A4	5'- TTCTTAGCAAGGAGACTTCC -3'

<i>KU80</i>	Fwd	5'- ATGTCAAGTGAGTCAACAACCTTTCATCGTGGATGTTTCACCA TCAATGATcgtacgctgcaggtcgac-3'
<i>KU80</i>	Rev	5'- CAAAGGATGTTAGACCTTTTTTAATTATTGCTATTGTTTGAC TTCCCCTatcgatgaattcgagctcg -3'
<i>KU80</i>	A1	5'- CCATTGCCAGGATTTCGACAA-3'
<i>KU80</i>	A4	5'- GATTAGAGAACCGCTCGACC -3'
<i>NEJ1</i>	Fwd	5'- CCCACAGAAAAAAGAAAATTTGGAAAGGTGAGAATAAAAA ACcgtacgctgcaggtcgac-3'
<i>NEJ1</i>	Rev	5'- TTGAAAGGTCCAACCTTAATTTTTGACGTTTAATTGACTTGCC atcgatgaattcgagctcg-3'
<i>NEJ1</i>	A1	5'-ACCAAATAGCCGCGGTAAAA-3'
<i>NEJ1</i>	A4	5'-TGGCATAACGTTACCATTCAA-3'
<i>ELG1</i>	Fwd	5'- CCACCATTACCATTACATCAATCGATTTTCCCAGTAGGTGAC AAAGAGCTGTCAGATCGGcgtacgctgcaggtcgac -3'
<i>ELG1</i>	Rev	5'- CTTTAACGCTTGAACCAAGAGTTGATGTTAGGACCGGTAGTC TGTTCTTGatcgatgaattcgagctcg -3'
<i>ELG1</i>	A1	5'- GGCACGTGTCTTTATCTG -3'
<i>ELG1</i>	A4	5'- CCGAGCTTCCAATTGTTCTTGG -3'
<i>SGS1</i>	Fwd	5'- ATGGTGACGAAGCCGTCACATAACTTAAGAAGGGAGCACAA ATGGTTAAAGGAAACGGCGcgtacgctgcaggtcgac-3'
<i>SGS1</i>	Rev	5'- TCACTTTCTTCTCTGTAGTGACCTCGGTAATTTCTAAAACCT CGTCTCCCATTAGCAGAatcgatgaattcgagctcg-3'

<i>SGS1</i>	A1	5' - CGTGCGTTTCGAAGTGGATTGC -3'
<i>SGS1</i>	A4	5' - GCACACCACAATATGTCGTGG -3'
<i>RIF1</i>	Fwd	5'- GCCTCTAATAGTGATAATGTATCTCCGGTTACAAAAAGTGTA GCTCGTACGCTGCAGGTCGAC-3'
<i>RIF1</i>	Rev	5'- CTTTGCAATCTTTTTTTCAATTTCTCTTTTATTTGGCTCTTTGA ATCGATGAATTCGAGCTCG-3'
<i>RIF1</i>	A1	5' - GGAAGCAGGCTAATGCAAAG -3'
<i>RIF1</i>	A4	5' - GAGGTGAATCTGGTGGCATT -3'
<i>RIF2</i>	Fwd	5'- CAATATGCTGGTATCGACCGTGCAGTTTCAGAAACACTGTCT TTAGTCGCGTACGCTGCAGGTCGAC -3'
<i>RIF2</i>	Rev	5'- GGCATGTGACAAGCGAGTTGTAAGACTGTAATAACTTGCTTC CGGAATCAAAGGATCGATGAATTCGAGCTCG-3'
<i>RIF2</i>	A1	5' - GGAGCATGTAGATTCCGA -3'
<i>RIF2</i>	A4	5' - ACTTAAGTGAAGACGCTGG-3'
<i>DCC1</i>	Fwd	5'- CTGTCCAAGCCGTACATGGACGTCGTGGGATTCGCCAAGAC TGAATCAGAcgtacgctgcaggtcgac -3'
<i>DCC1</i>	Rev	5'- AGCCTCGCAGCATGTCAATGTCAATGTACATGGGAAGAAA GGTGGGAAAtcgatgaattcgagctcg-3'
<i>DCC1</i>	A1	5' - CCATCAACCTACATTCCGCA -3'
<i>DCC1</i>	A4	5' - AGAGGCTTGATATCCTCC-3'
<i>HUR1</i>	Fwd	5'- ATGTTTATCTTAGTATCCGTTGTAATATATGTACATATATAC ATCTACAcgtacgctgcaggtcgac -3'

<i>HUR1</i>	Rev	5'- TCAAATGTGCGCTATATATATACCATTTTTCCAAAGTATCTTT AAAAC TGatcgatgaattcgagctcg -3'
<i>HUR1</i>	A1	5'- GGAAGGAGAAGGATTAGCTG -3'
<i>HUR1</i>	A4	5'- ATGTTCAACTACGCCGTTGG -3'
<i>UPF1</i>	Fwd	5'- ACATTGTTAATCACTTAGTTTTATCCCACCATAACGTAGTTTC TTTACATcgtacgctgcaggtcgac-3'
<i>UPF1</i>	Rev	5'- AACTAATTGAACAGTGCATAACTGTAAGTTATCCAACGTACC TTCGACTAatcgatgaattcgagctcg -3'
<i>UPF1</i>	A1	5'- CCGTTCTCACACTCCTTAT -3'
<i>UPF1</i>	A4	5'- AATGAGAAGCCTCCTCCTGG -3'
<i>SRB2</i>	Fwd	5'- ATGGGAAAATCAGCCGTTATATTCGTGGAAAGAGCCACTCC CGCTACACTcgtacgctgcaggtcgac -3'
<i>SRB2</i>	Rev	5'- TCACAGCTCCAGAGCACGAACATACTGGTACGCCAAATCAC ATATTTTCGTatcgatgaattcgagctcg-3'
<i>SRB2</i>	A1	5'- GGACGAACAGTGTGCGTTT -3'
<i>SRB2</i>	A4	5'- AATCCGGGCTTATCCATGG-3'
<i>GTR1</i>	Fwd	5'- CGACGCCAAAATTTTTGTTCTTCTGCATAAGATGGATCTTGTT CAGTTGGcgtacgctgcaggtcgac -3'
<i>GTR1</i>	Rev	5'- TCATTGGAAAACTCTTTGGCTTTTTTGATGTTTTCCAATACT AATTCTTatcgatgaattcgagctcg-3'
<i>GTR1</i>	A1	5'- GGAGATTGGGTGCCACCATT-3'
<i>GTR1</i>	A4	5'- AAACCCCGACAAGATTGG-3'
<i>OGG1</i>	Fwd	5'- ATGTCTTATAAATTCGGCAAACCTTGCCATTAATAAAAAGTGAG CTATGTCTAGCAAATGTGcgtacgctgcaggtcgac -3'



<i>OGG1</i>	Rev	5'- CTAATCTATTTTTGCTTCTTTGATGTGAAGATCAGACAATTCA ACTTTCAGTTTCATTTGatcgatgaattcgagctcg -3'
<i>OGG1</i>	A1	5'- CCTGAAACCACGACTACTCA -3'
<i>OGG1</i>	A4	5'- CATGTGGGTGTAGACTATGG -3'
<i>RPB4</i>	Fwd	5'- ATGAATGTTTCTACATCAACCTTCAAACAAGACGGAGAAGA TTGAAGAAAGTGGAGGAAcgtacgctgcaggtcgac-3'
<i>RPB4</i>	Rev	5'- TTAATAGAGTGTCTAGGTTTGACAATTCCTTTAGTATCCTT TCCAACCTCATCGTCTGAatcgatgaattcgagctcg -3'
<i>RPB4</i>	A1	5'- GCAACGCGACAATAGTGA -3'
<i>RPB4</i>	A4	5'- CGCACCTGTACATTCAATTGG -3'
<i>RPB9</i>	Fwd	5'- ATGACTACGTTTAGATTTTTGTCGTGACTGCAACAATATGTTG TACCCTCGTGAGGATAAAcgtacgctgcaggtcgac-3'
<i>RPB9</i>	Rev	5'- TCATGAAAACCTGCGTCCTTTTGTTTTTTTGATCTGAAGTAAAT ATGTGTGAGCAAGATAatcgatgaattcgagctcg-3'
<i>RPB9</i>	A1	5'- GGCTGCTTGT CCTGTTTCCTT -3'
<i>RPB9</i>	A4	5'- CTTAGACGTATCTCTCGTCC -3'
<i>CAX4</i>	Fwd	5'- ATGAATAGTACCGCCGCTGCAATAAATCCAAATCCAAATGTT ATACCATTcgtacgctgcaggtcgac -3'
<i>CAX4</i>	Rev	5'- CTAATCCCTTTTGGATTTATCATTGAAAGATCGCTGATTTATT CTTTTCCatcgatgaattcgagctcg -3'
<i>CAX4</i>	A1	5'- GGATGAAATAGGAGAATTCGGG -3'
<i>CAX4</i>	A4	5'- CGTCAGAGGGTATTATCTCC -3'

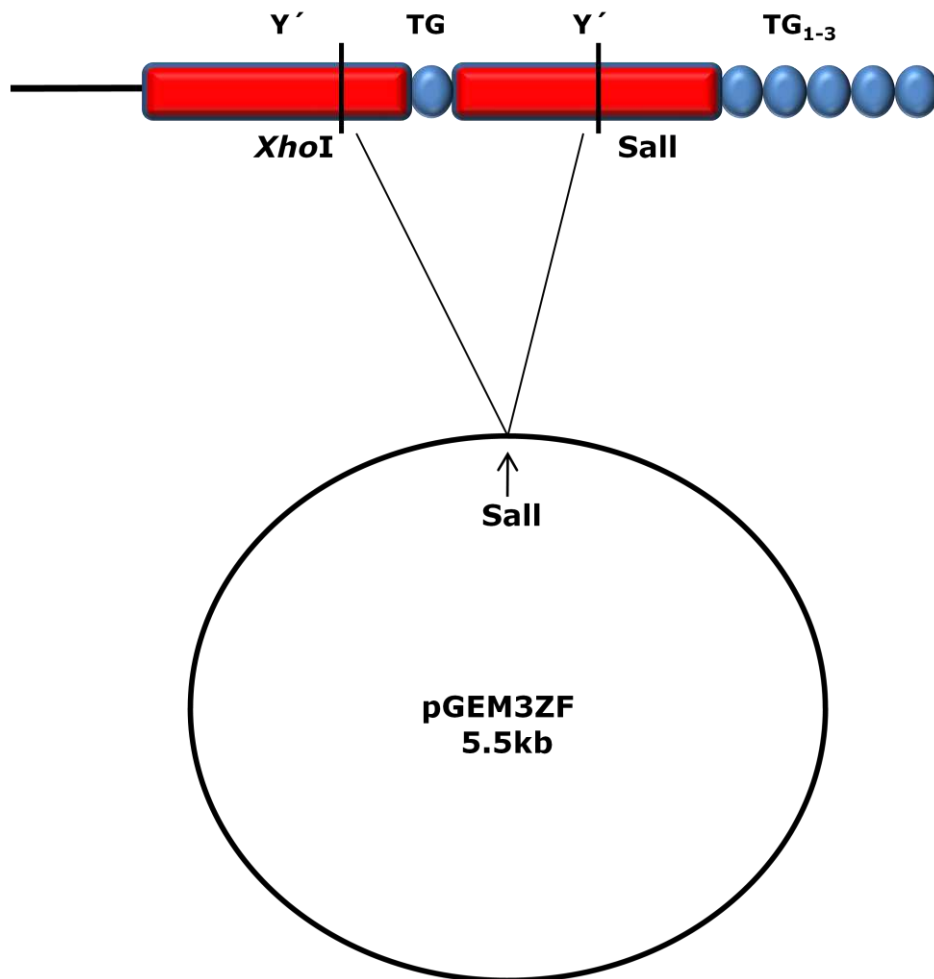
<i>RPP1A</i>	Fwd	5'- ATGTCTACTGAATCCGCTTTGTCTTACGCCGCCTTGATTTTG GCTGACTCcgtagcgtgcaggtcgac -3'
<i>RPP1A</i>	Rev	5'- CTAATCAAATAAACCGAAACCCATGTCGTCATCGGATTCTTC TTTAGCTTatcgatgaattcgagctcg -3'
<i>RPP1A</i>	A1	5'- CCTTAACCCTGTAGCCTCAT -3'
<i>RPP1A</i>	A4	5'- GTCTGGGTGAGAATATGG -3'
<i>MET7</i>	Fwd	5' CAATAAATTAATATTGTTTCATATCACTGGAACAAAAGGTA AAGGTTCTAcgtacgctgcaggtcgac -3'
<i>MET7</i>	Rev	5'- AATCGAACCATATCTCTGAACCATGTTGATGCAGCTACCATA CTATCTTTatcgatgaattcgagctcg -3'
<i>MET7</i>	A1	5'- CCATGACCGATTTAGCCATC -3'
<i>MET7</i>	A4	5'- GCTTCCTCTATACTAGCC -3'
<i>RAD52</i>	Fwd	5'- GGAAGATATTGGGTATGGTACCGTGGAGAACGAAAGACGGA AACCTGCCGCCGTACGCTGCAGGTCGAC -3'
<i>RAD52</i>	Rev	5'- CGCTGGAATATGCTTGGACGTAGTCTGATCAACGGTGTGCC TAATGGATCGATGAATTCGAGCTCG -3'
<i>RAD52</i>	A1	5'- GGATGAGAAGAAGCCCGT -3'
<i>RAD52</i>	A4	5'- TAGGCTTGCGTGCATGCAGG -3'

**Table 2.3: *S. cerevisiae* Strains used in this study**

All strains derived from the Y55 diploid strain that named YGL9. It is a telomerase-positive that a heterozygous for *EST2* gene. Type II survivor strains were generated and the target genes were deleted individually in one strain (YGL2.15). Two genes (*RAD50* and *RPB9*) were deleted in two more different type II strains (YGL9.12 and YGL9.17). *KU80* was deleted in YGL38 type II strain.

<b>Strain</b>	<b>Relevant genotype</b>	<b>source / reference</b>
YGL9	<i>HO/HO, MAT a/α ura3-n/ura3-n, leu2-r/leu2-r, ade1-1/ade1-1, can1R/can1R, EST2/est2::KANMX</i>	(Liti and Louis, 2003)
YGL2.15	<i>est2::KANMX/est2::KANMX</i>	Liti and Louis
YGL38	<i>est2::KANMX/est2::KANMX</i>	Liti and Louis
YGL9.12	<i>est2::KANMX/est2::KANMX</i>	This study
YGL9.17	<i>est2::KANMX/est2::KANMX</i>	This study
YGL2.15RAD50	<i>est2::KANMX/est2::KANMX, RAD50/rad50::hphMX</i>	This study
YGL9.12RAD50	<i>est2::KANMX/est2::KANMX, RAD50/rad50::hphMX</i>	This study
YGL9.17RAD50	<i>est2::KANMX/est2::KANMX, RAD50/rad50::hphMX</i>	This study
YGL2.15MRE11	<i>est2::KANMX/est2::KANMX, MRE11/mre11::hphMX</i>	This study
YGL2.15XRS2	<i>est2::KANMX/est2::KANMX, XRS2/xrs2::hphMX</i>	This study
YGL38KU80	<i>est2::KANMX/est2::KANMX, KU80/ku80::natMMX</i>	This study
YGL2.15NEJ1	<i>est2::KANMX/est2::KANMX, NEJ1/nej1::hphMX</i>	This study
YGL2.15ELG1	<i>est2::KANMX/est2::KANMX, ELG1/elg1::hypMX</i>	This study

YGL2.15SGS1	<i>est2::KANMX/est2::KANMX, SGS1/sgs1::hphMX</i>	This study
YGL2.15RIF1	<i>est2::KANMX/est2::KANMX, RIF1/rif1::hphMX</i>	This study
YGL2.15RIF2	<i>est2::KANMX/est2::KANMX, RIF2/rif2::hphMX</i>	This study
YGL2.15DCC1	<i>est2::KANMX/est2::KANMX, DCC1/dcc1::hphMX</i>	This study
YGL2.15HUR1	<i>est2::KANMX/est2::KANMX, HUR1/hur1::hphMX</i>	This study
YGL2.15UPF1	<i>est2::KANMX/est2::KANMX, UPF1/upf1::hphMX</i>	This study
YGL2.15SRB2	<i>est2::KANMX/est2::KANMX, SRB2/srb2::hphMX</i>	This study
YGL2.15GTR1	<i>est2::KANMX/est2::KANMX, GTR1/gtr1::hphMX</i>	This study
YGL2.15OGG1	<i>est2::KANMX/est2::KANMX, OGG1/ogg1::hphMX</i>	This study
YGL2.15RPB4	<i>est2::KANMX/est2::KANMX, RPB4/rpb4::hphMX</i>	This study
YGL2.15RPB9	<i>est2::KANMX/est2::KANMX, RPB9/rpb9::hphMX</i>	This study
YGL9.12RPB9	<i>est2::KANMX/est2::KANMX, RPB9/rpb9::hphMX</i>	This study
YGL9.17RPB9	<i>est2::KANMX/est2::KANMX, RPB9/rpb9::hphMX</i>	This study
YGL2.15CAX4	<i>est2::KANMX/est2::KANMX, CAX4/cax4::hphMX</i>	This study
YGL2.15RPP1A	<i>est2::KANMX/est2::KANMX, RPP1A/rpp1a::hphMX</i>	This study
YGL2.15MET7	<i>est2::KANMX/est2::KANMX, MET7/met7::hphMX</i>	This study
YGL2.15RAD52	Ho::HYGMX,est2::KANMX,rad52::NAT	This study



**pEL30 construction**

**Figure 2.1. pEL30 map**

The pEL30 plasmid was used to probe telomeres in wild-type and survival strains. It contains Y' and TG sequence from *S. cerevisiae* in a pGEM3ZF vector.

**KAc medium:**

KAc medium were used for yeast sporulation. It consist of 2.5% w/v of Potassium Acetate, 0.27% w/v of Yeast Extract, 0.06% w/v of Dextrose-d-Glucose. Prior to autoclaving, the pH was adjusted to 7.0. 0.1% w/v of COM drop out powder (see table below) and 2.5% of bacto-agar was added.

**Table 2.2. Complete media**

<b>Amino acids</b>	<b>Mg</b>
Adenine	800mg
Arginine	800m
Aspartic acid	4000mg
Histidine	800mg
Leucine	800mg
Lysine	1200mg
Methionine	800mg
Threonine	800mg
Trptophan	800mg
Tyrosine	1200mg
Uracil	800mg
Phenylalanine	2000mg

## Drug containing media

**Table 2.3. Drugs used with concentration and supplier**

Name	Concentration	Supplier
Hygromycin B ( HYG)	6 µl/ml	Invitrogen
Neorseoethricin (NAT)	100µg/ml	Invitrogen
Geneticin (G418)	0.4 µg/ml	Invitrogen

### 2.1.8. *E. coli* growth media and storage

Solid and liquid Luria Bertani (LB) media consist of 0.5% w/v of Yeast Extract, 1% w/v of Tryptone, 0.5% w/v of Sodium Chloride, and autoclaved at 120°C for 35 minutes. When cool, 1% v/v of ampicillin was added. For plates, 2.5% w/v bacto-agar was added. *E. coli* cells were stored at minus 80°C in 15% v/v glycerol in dH<sub>2</sub>O.

## 2.2. Methods

### 2.2.1. PCR- mediated gene disruption

PCR-mediated gene disruption was conducted in an MJ Research DNA Engine Tetrad 2 thermal cycler as described in (Wach et al., 1994) ( Fig. 2.1), using ~ 65 bp oligonucleotides consisting of 45 bp homology to the upstream and downstream region of the target gene and 20 bp of the marker cassette on both sides. The three markers used were - *kanMX*, *natMX* and *hphMX* cassettes, which were amplified from plasmids, pFAKanMX4, pNatCre and

pAG26, respectively (Wach et al., 1994, Guldener et al., 1996, Goldstein and McCusker, 1999), and conferred resistance to Geneticin, Nourseothricin and Hygromycin B, respectively. The PCR reaction was performed in a 50  $\mu$ l volume consisting of  $\sim$  50ng template plasmid DNA (containing one of the above marker cassette), 5 $\mu$ l 10X buffer (200mM Tris-HCL pH 8.4), 2  $\mu$ l 50mM MgCl<sub>2</sub>, 2.5 $\mu$ l each primer (at 10mM), 2 $\mu$ l dNTPs (all four at 5mM each) and 0.5  $\mu$ l TAQ polymerase (5U/  $\mu$ l). This reaction was subjected to: 95°C for 5 minutes followed by 35 cycles of 94°C for 30 seconds, 58°C for 30 seconds and 72°C for 5 minutes, followed by 72°C for 10 minutes. The PCR product was used in a yeast transformation.

### **2.2.2. Transformation of yeast**

Diploid type II survivors were transformed using the Lithium Acetate method as described in (Gietz and Schiestl, 2007) (Fig. 2.1). Yeast cells were grown overnight in 5ml of YEPD media at 30°C with shaking. Subsequently, the cells were diluted 1:10 in liquid YEPD and grown for 5 hours at the same conditions. Yeast cells were harvested by table top centrifugation at 3000 rpm for 5 minutes. The supernatant was discarded and the pellet was resuspended in 1 ml sterile dH<sub>2</sub>O, and then transferred to a 1.5 ml Eppendorf tube. The cells were washed twice in sterile dH<sub>2</sub>O by centrifugation at 13000 rpm for 1 minute and then twice re-suspended in 1ml 100mM Lithium Acetate. The cells were re-suspended in 1 ml of 100 mM LiAc, and split in two aliquots, one of which was used as negative control, and the volume was adjusted with dH<sub>2</sub>O. The cells were harvested by centrifugation at 13000 rpm for 15 seconds and the following components added: 240  $\mu$ l of polyethylene glycol (PEG) (50% w/v), 36 $\mu$ l of 1 M LiAc, 20  $\mu$ l (2.5 mg/ml) of denatured salmon sperm



DNA (Invitrogen), and 1 µg of transforming DNA in 55 µl dH<sub>2</sub>O. The cells were completely re-suspended by vortexing and incubated at 30°C for 30 minutes, then heat shocked at 42°C for 20 - 25 minutes. The cells were harvested by centrifugation at 7000 rpm for 20 seconds, re-suspended in 1 ml of YEPD and incubated at 30°C for three hours with shaking. Cells were pelleted by centrifugation at 7000 rpm for 20 seconds and re-suspended in 800 µl of sterile dH<sub>2</sub>O. The cells were plated on appropriate selective media. Cells were incubated at 30°C for 2 or 3 days.

### 2.2.3. Yeast Colony PCR

Colony PCR was used to confirm the deletion of the target gene. A yeast colony was suspended in 10 µl of 1% (w/v) zymolyase and incubated at 37°C for 60 minutes. 15 µl PCR mix was added (1 µl of 5 mM gene-specific primer (A1), 1 µl of 5 mM marker-specific primer (H2 for HYG, primers for all markers are shown in Table 2.4), 1 µl of 2.5 mM of dNTPs, 0.25 µl of Taq polymerase, 2.5 µl of 10X Taq buffer, 1 µl of Mg<sup>++</sup> (Magnesium), 2 µl rediload (Invitrogen) and 6.25 of PCR water. The PCR reaction was performed at: 95°C for 5 minutes, followed by 40 cycles of 95°C for 30 seconds, 58°C for 30 seconds and 72 °C for 3 minutes, with a final extension of at 72°C for 10 minutes.

**Table 2.4. Primers for used markers**

Marker	Primer	Sequence
hphMX	H2	CGGCGGGAGATGCAATAGG
hphMX	H3	TCGCCCCGAGAAGCGCGGCC
NATMX	N2	GATTCGTCGTCCGATTCGTC
NATMX	N3	AGGTCACCAACGTCAACGCA
KANMX	K2	ttcagaacaactctggcgca
KANMX	K3	catcctatggaactgcctcgg

### **Agarose gel electrophoresis:**

Agarose gel electrophoresis was performed using 1% w/v agarose in 1 x TBE (10 x TBE buffer: 0.89 M Tris-HCl, 0.89 M boric acid, 100 mM EDTA). Colony PCR product was run in 1% w/v of agarose gel to confirm product size.

#### **2.2.4. Yeast sporulation**

Diploid yeast cells were grown at 30°C on solid YEPD medium overnight, replica-plating onto KAc medium. Cells were incubated at room temperature for at least 3 days.

#### **2.2.5. Yeast -tetrad- dissection**

Yeast cells sporulation was confirmed by microscopic examination. A small patch of cells was transferred into 100 µl of dissecting buffer (1 M sorbitol, 10 mM EDTA, 10 mM NaPO<sub>4</sub> pH7.5) and of 5 µl of 1% (w/v) zymolyase (20T ) and incubated at 30°C for 30 minutes. A further 400 µl of the dissecting buffer was added to stop the reaction. Spores were dissected- using a micromanipulator, (Zeiss Axiolab, from Singer Instruments). The dissected spores were incubated at 30°C for two days on YEPD plate. Spores were transferred to media containing the appropriate drug to determine the genotype.

#### **2.2.6. Single colony propagation**

After spores were genotyped, six serial rounds of propagation of a single colony (containing the deleted gene) were conducted on solid YEPD medium at

30°C for two days (which equals ~ 150 generations of yeast generations). Twenty different cell lines for each deleted gene were propagated

### **2.2.7. Preparation of yeast genomic DNA**

Yeast cells were grown overnight at 30°C with shaking in 5 ml YEPD, and subsequently harvested by centrifugation at 3500 rpm for 5 minutes. The cells were re-suspended in 0.5 ml of spheroplasting solution consist of 1.2M sorbital, 200mM of Tris-HCl, pH 7.5, and 20mM, 1% v/v of  $\beta$ -mercaptoehanol in dH<sub>2</sub>O. Cell were transferred to Eppendorf tubes containing 50  $\mu$ l of 1% (w/v) zymolyase 20T in spheroplasting solution, incubated at 37°C for 30 minutes, and harvested by centrifugation at 13000 rpm for 3 minutes. The cells were re-suspended in 50  $\mu$ l of 1M sorbital and 0.5 ml of lysis solution (50 mM of Tris-HCl pH7.5, 100 mM of NaCl, 100 mM EDTA, 0.5%SDS) to which 10  $\mu$ l of proteinase K (20mg/ml), 5  $\mu$ l of RNase (10mg/ml), was added. Cells were incubated at 65°C for at least two hours and up to overnight. 500 $\mu$ l of phenol: chloroform (1:1, v/v) was added, the solution vigorously mixed and pellet collected by centrifugation at 13000 rpm for 5 minutes. The aqueous upper layer was transferred to a fresh tube and the extraction repeated twice. 1 ml of ethanol was added, and until the DNA spooled by inversion of the tube. The supernatant was carefully removed. Samples were centrifuged briefly for ~ 5 seconds and the remaining supernatant removed. 200 $\mu$ l of 70 % of ethanol was added to wash the DNA, and after brief centrifugation the supernatant removed. The DNA was air dried - and dissolved in 200  $\mu$ l of 1X TE pH 8.0 (10X TE consist of 1M Tris, pH 8.0, 0.5M of EDTA in dH<sub>2</sub>O). DNA samples were stored at -20°C.

### **2.2.8. Restriction enzyme digest**

100 ng yeast genomic DNA was digested overnight at 37°C with 20 units of the restriction enzyme *Xho* I. This enzyme cuts once within the Y' element. 2.5µl of appropriate restriction buffer (New England Biolabs), supplemented with 0.5 µl of RNase (10mg/ml) were added. To ensure complete digestion, 10 units of *Xho* I was added the next morning and incubation continued for further four hours.

### **2.2.9. Agarose gel electrophoreses of genomic DNA**

Agarose gel electrophoresis was performed to separate genomic DNA. Agarose gels were typically made using 0.9% (w/v) agarose in 1 x TBE (10 x TBE buffer: 0.89 M Tris-HCl, 0.89 M boric acid, 100 mM EDTA) containing 10 µl /100 ml Ethidium bromide (Sigma). Before loading digested yeast DNA, 5µl of RediLoad buffer (Invitrogen) was added to each sample. The DNA was separated at 40V for 16 Hours in 1X TBE buffer.

### **2.2.10. Southern analysis**

A non-radioactive method was used to perform Southern blot analysis of telomere structure. Fluorescein-labelled probes were made using the Fluorescein High-prime kit (Roche). The gel was photographed to confirm the DNA was fully digested and correctly separated. The 0.9% w/v agarose gel was rinsed in dH<sub>2</sub>O and incubated for 30 minutes in denaturation buffer (5.8% w/v of NaCl, 2% w/v of NaOH, in dH<sub>2</sub>O) , and rinsed again in water. The gel was incubated in neutralization buffer (6% w/v of Trizma Base, 17.5% w/v of NaCl in dH<sub>2</sub>O, pH: 7.5 for 2X 15 minutes. The DNA was transferred onto a

positively charged Nylon membrane (Amersham), using 20X SSC (17.6% w/v of NaCl, 8.8% w/v of Tri Sodium Citrate, in dH<sub>2</sub>O, pH. 7.0) buffer and Whatman paper. The gel was blotted overnight using 20X SSC buffer. Both sides of the membrane were exposed to 12 J/m<sup>2</sup> of UV to cross-link the DNA to the membrane. The membrane was rinsed in 4X SSC and placed into a hybridisation tube, 15 ml of hybridisation solution (0.1% (w/v) of lauroyl-sarcosine, 0.02% (w/v) of SDS and 1/10 volume of 10X blocking solution (Roche) was added and the membrane incubated in an hybridisation oven at 60°C for at least 1 hour. The fluorescein -labelled probe was denatured at 100°C for 5 minutes, and incubated for 5 minutes in ice. The solution was replaced with 15 ml of fresh hybridisation solution containing 5 µl of denatured Y'-TG<sub>1-3</sub> probe and 0.5 µl of lambda ladder probe, incubated overnight at 60°C. Subsequently, the membrane was washed 2X 10 minutes with 2XSSC containing 0.1 of %SDS at 60°C, followed by 2X 10 minutes with 0.1XSSC, 0.1%SDS at 60°C. The membrane was washed once in washing buffer (10X maleic acid buffer, 0.3 % Tween) and incubated at room temperature with shaking for 3-5 minutes. 10 X Maleic acid(MA) buffers consist of 1.16% w/v of Maleic Acid, 0.87 % w/v of NaCl in dH<sub>2</sub>O , pH 7.5 autoclaved for 35 minutes at 120°C. The membrane was incubated at room temperature with shaking for 30 minutes in 200 ml of blocking solution (dilute 10X blocking solution 1:10 in 10XMA buffer). 10X blocking solution consist of 10% w/v of blocking reagent (Roche) in 10X MA ,prepared by dissolving gradually 10 g of blocking reagent in 100 ml of MA buffer with heating at ~60°C and stirring. Then the membrane was incubated for 30 minutes in antibody solution, 5 µl of Antifluorescein-AP in 200 ml of blocking solution. The membrane was washed 2X 15 minutes in washing buffer at room

temperature. 3-4 ml of CDP star detection buffer (PerkinElmer life science) was spread over the membrane's side containing the DNA, and the membrane was wrapped in Saran wrap, in dark place for 5 minutes ( the cassette was used for that). The membrane was placed in fresh Saran wrap; the Fuji film was exposed for 60 minutes, and developed using a Xograph SRX-201.

### **2.2.11. CHEF plug preparation and CHEF gel electrophoresis**

Cells were grown overnight in 5 ml of liquid YEPD. Cells were spun down in Sorvall centrifuge for 5 minutes at 3000 rpm. The supernatant was discarded, and the pellet was resuspended in 1 ml of 50 mM cold EDTA, and transferred to an eppendorf tube. The pellet was spun down for 30-60 seconds at 13000 rpm. The pellet was re-suspended in 200  $\mu$ l of 50 mM cold EDTA. 100  $\mu$ l of SCE / zymolyase/  $\beta$ -mercaptoEhanol solution was added. SCE solution consists of 1 M of sorbital, 0.1 M of sodium citrate and 0.05M of EDTA, 3 mg of zymolyase (20T) and 25  $\mu$ l of  $\beta$ -mercaptoEhanol were added to 100 $\mu$ l of SCE solution. 1% of low melt agarose was prepared in 0.125 M of EDTA. 0.5 ml of agrose was added, piptted up and down, then 100  $\mu$ l of it was loaded into the plug mold. The plug was set in ice for 60 minutes. When the plugs set, they were put into clean eppendorf tubes and overlayed with 0.5 ml of 0.45 M EDTA, 0.1 M Tris, and 5 $\mu$ l/ml of  $\beta$ -mercaptoEhanol, incubated at 37°C for at least four hours. This is to digest cells walls. the overlay was removed and replaced with 0.5 ml of 1% sarkosyl , 1 mg/ml proteinase K, 1 mg/ml RNase, and 0.4 M EDTA, then incubated overnight at 37°C to digest the protein or RNA. The next day, tubes were placed at room temperature for 30 minutes, and the overlay was removed, then the plugs were rinsed with cold 0.5 M of EDTA. Finally, the plugs were stored in 0.45 M of EDTA and 0.1 M of Tris at 4°C. To separate yeast cells chromosomes, 1% of agrose gel were

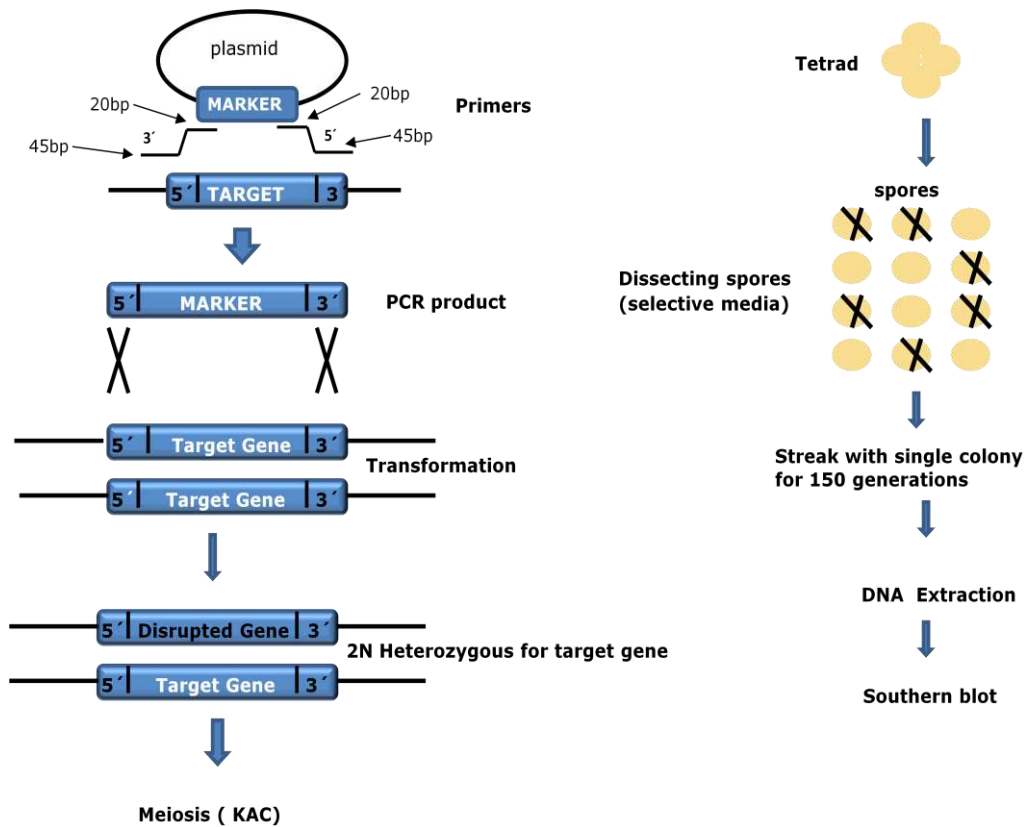
melted in 0.5X TBE. The plugs were loaded in the gel. The 0.5 TBE buffer was cooled to 14°C, then the gel was run at 120 angle and 6 Volts. The program was a 15 hours with switching every 60 seconds, followed by 9 hours with switching every 90 seconds. Finally, the gel was washed in 400 ml of water supplemented with 20µl (10mg/ml stock solution, Sigma) ethidium bromide for visualisation.

### **2.2.12. Temperature sensitivity test**

Yeast Strains were incubated at 30°C for two days. A single colony of wild-type, type II and mutant type II (after each gene has been deleted, ~150 generations) was each resuspended in 45 µl of distilled water and six ten-fold serial dilutions were produced per yeast strain. Three independent cell lines were examined for each deleted gene. 5 µl of each - yeast suspension was spotted onto YEPD plates and incubated at either 30°C or 37°C for 48 hours for each gene-deletion strain.

### **2.2.13. UV sensitivity test**

The serial diluted yeast strains (as in 2.2.12) were each exposed to either 20 m<sup>2</sup>/J or 80 m<sup>2</sup>/J in the dark and subsequently incubated at 30°C for 48 hours.



**Figure 2.2. Summary of gene deletion method in diploid *S. cerevisiae***

The disrupted target gene amplification, transformed to yeast cells. Then the tetrads were dissected, followed by single colony streaking. Finally, DNA was extracted, southern blotted to define telomere phenotype. X represents spores that cannot grow in the selective media. These spores have undeleted copy of the target gene.



#### **2.2.14. *E. coli* growth media and stocks**

*E. coli* cells containing the plasmid of interest were grown from frozen glycerol stocks. Cells were incubated at 37°C for two days in Luria Bertani (LB) plates with supplementation of 50 mg /L ampicillin. A single colony was transferred into 3 ml of liquid LB media supplemented with 1 µl/ml of ampicillin and incubated overnight with shaking at 37°C. A Qiagen miniprep kit was used to extract DNA plasmid that contains Y'-TG probe. Protocol was provided with the Kit. Finally, the plasmid was labelled with fluorescein-high prime.

## **Chapter 3: Genetic requirements for type II survivor maintenance post-senescence**

### **3.1. Introduction**

*S. cerevisiae* cells are able to maintain their telomeres in the absence of telomerase, utilizing the homologous recombination (HR) pathway (Lundblad and Blackburn, 1993). At least two types of survivors have been described previously, type I and type II, amplifying their telomeres by two different mechanisms. Type I survivors are characterised by the amplification of Y' elements, while type II survivors amplify the TG<sub>1-3</sub> repeats to elongate their telomeres (Le et al., 1999, Chen et al., 2001, Teng and Zakian, 1999). The *RAD52* gene is required to establish both types of survivors (Le et al., 1999, Chen et al., 2001). *Rad52*, a DNA repair gene is essential for all types of recombination (Rudin et al., 1989). Similarly, some human cancer cells (~10%) can extend their telomeres in the absence of telomerase utilising homologous recombination. This pathway is termed Alternative Lengthening of Telomeres (ALT) (Bryan et al., 1997, Henson et al., 2005). Genes required to establish types of *S. cerevisiae* survivors have been previously investigated (Le et al., 1999, Chen et al., 2001, Huang et al., 2001, Cohen and Sinclair, 2001). A central question that remains unanswered is what genes are required to maintain telomerase-negative survivors? In this chapter, genes were deleted in established survivors to address this question. Candidate genes were investigated to see if they are required for *S. cerevisiae* diploid type II telomere maintenance. Diploid yeast cells are more relevant to mammalian cells than haploid. Moreover, diploid survivors yeast strains were found to be unstable compared to haploid strains (Liti and Louis, 2003). Therefore the

survivors diploid strains behaved similarly to human cancer cells. Diploid yeast cells are generated as result of *HO* gene function. *HO* encodes an endonuclease that initiates mating-type switching. There are two transcription silent loci (HML alpha and HMR a) storing the information for mating type (Nasmyth, 1982, Herskowitz, 1988). *HO* recognises and cleaves a 24 bp site at *MAT* making a DSB. Then the *MAT* locus sequence is replaced with a new copy from HML or HMR. Cells that express *MAT*  $\alpha$  are able to mate with cells that express *MAT*  $a$  to generate a diploid strain. The *HO* gene is activated once after sporulation (Jin et al., 1997). Type II survivors were chosen for two reasons. First, *S. cerevisiae* type II survivors are more stable than type I (Teng and Zakian, 1999, Lundblad and Blackburn, 1993). Second, type I survivors have difficulty in meiosis (Liti and Louis, unpublished). Therefore, it is more amenable to study these genes with type II survivor strains. Candidate genes were chosen based on the following reasons. First, some of these genes were required to establish type II survivors (Le et al., 1999, Chen et al., 2001, Huang et al., 2001). Therefore they may be required for the maintenance of these survivors but perhaps not. Second, the majority of these genes affect telomere length in the telomerase-positive yeast background (Askree et al., 2004). Consequently, they may have the same role in survivor strains.

### ***3.2. General approach to test post-senescence gene requirement***

The general method used for gene requirement at post-senescence will be explained here. First, the catalytic subunit of telomerase, *EST2* was deleted in a *S. cerevisiae* wild-type diploid to generate a heterozygous knockout strain, using a KANMX cassette as described previously (Wach et al., 1994). Strains

used here are HO+. The *HO* gene encodes an endonuclease that creates a DSB at the *MAT* locus (Haber, 2006). As result, spores that have MAT a-type can generate MAT  $\alpha$ -type cells. Conjugation occurs between these two different MAT types, generates diploid cells. Cells from the heterozygous deletion were grown on YEPD plates for two days at 30°C, replicated to KAc plates and grown at room temperature for at least three days. Spores were micro-manipulated to separate the four meiotic products, grown on YEPD plates for two to three days at 30°C, then replicated to G418 containing media to detect their genotype. Only cells which have the deleted gene mutated by KANMX were grown. *est2* $\Delta$  cells derived from independent spores were patched on YEPD plates, incubated at 30°C for two days. Single colonies were then restreaked further. At  $\sim$  75 generations (usually at plate three or four of streaking), only a small population of these cells grew, while the majority stopped growing and died due to telomere shortening at this stage, called senescence (Fig.3.2. A) (Lundblad and Blackburn, 1993). Cells which escaped senescence were streaked a further six times, each time they were incubated at 30°C for two days. A *S. cerevisiae* mother cell needs approximately two hours to generate a bud. In other words, one generation of *S. cerevisiae* is roughly two hours. Therefore, incubation for two days allows cells to grow for  $\sim$  25 generations. Genomic DNA of these cells was isolated and digested with the restriction enzyme *Xho*I, which cuts once within Y' elements. Digested DNA was run at 45V for 16 hours to separate its fragments. The DNA was blotted overnight to transfer DNA fragments to a positive charged nylon membrane. The separated fragments DNA were probed with Y'-TG probe to distinguish between the types of survivors, type I and II (Fig. 3.4). Type II

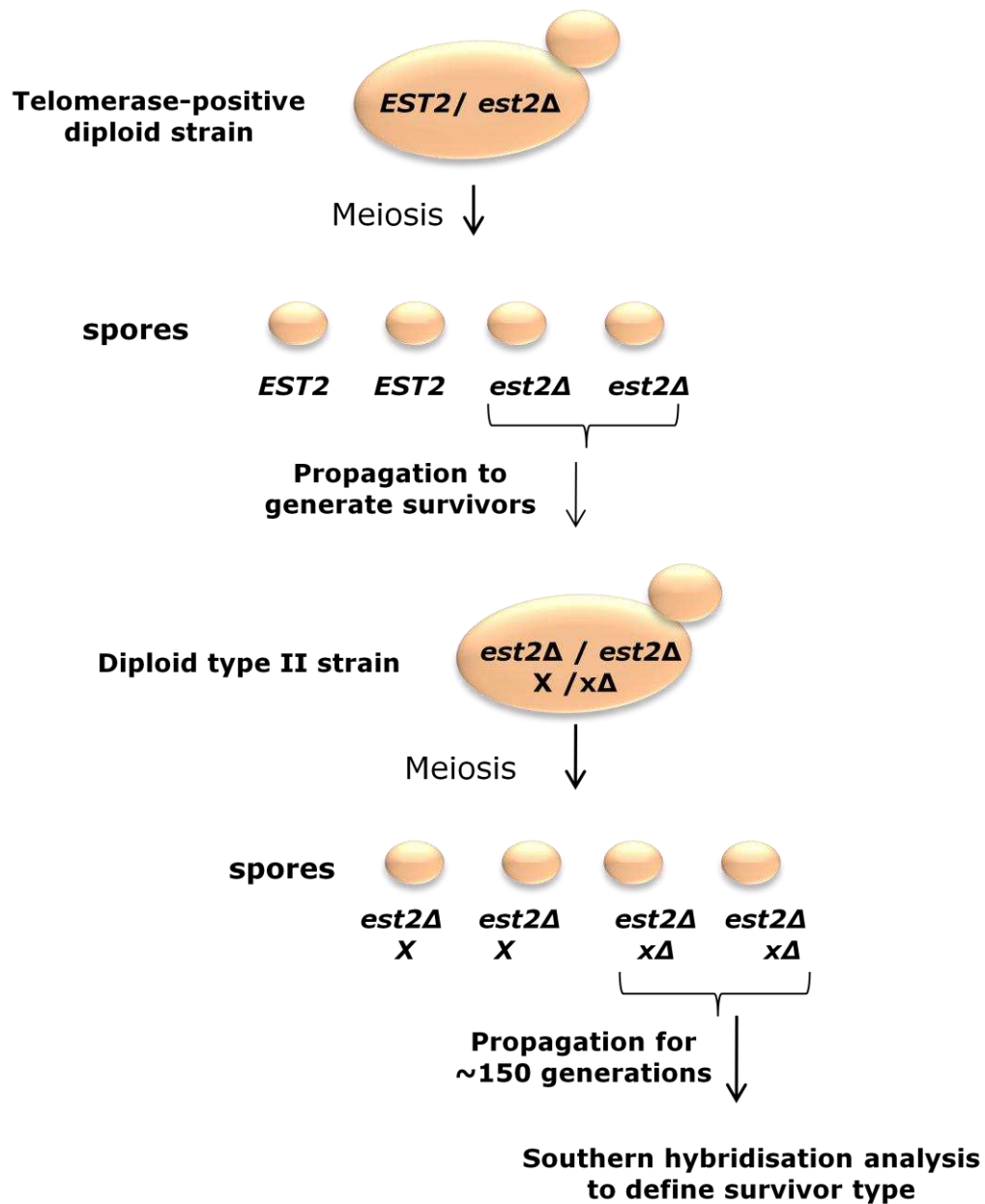
survivors were labelled, stored in 25% glycerol at -80 for the next part of this experiment.

Candidate genes were deleted in a type II strain background using the HYG cassette as explained in Chapter 2 (Fig.3.1). The HYG cassette is 1.7 kb. Colony PCR is performed to ensure that the target gene is successfully disrupted. Two primers were used for this purpose. A1, a primer for the target gene (any gene in this study), is located at ~300 bp upstream from the start point of this gene. H2, a primer for the HYG cassette, is located at ~700 bp in the cassette. Consequently, the expected size for the colony PCR product is ~ 1.0 kb (Fig. 3.3 A-B). Correct transformants that were now heterozygous for the candidate gene $\Delta$  were grown for two days on YEPD plates at 30°C, then replicated to KAc media. Spores were micro-manipulated to separate the four spores, incubated at 30°C for two to three days, and replicated to hygromycin containing media. Two spores from each tetrad which have the deleted gene should grow due to drug resistance. 20 different cell lines derived from independent spores were streaked for six times (~ 150 generations) for each candidate gene; they were incubated at 30°C for two days at each streak. Genomic DNA was isolated and digested with *Xho*I. Southern blots of separated fragments were probed with a Y'-TG probe, developed to identify telomere structure. Cells were expected to behave in one of four ways. First, cells may continue as type II if they do not require the candidate gene, they will continue to elongate their telomeres, amplifying the TG repeat. Second, cells may die, if this gene is required for telomere elongation. Third, cells may switch to type I indicating a requirement for this gene for type II but not type

I. Fourth, cells may exhibit a new type of survivor, if they grow and display different telomere structures than previous known types (Fig. 3.2. B).

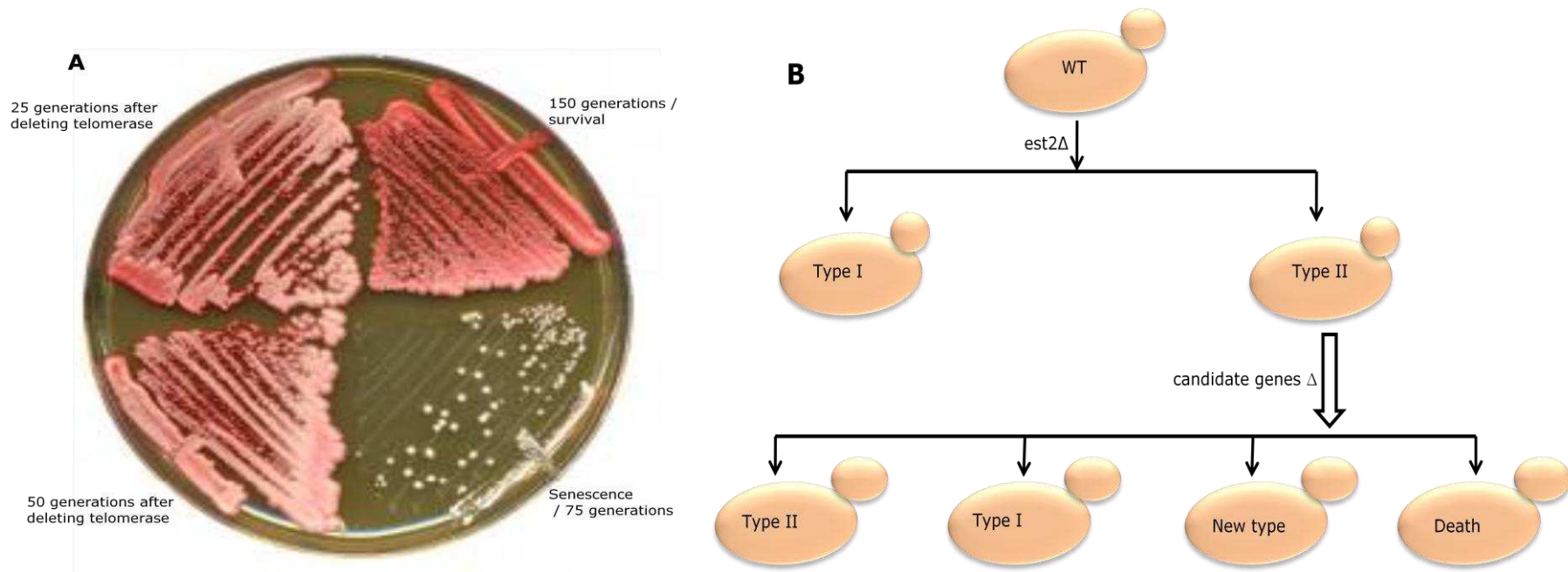
### ***3.3. The MRX-complex and SGS1 are not required for telomere maintenance in diploid type II survivors, despite being required to establish this survivor state***

The MRX- complex (*RAD50*, *MRE11* and *XRS2*) and *SGS1* were investigated to see if they are required for telomere maintenance in type II survivor background strains. First, the *EST2* gene, *S. cerevisiae* catalytic subunit telomerase (Lendvay et al., 1996, Lundblad and Blackburn, 1993) was deleted in a Y55 diploid strain. the heterozygous strain of telomerase component *EST2* knock out, named YGL9 was generated (Liti and Louis, 2003). This strain can be considered as wild type for telomerase maintenance because it still has a copy of the *EST2* gene. Second, the YGL9 stain was replica plated on G418 containing medium. Strains which have the deleted gene were streaked until survivors were generated (the genotype for these is *est2::KANMX / est2::KANMX*). Most of these cells died at ~ 75 generations, but a small number of cells bypassed this time point to generate survivors, as described previously (Lundblad and Blackburn 1993). Survivor strains were stored in 25% glycerol at -80 until the next step of the experiment. Third, MRX complex genes and *SGS1* were deleted individually in diploid type II survivor strains, named YGL2.15, derived from YGL9, using the HYG cassette. The deletion cassette for each gene was transformed into the YGL2.15 strain; spores were dissected and replicated to hygromycin containing media. For the *RAD50* gene, the genotype is *rad50::HYGMX / rad50::HYGMX*. 22 spores (independent cell lines) were streaked on YEPD plates and incubated at 30°C



**Figure 3.1. *S. cerevisiae* survivor establishment and the candidate genes deletion**

*EST2/est2Δ* heterozygous strain was sporulated. *est2Δ* Spores were propagated to generate survivor strains. *est2Δ* spores are HO+, thus these survivors are diploid. The candidate genes were deleted individually in diploid type II survivor strains. Cells were propagated for ~150 generations and Southern hybridisation analysis was performed to define survivor type. X indicates to any target gene.

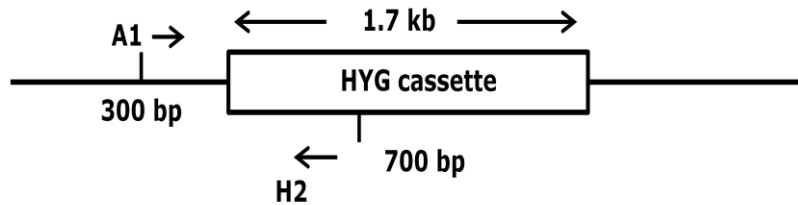


**Figure 3.2. *S. cerevisiae* senescence stage and survivors cells after deleting telomerase, then deleting the candidate genes**

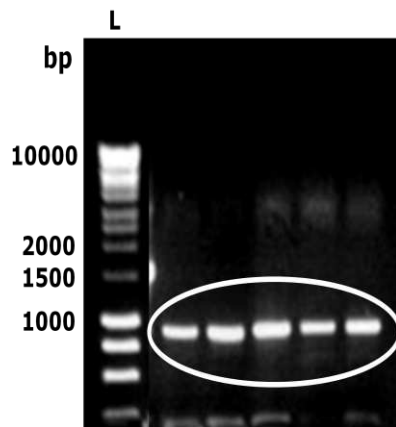
**A-** Plate shows senescence stage at  $\sim 75$  generations and survivors cells at 150 generations after deleting telomerase (*EST2*) in wild type strain (WT). **B-** Two types of survivors cells (Type I, II) are arising after telomerase deletion, and four expected possibilities after deleting the candidate genes in type II survivors. Strains are HO+; therefore, diploid cells were generated immediately after dissection.



**A**

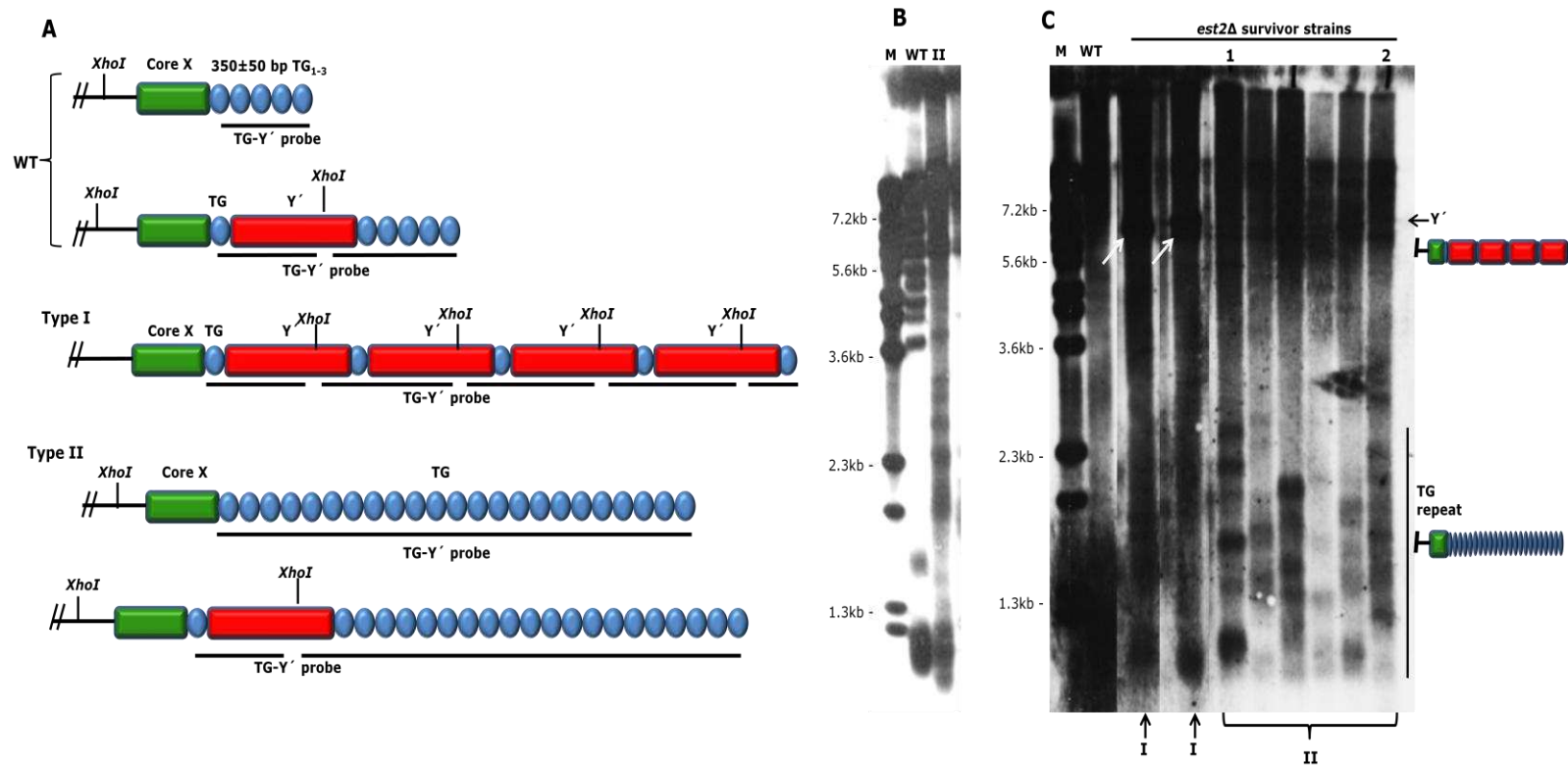


**B**



**Figure 3.3: Colony PCR to confirm target gene disruption**

**A-** Scheme for primers used in colony PCR. A1 represent the primer of target gene. H2 represent the primer of HYG cassette. The expected size for the PCR produce is ~ 1000 bp. **B-** Colony PCR photo for different *S.cerevisiae* cells that have the disrupted target gene. L represents the HyperLadder I (from Bioline). Bands in the white circle show the expected size for one gene (*met7Δ*). Samples in B represent an example for all used genes.



**Figure 3.4: *S. cerevisiae* wild type and *est2Δ* survivors telomere patterns**

**A-** Scheme for *S.cerevisiae* wild type, type I, and type II survivors telomere structure. Green boxes represent core X, blue circles represent TG<sub>1-3</sub> repeats, red boxes Y' elements. *XhoI* cuts within Y' element as indicated in A. DNA was probed with the TG-Y' probe as indicated by black lines under subtelomeric and telomeres structure. **B-** Southern blots for WT and type II (YGL2.15). **C-** Southern blot for *est2Δ* (type I and type II) survivor strains. White arrows showed Y' elements, 1 refer to type II (YGL9.12), 2 refer to type II (YGL9.17). (See the text for more details).

for two days. Cells were streaked for single colonies on YEPD seven times (~ 175 generations), in the same conditions as the first streaking. Genomic DNA was isolated and digested with the restriction enzyme *Xho* I, which cuts once within Y' elements. Fragments of the digested DNA were separated at 45V for 16 hours. The digested DNA was transferred to a positive charged nylon, and then probed with Y' - TG probe, developed. 22 out of 22 (100%) of *rad50*Δ independent cell lines displayed the type II telomere structure (Fig. 3.6, Table 3.1) thus, *RAD50* is not required to maintain telomeres as type II. *mre11*Δ and *xrs2*Δ displayed the same results as *rad50*Δ. Both genes were deleted using the HYG cassette and their genotypes were *mre11::HYG / mre11::HYG* and *xrs2::HYG / xrs2::HYG*. Twenty out of twenty (100%) *mre11*Δ independent strains displayed type II telomeres (Fig. 3.5 and table 3.1). YGL2.15*xrs2*Δ strains (21 independent strains) remain as type II for ~175 generations after the deletion of this gene (Fig.3.7, Table 3.1). In addition, *sgs1*Δ displayed type II telomere structure at ~ 150 generations showing no requirement for this gene to maintain this type of survivors (Fig. 3.8 and table 3.1).

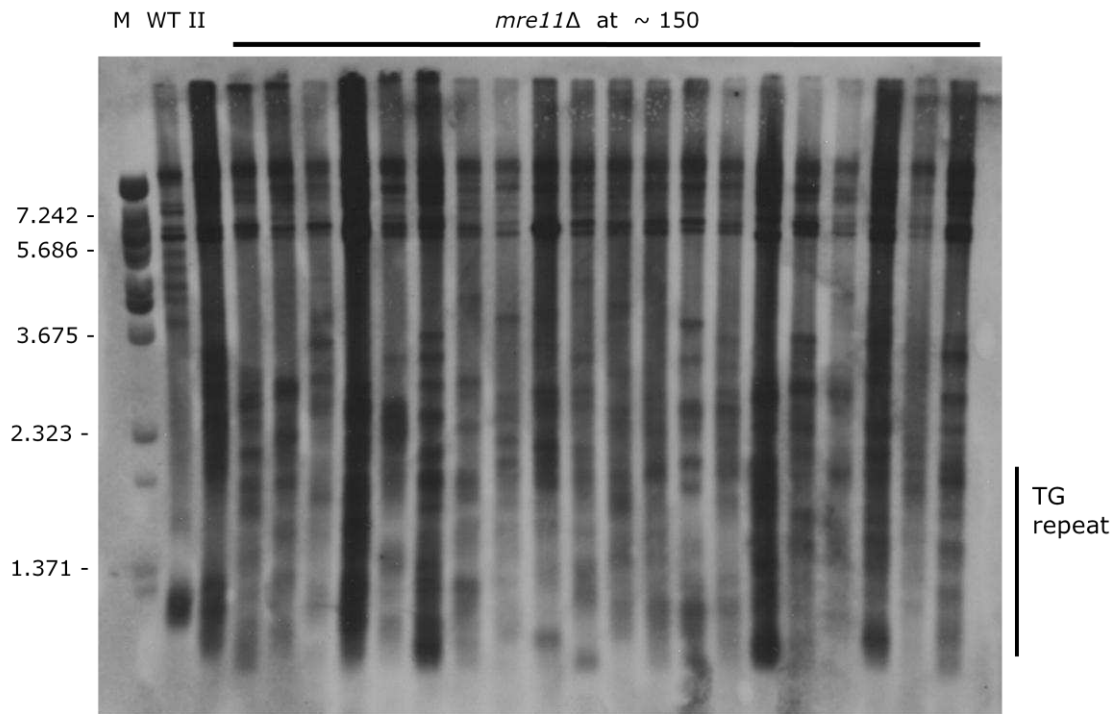
### ***3.4. RAD52 is required for telomere maintenance in haploid type II survivors***

*RAD52* was investigated to see if it required for telomere maintenance in survivors. It is an essential gene for all recombination pathways, implicated in DNA repair (Symington, 2002). However, the *HO* gene encodes an endonuclease enzyme to introduce a double strand break (DSB) within the *MAT* locus (Strathern et al., 1982, Nickoloff et al., 1986). Therefore, *HO* was deleted to prevent DNA cleavage, followed by *RAD52* deletion to examine its

role in telomere maintenance. Thus, *HO* was deleted in the YGL2.15 strain (type II); using the HYG MX cassette as described in chapter 2. Cells were grown in YEPD at 30°C for two days. Colony PCR was done to ensure gene deletion. Cells of this strain, containing *ho*Δ were stored at -80 in 25% glycerol for further study. *RAD52* was deleted in this strain, using NAT MX cassette as presented in Chapter 2. Cells were grown at 30°C for two days, replica-plated to KAc plate. Spores were dissected, incubated at 30°C for two to three days, replica-plated to NAT and HYG plates. 20 cell lines derived from independent spores, involved *ho*Δ and *rad52*Δ, were streaked on YEPD plates, incubated at 30°C for two days. 12 out of 20 cells died after first streaking. The rest displayed slow growth, they were restreaked on YEPD at the same conditions above, but seven of these cell lines died at second streaking. The last cell line was propagated for a further two days, but it died immediately after the fourth streaking. Consequently, it was hard to do any further analyses, but it is clear that *RAD52* is required for telomere maintenance in survivors in this strain (table 3.1).

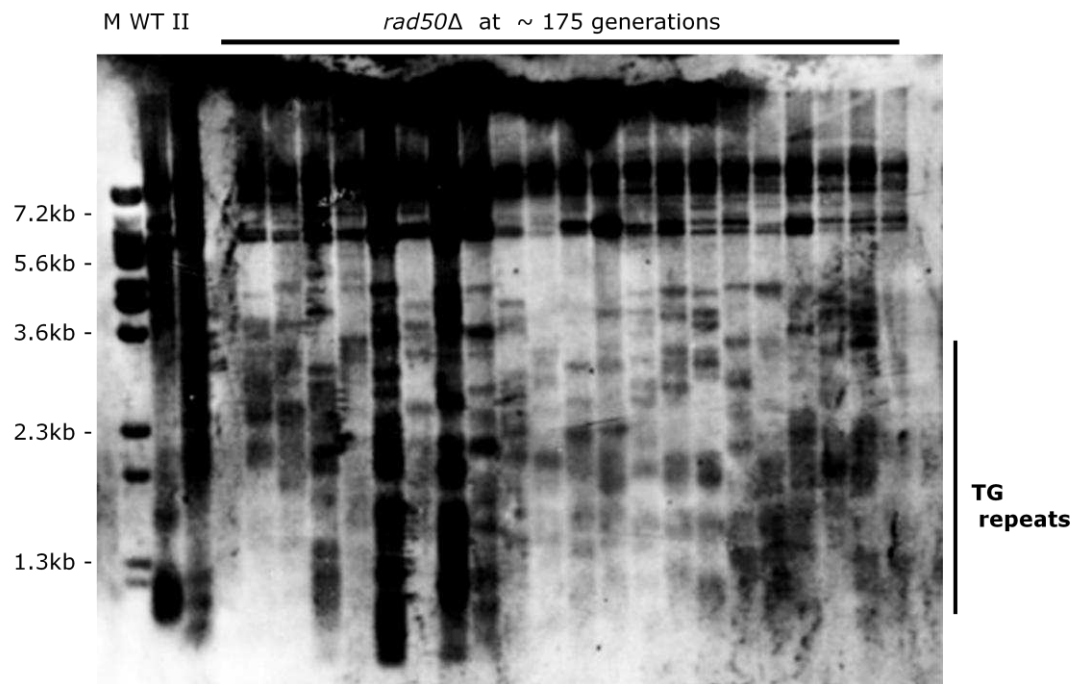
### ***3.5. Deletion of KU80 in type II background switches to new type***

*KU80* was examined for diploid type II survivor maintenance. First, a type II survivor strain was generated as described above. This strain was derived from the YGL9 strain (YGL38). *KU80* was deleted in this strain using NAT MX cassette as described in chapter 2, as a result of that, a heterozygous strain of *KU80* (YGL38) was generated. Spores of this strain were dissected, grown on a YEPD plate for two to three days at 30°C, replicated to a NAT containing plate, incubated at the same conditions for two days. Two spores of each tetrad were grown as a result of drug resistance, these have *KU80* deleted.



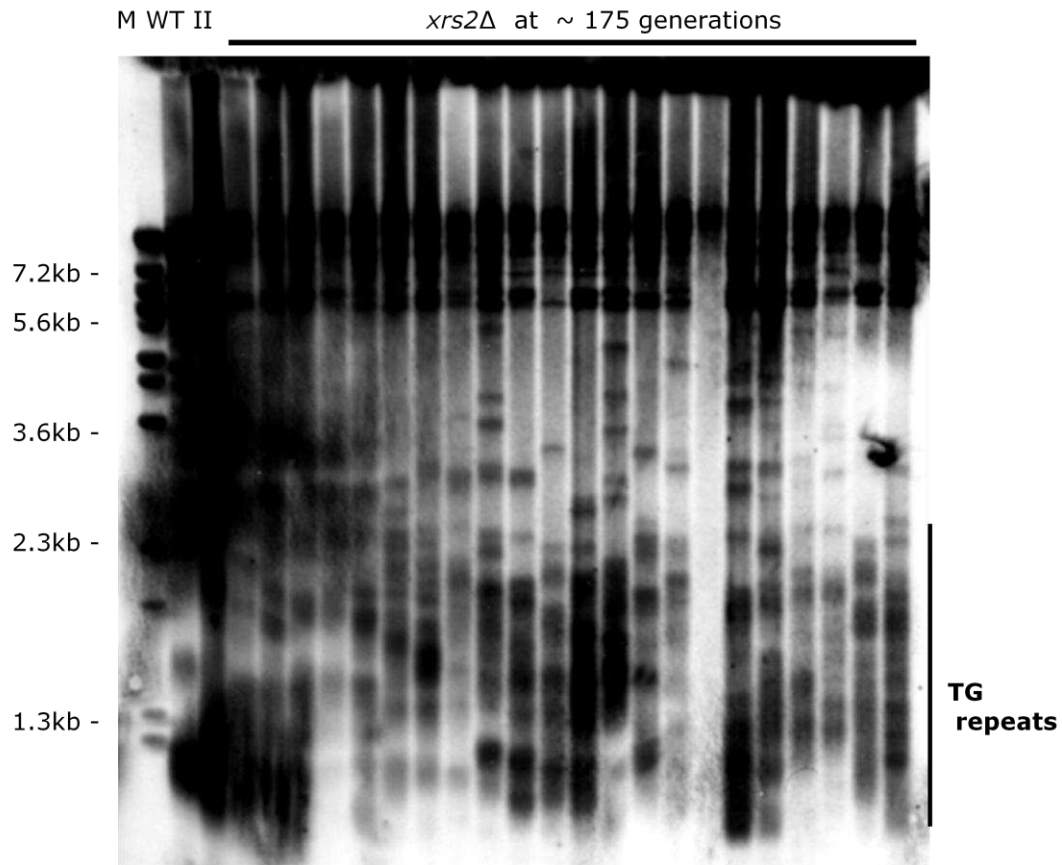
**Figure 3.5. Southern blot for *mre11*Δ in diploid type II strain**

Genomic DNA digested with *Xho* I, probed with Y'-TG<sub>1-3</sub> repeat probe. 20 independent cell lines from the same transformation displayed type II telomere pattern at ~150 generations from deleting this gene. First lane shows the DNA ladder, M ( $\lambda$ /Bste II), second lane shows *S. cerevisiae* Wild Type (WT), third lane shows type II strain before deleting the candidate gene. The rest of the lanes are *mre11*Δ independent strains at ~ 150 generations after deleting this gene, displaying TG repeat (Type II).



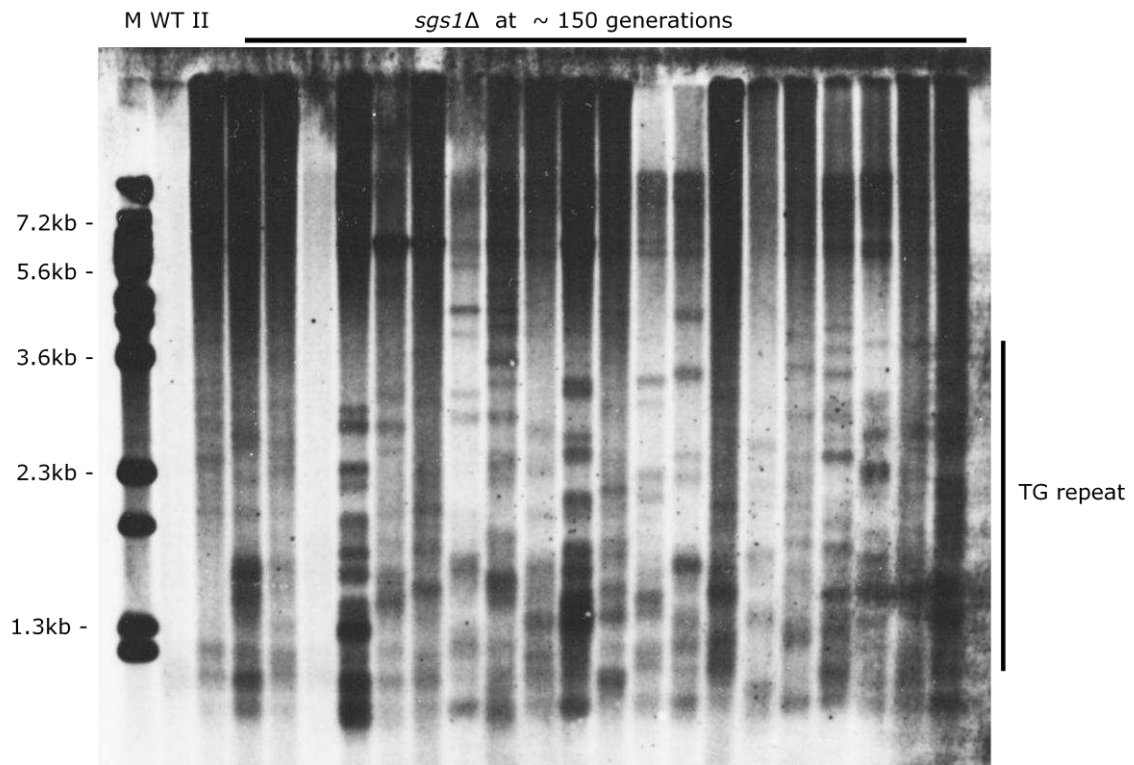
**Figure 3.6. Southern blot for *rad50Δ* in diploid type II strain**

Genomic DNA digested with *Xho* I, probed with Y'-TG<sub>1-3</sub> repeat probe. 22 independent cell lines from the same transformation displayed type II telomere pattern at ~175 generations from deleting this gene. First lane shows the DNA ladder, M ( $\lambda$ /Bste II), second lane shows *S. cerevisiae* Wild Type (WT), third lane shows type II strain before deleting the candidate gene. The rest of the lanes are *rad50Δ* independent strains at ~ 150 generations after deleting this gene, displaying TG repeat (Type II).



**Figure 3.7. Southern blot for *xrs2Δ* in diploid type II strain**

Genomic DNA digested with *Xho* I, probed with Y'-TG<sub>1-3</sub> repeat probe. 21 independent cell lines from the same transformation were displayed type II telomere pattern at ~175 generations from deleting this gene. First lane show the DNA ladder, M ( $\lambda$ /Bste II), second lane show *S. cerevisiae* Wild Type (WT), third lane show type II strain before deleting the candidate gene. The rest lanes are *xrs2Δ* independent strains at ~ 175 generations after deleting this gene, displaying TG repeat (Type II).



**Figure 3.8. Southern blot for *sgs1Δ* in diploid type II strain**

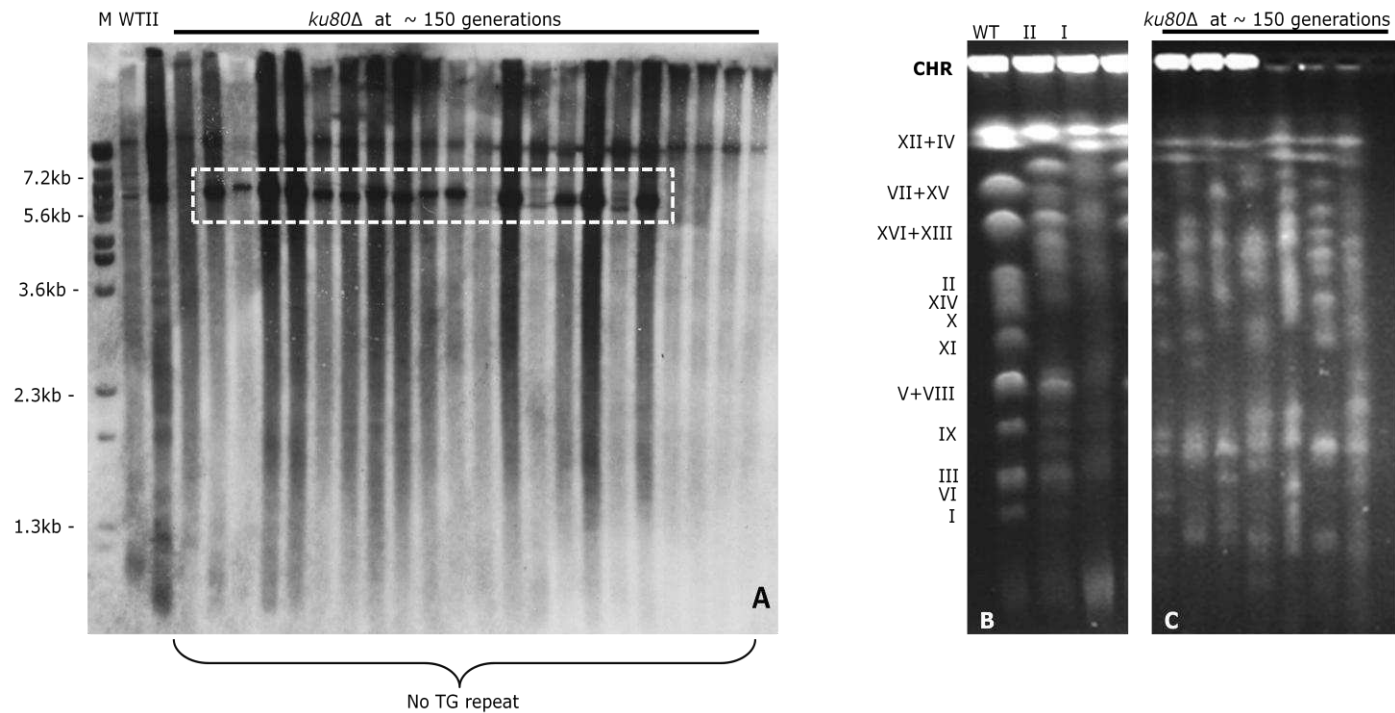
Genomic DNA digested with *Xho* I, probed with Y'-TG<sub>1-3</sub> repeat probe. 19 independent cell lines from the same transformation displayed type II telomere pattern at ~150 generations from deleting this gene. First lane shows the DNA ladder, M ( $\lambda$ /Bste II), second lane shows Wild Type (WT), third lane shows type II strain before deleting *SGS1*, the rest of the lanes are *sgs1Δ* displaying TG repeats.



22 independent colonies were streaked on YEPD plates, incubated at 30°C for two days, then a single colony of each one was re-streaked for further five times at the same conditions to bring cells to approximately 150 generations of deleting this gene. Cells were stored in 25% glycerol at -80 for further experiments. Genomic DNA was extracted at this stage, digested with *XhoI* enzyme, probed with Y' - TG probe, developed to display telomere type. All 22 independent cell lines displayed a completely different telomere pattern than type I and II survivors. The bands which indicate TG<sub>1-3</sub> telomeres and the terminal fragment were totally absent in all 22 independent cell lines. However, some of these cell lines exhibited amplified bands close to the Y' element long position, indicated by the white box in the Southern blot, therefore cells are not type II survivors (Fig. 3.9. A, Table 3.1). A CHEF gel was run for some independent cell lines (3 to 9) of the *ku80Δ* strain. Chromosomes of all these cell lines strains entered the gel. Thus, they behaved different to type I as in type I the chromosome do not enter pulsed field gels (Liti and Louis, 2003) (Fig.3.9 B, C). Therefore, this result confirms that all 22 *KU80Δ* strains displayed a new telomere structure.

### ***3.6. Candidate genes are not required for telomere maintenance in diploid type II survivors***

The rest of the candidate genes were examined for telomere maintenance in type II survivors. All these genes were deleted individually in the same strain (YGL2.15), using the HYG<sup>R</sup> cassette as described in chapter 2. Thus, strains are heterozygous for each gene. Spores were dissected, replica-plated to HYG media, incubated at 30°C for two days. Only two spores of each tetrad were grown as result of drug resistance. 20 independent colonies were streaked on



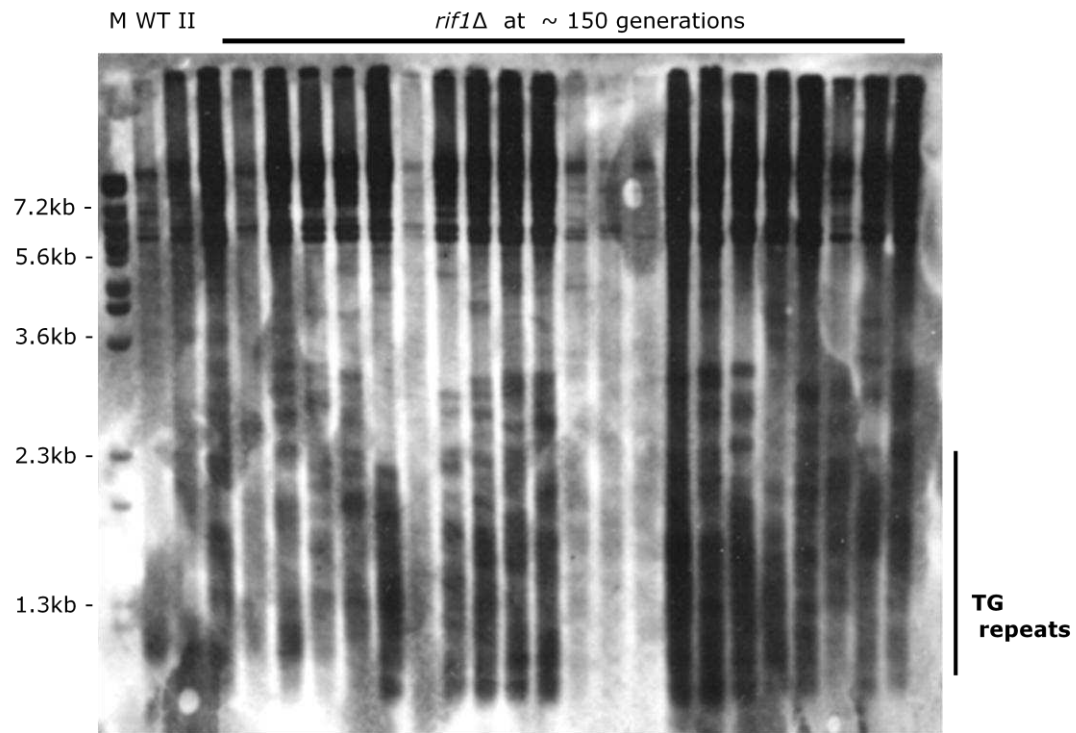
**Figure 3.9. Southern blot and CHEF gel for *ku80Δ* in diploid type II survivors at ~ 150 generations**

**A-** Genomic DNA digested with *Xho* I, probed with Y'-TG<sub>1-3</sub> repeat probe. First, second, and third lanes are the same as the first three lanes in Figure 3.3. All 22 independent cell lines do not display TG repeat bands, and almost of these cell lines exhibit a band close to long Y position. **B-** Chef gel for wild type (WT), type II, and type I strains. Chromosomes in type I survivors did not enter the gel. **C-** Chef gel for *ku80Δ*, samples 3 to 9 in A, chromosomes enter the gel to confirm they are not type I.

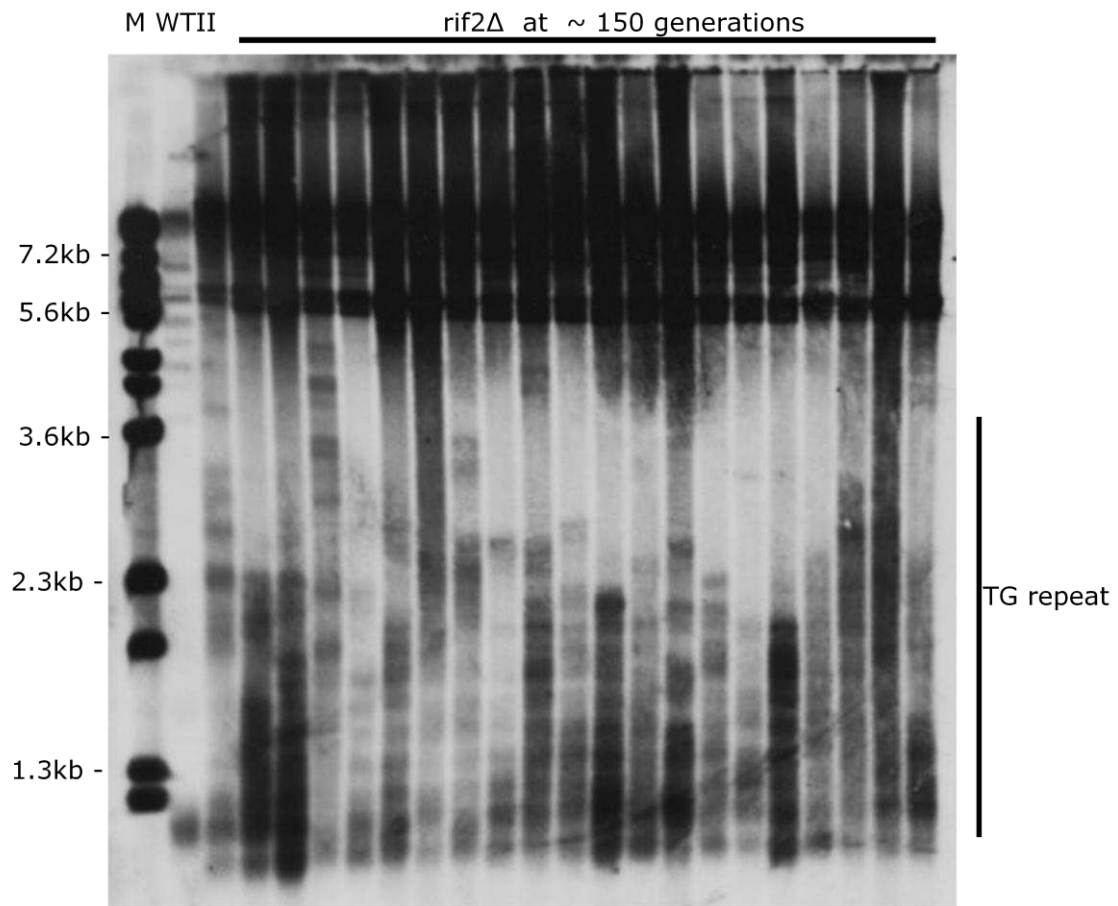
YEPD plates and incubated at 30°C for two days. Single colonies for each independent spore were restreaked at the same conditions above for further five times, growing until approximately 150 generations. Genomic DNA was prepared and digested with *Xho* I enzyme. Gel run to separate fragments and Southern blot was obtained; membrane was probed with Y'-TG probe, developed. All 20 independent cell lines for each gene displayed a TG repeats telomere pattern. Therefore, strains maintained as type II, displaying no requirement for these genes at this time point (Table 3.1). Investigated genes here are *RIF1*, *RIF2*, *NEJ1*, *ELG1*, *DCC1*, *HUR1*, *UPF1*, *SRB2*, *GTR1*, *OGG1*, *RPB4*, *RPP1A*, *CAX4*, and *MET7*, southern blots are shown respectively in the figures 3.10 to 3.23.

### **3.7. Discussion**

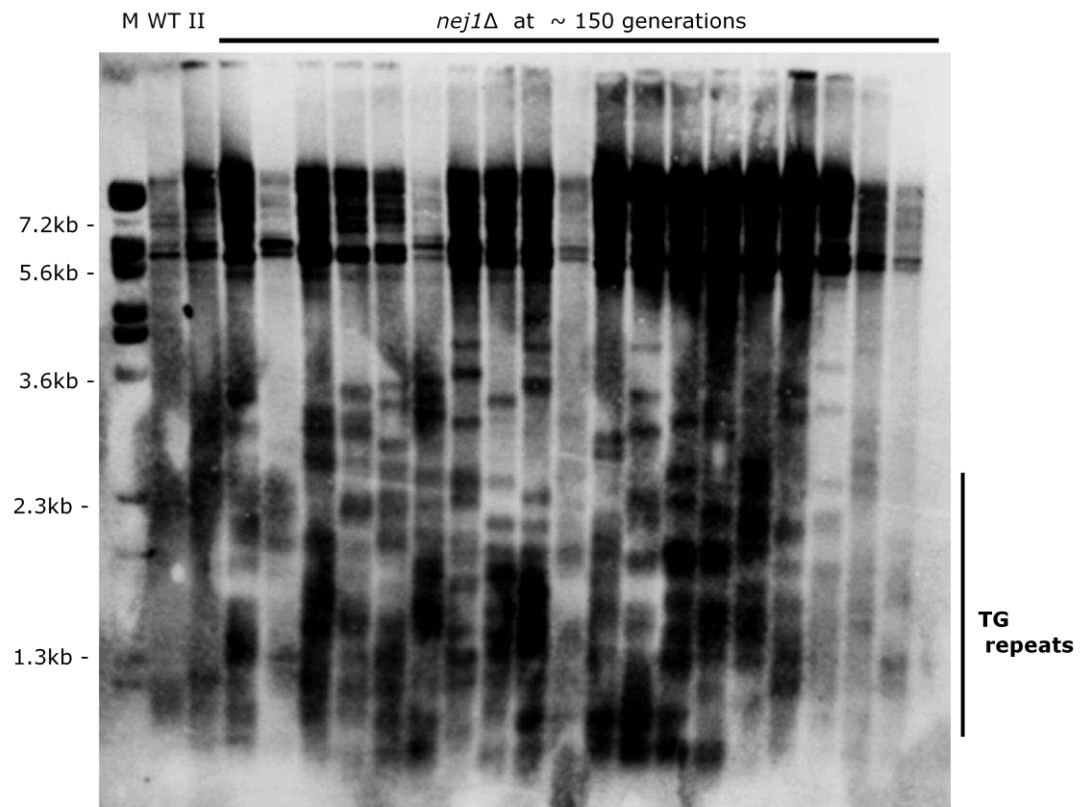
The aim of this study was to test several genes to determine if they are required for telomere maintenance in type II survivor strains at usual propagation (~150 generations after deleting the candidate genes). Genes were deleted individually in type II survivor strains. All strains were derived from the Y55 background strain, named YGL9, used in previous work (Liti and Louis, 2003). The majority of the candidate genes which were investigated here were not required for telomere maintenance as type II, at least until ~ 150 generations of genes deletion. MRX- complex (*RAD50*, *MRE11*, and *XRS2*) genes are involved in telomere maintenance, DSB repair by HR and non-homologous end joining (NHEJ), cell cycle response to DNA damage (reviewed in (Borde, 2007)). In addition, it has been found that MRX - complex involved in base excision repair (BER) pathway (Steininger et al., 2009). The findings here do not seem consistent with the complex functions. Thus, type II



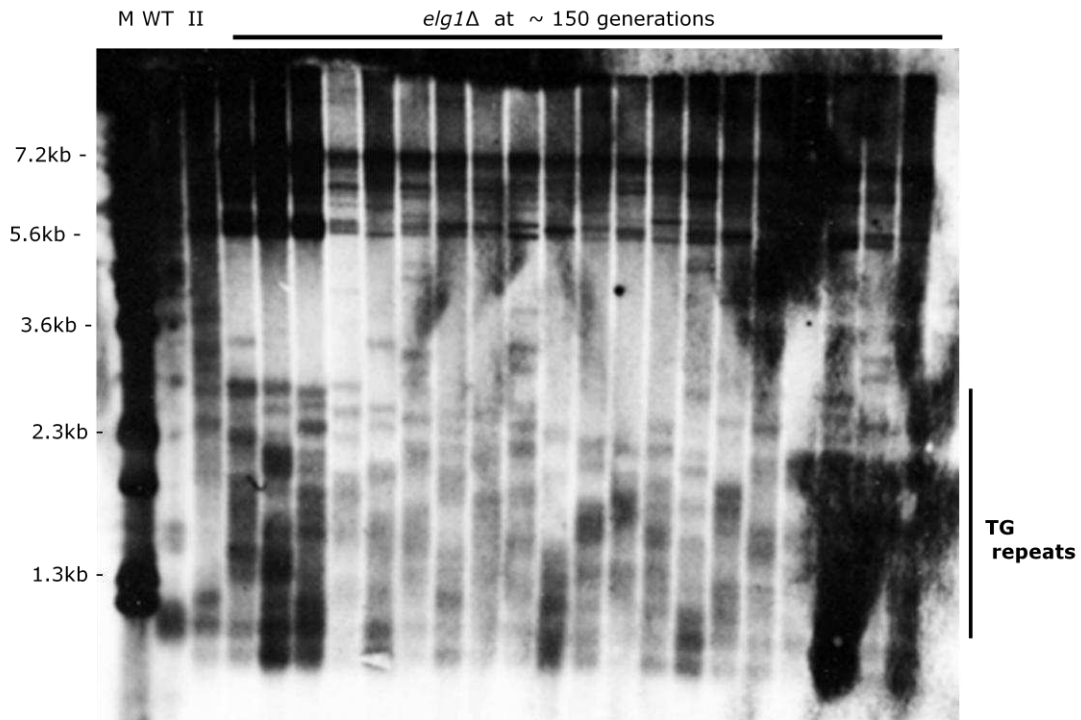
**Figure 3.10. Blot for *rif1*Δ in diploid type II survivors at ~ 150 generations**  
 Genomic DNA digested with *Xho* I, probed with Y' - TG probe. The first three lanes are the same as the first three in figure 3.5. The rest of the lanes (22 samples) are independent cell lines for *rif1*Δ, exhibiting TG repeats to confirm type II maintenance.



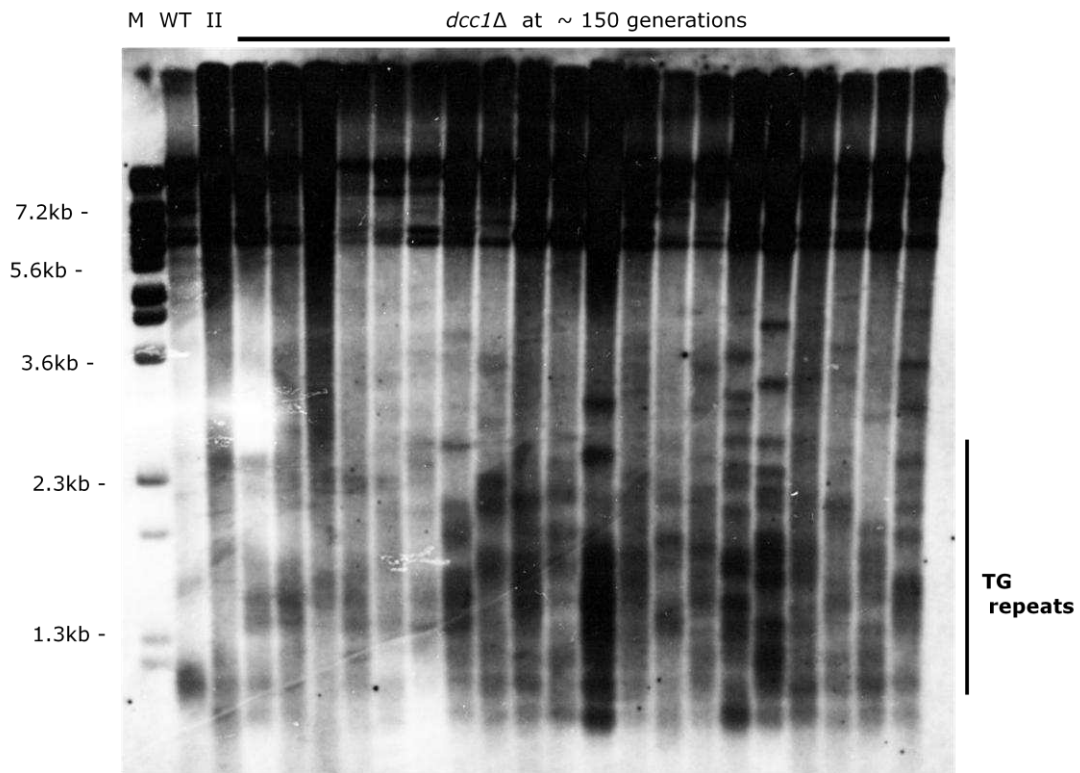
**Figure 3.11. Blot for *rif2*Δ in diploid type II survivors at ~ 150 generations**  
 Genomic DNA digested with *Xho* I, probed with Y' - TG probe. The first three lanes are the same as the first three in figure 3.5. The rest of the lanes (20 samples) are independent cell lines for *rif2*Δ, exhibiting TG repeats to confirm type II maintenance.



**Figure 3.12. Blot for *nej1Δ* in diploid type II survivors at ~ 150 generations**  
 Genomic DNA digested with *Xho* I, probed with Y' - TG probe. The first three lanes are the same as the first three in figure 3.5. The rest of the lanes (20 samples) are independent cell lines for *nej1Δ*, exhibiting TG repeats to confirm type II maintenance.

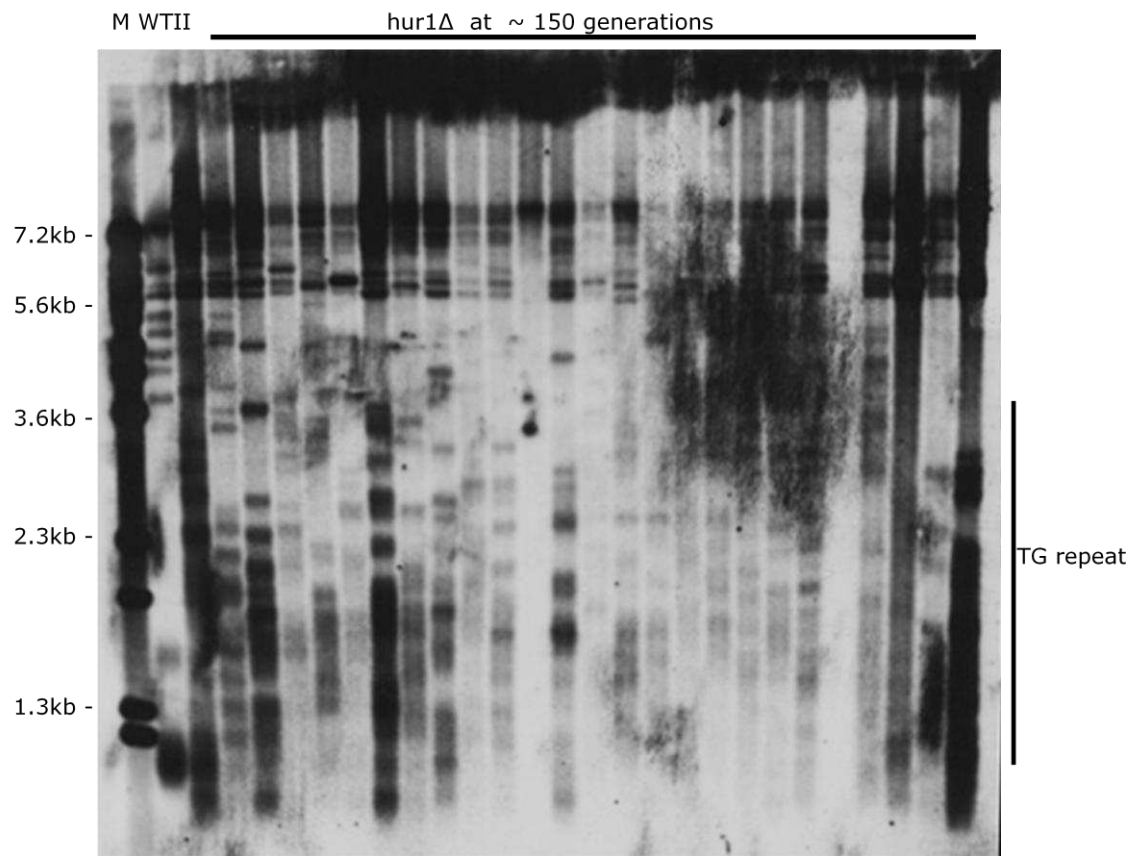


**Figure 3.13. Blot for *elg1Δ* in diploid type II survivors at ~ 150 generations**  
 Genomic DNA digested with *Xho* I, probed with Y' - TG probe. The first three lanes are the same as the first three in figure 3.5. The rest of the lanes (20 samples) are independent cell lines for *elg1Δ*, exhibiting TG repeats to confirm type II maintenance.

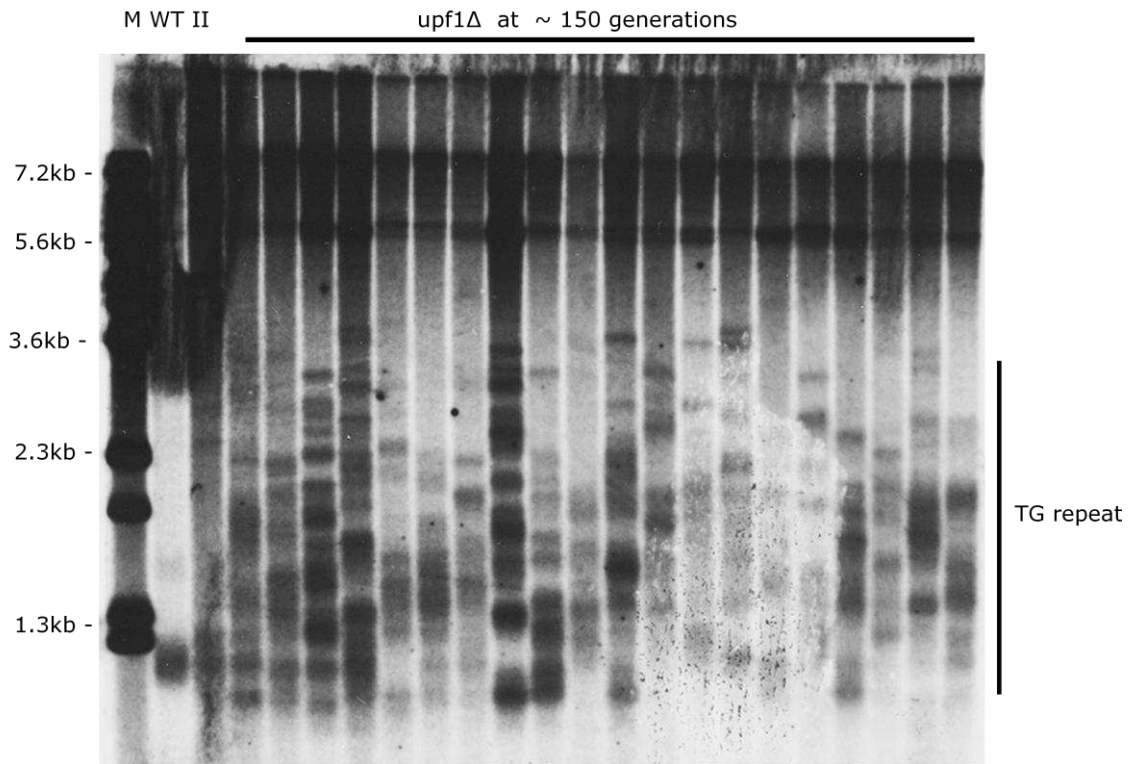


**Figure 3.14. Blot for *dcc1Δ* in diploid type II survivors at ~ 150 generations**  
 Genomic DNA digested with *Xho* I, probed with Y' - TG probe. The first three lanes are the same as the first three in figure 3.5. The rest of the lanes (20 samples) are independent cell lines for *dcc1Δ*, exhibiting TG repeats to confirm type II maintenance.

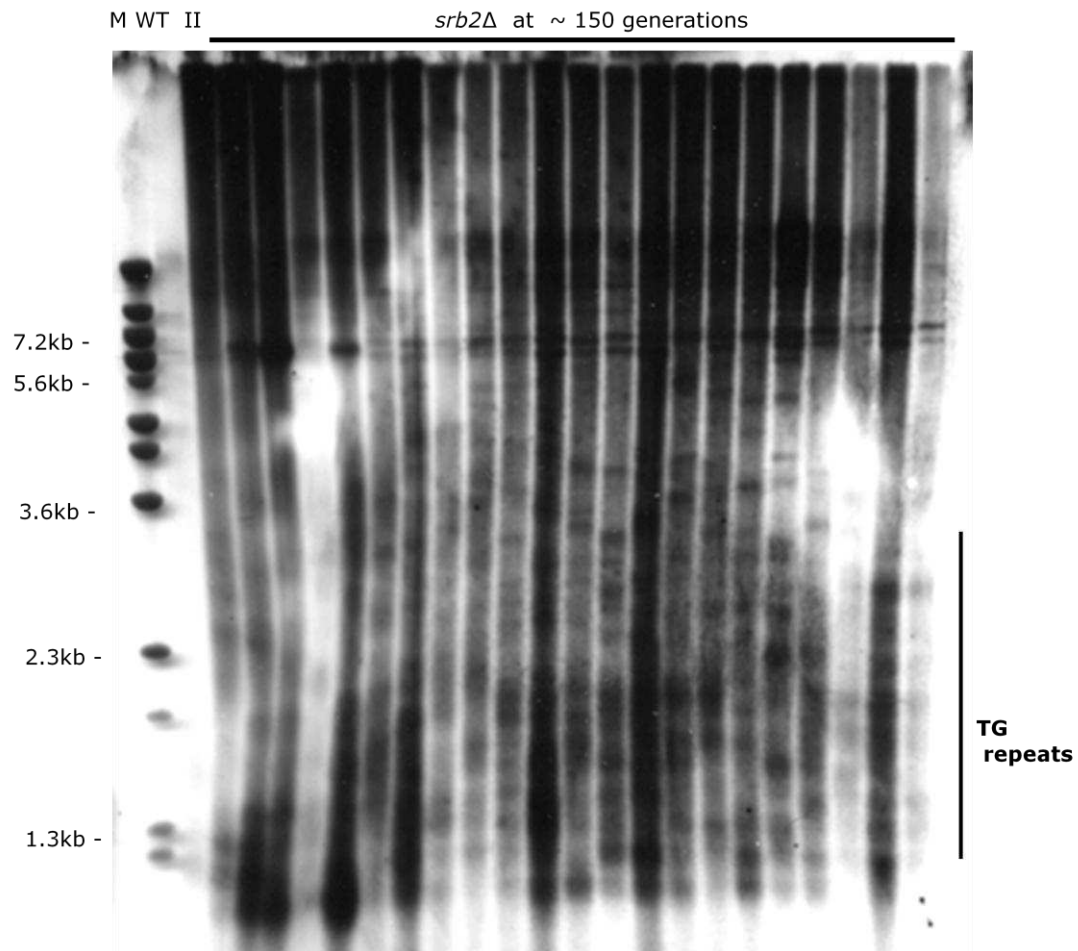




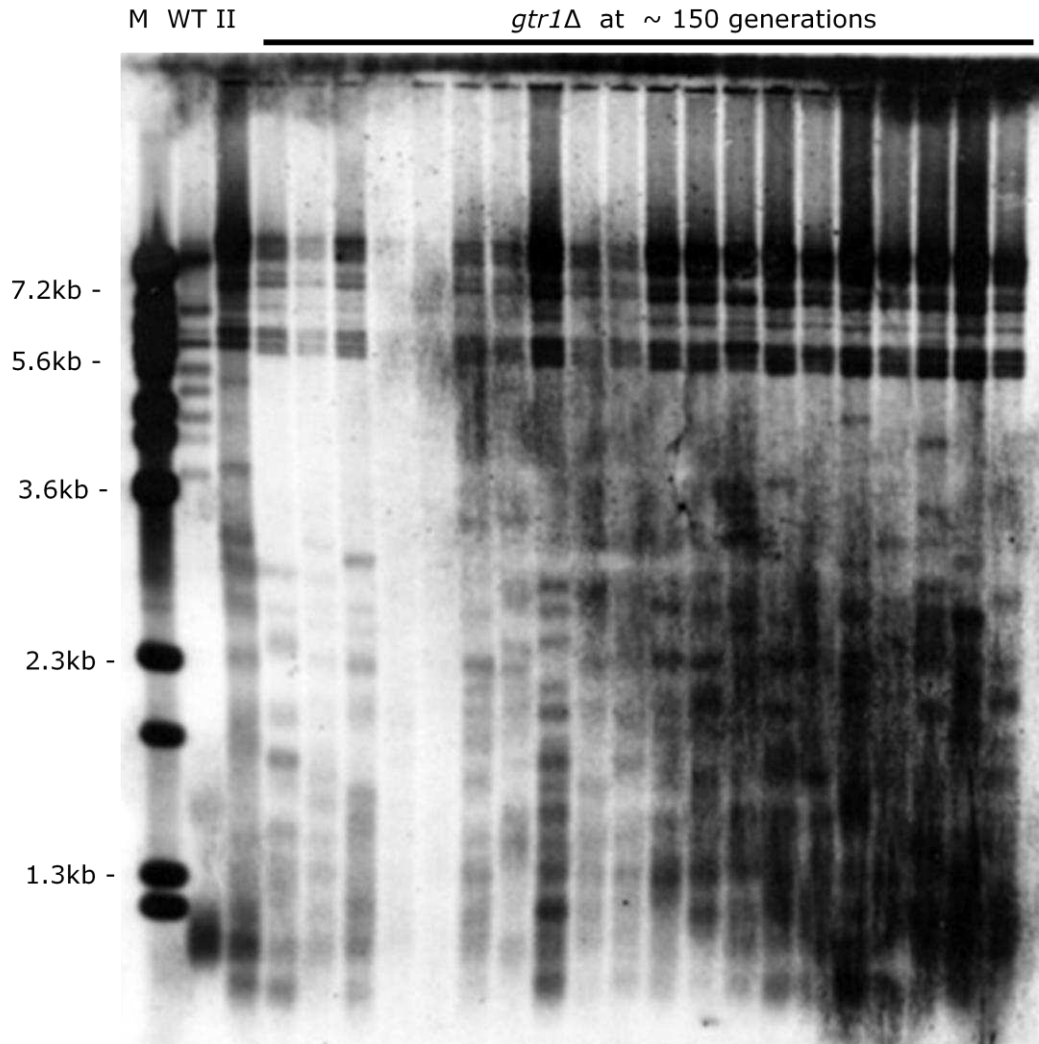
**Figure 3.15. Blot for *hur1Δ* in diploid type II survivors at ~ 150 generations**  
 Genomic DNA digested with *Xho* I, probed with Y' - TG probe. The first three lanes are the same as the first three in figure 3.5. The rest of the lanes (20 samples) are independent cell lines for *hur1Δ*, exhibiting TG repeats to confirm type II maintenance.



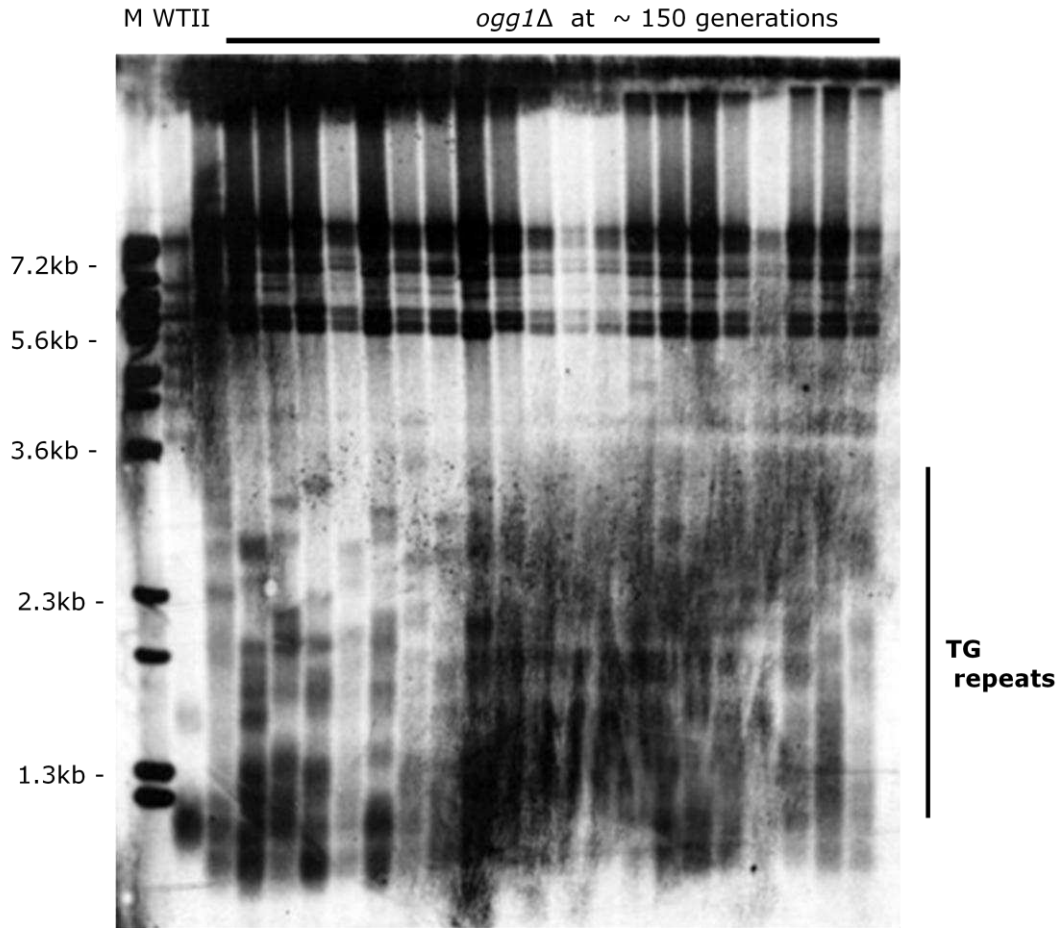
**Figure 3.16. Blot for *upf1*Δ in diploid type II survivors at ~ 150 generations**  
 Genomic DNA digested with *Xho* I, probed with Y' - TG probe. The first three lanes are the same as the first three in figure 3.5. The rest of the lanes (20 samples) are independent cell lines for *upf1*Δ, exhibiting TG repeats to confirm type II maintenance.



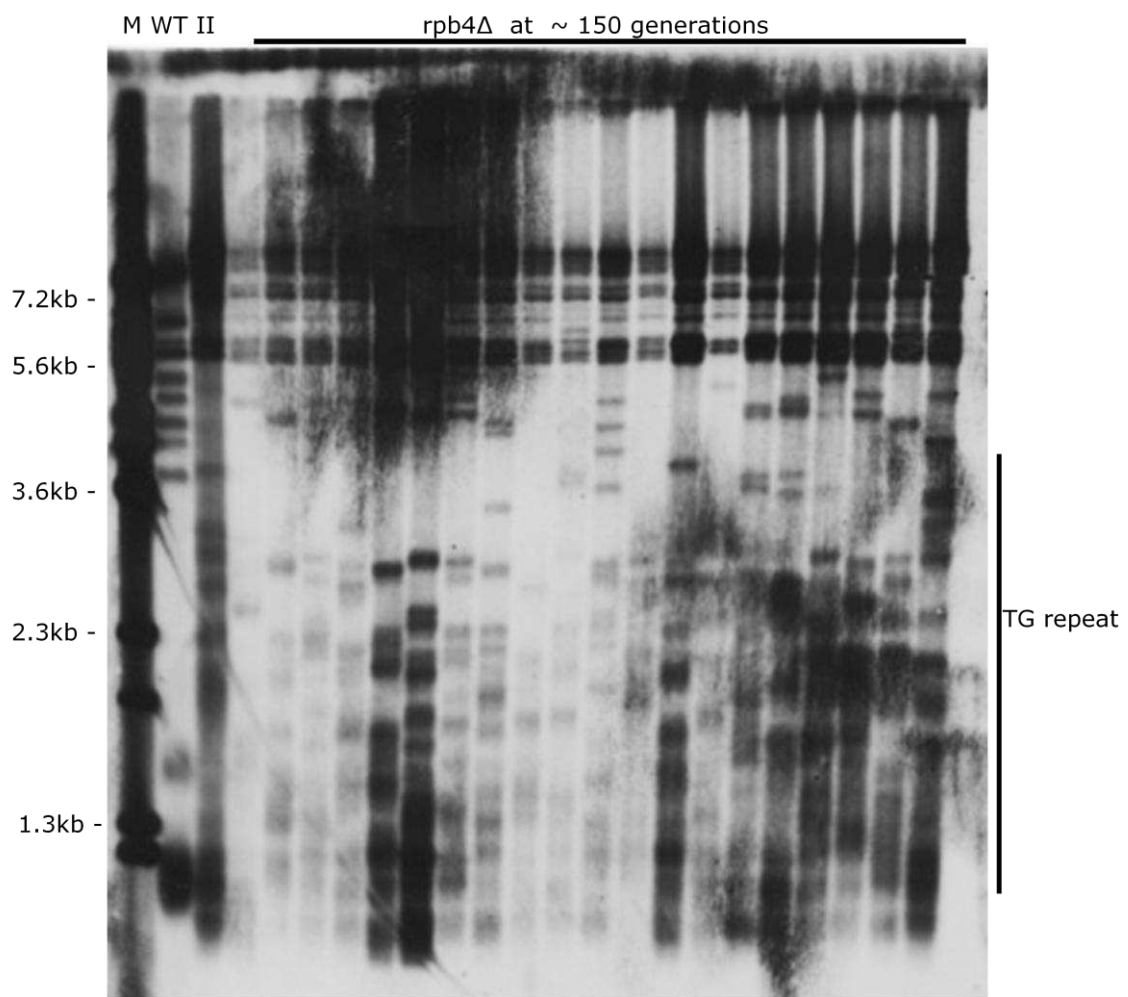
**Figure 3.17. Blot for *srb2Δ* in diploid type II survivors at ~ 150 generations**  
 Genomic DNA digested with *Xho* I, probed with Y' - TG probe. The first three lanes are the same as the first three in figure 3.5. The rest of the lanes (20 samples) are independent cell lines for *srb2Δ*, exhibiting TG repeats to confirm type II maintenance.



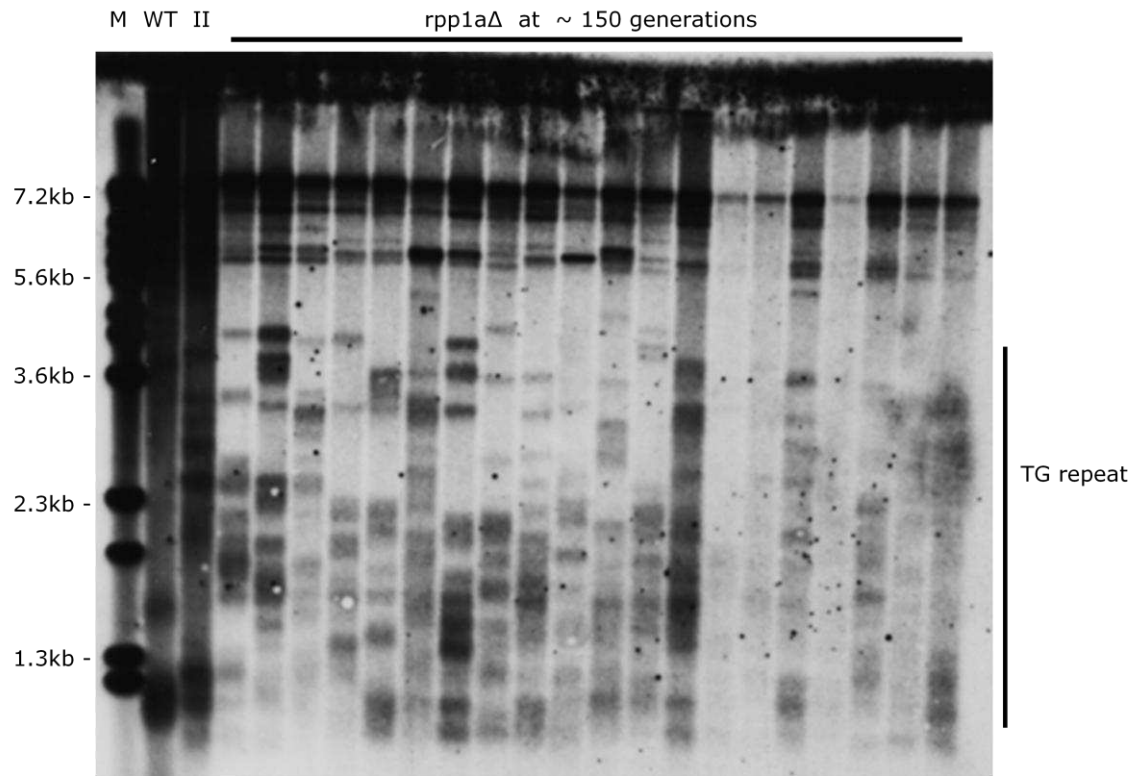
**Figure 3.18. Blot for *gtr1Δ* in diploid type II survivors at ~ 150 generations**  
 Genomic DNA digested with *Xho* I, probed with Y' - TG probe. The first three lanes are the same as the first three in figure 3.5. The rest of the lanes (20 samples) are independent cell lines for *gtr1Δ*, exhibiting TG repeats to confirm type II maintenance.



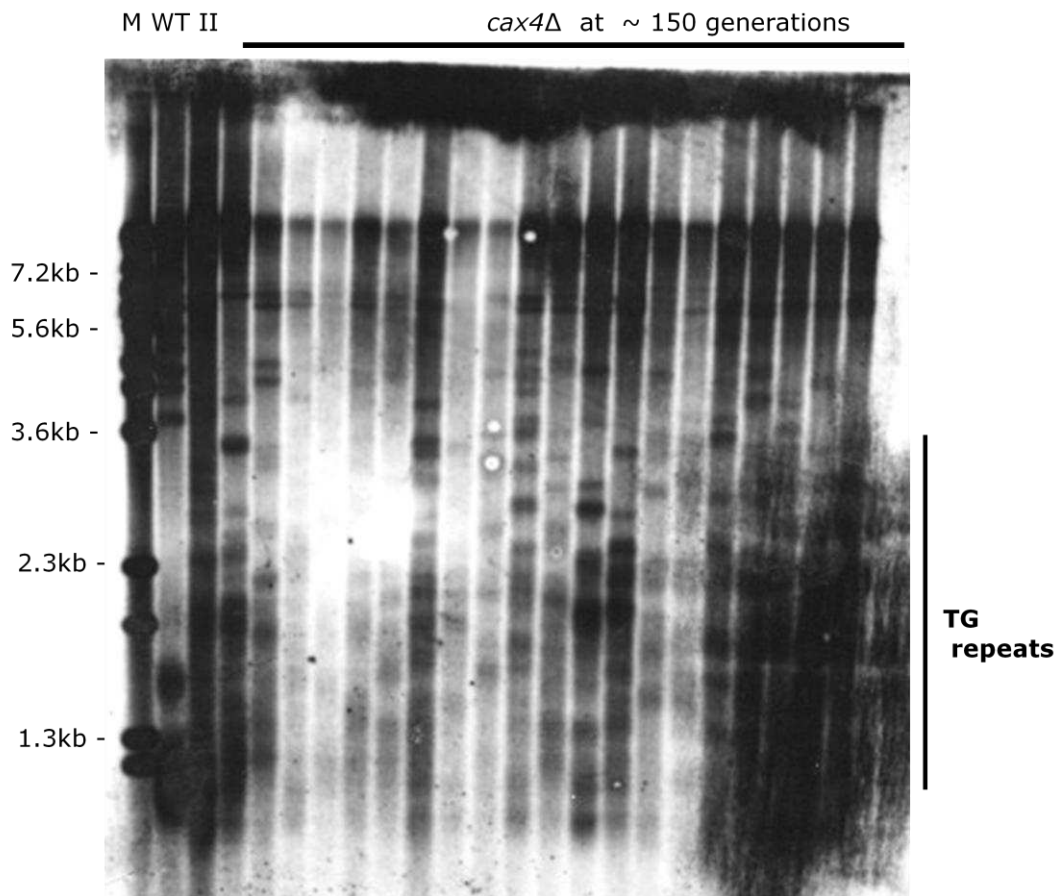
**Figure 3.19. Blot for *ogg1*Δ in diploid type II survivors at ~ 150 generations**  
 Genomic DNA digested with *Xho* I, probed with Y' - TG probe. The first three lanes are the same as the first three in figure 3.5. The rest of the lanes (20 samples) are independent cell lines for *ogg1*Δ, exhibiting TG repeats to confirm type II maintenance.



**Figure 3.20. Blot for *rpb4*Δ in diploid type II survivors at ~ 150 generations**  
 Genomic DNA digested with *Xho* I, probed with Y' - TG probe. The first three lanes are the same as the first three in figure 3.5. The rest of the lanes (20 samples) are independent cell lines for *rpb4*Δ, exhibiting TG repeats to confirm type II maintenance.



**Figure 3.21. Blot for *rpp1a*Δ in diploid type II survivors at ~ 150 generations**  
 Genomic DNA digested with *Xho* I, probed with Y' - TG probe. The first three lanes are the same as the first three in figure 3.5. The rest of the lanes (20 samples) are independent cell lines for *rpp1a*Δ, exhibiting TG repeats to confirm type II maintenance.



**Figure 3.22. Blot for *cax4*Δ in diploid type II survivors at ~ 150 generations**  
 Genomic DNA digested with *Xho* I, probed with Y' - TG probe. The first three lanes are the same as the first three in figure 3.5. The rest of the lanes (20 samples) are independent cell lines for *cax4*Δ, exhibiting TG repeats to confirm type II maintenance.





Gene	Yeast strain	Total colonies	No. Colonies (%)		
			I	II	new type
<i>RAD52</i>	YGL2.15	20	Spores died early		
<i>RAD50</i>	.....	22	0(0%)	22(100%)	0(0%)
<i>MRE11</i>	.....	20	0(0%)	20(100%)	0(0%)
<i>XRS2</i>	.....	20	0(0%)	20(100%)	0(0%)
<i>KU80</i>	YGL38	22	0(0%)	0(0%)	22(100%)
<i>NEJ1</i>	YGL2.15	20	0(0%)	20(100%)	0(0%)
<i>ELG1</i>	.....	20	0(0%)	20(100%)	0(0%)
<i>SGS1</i>	.....	19	0(0%)	19(100%)	0(0%)
<i>RIF1</i>	.....	20	0(0%)	20(100%)	0(0%)
<i>RIF2</i>	.....	20	0(0%)	20(100%)	0(0%)
<i>DCC1</i>	.....	20	0(0%)	20(100%)	0(0%)
<i>HUR1</i>	.....	20	0(0%)	20(100%)	0(0%)
<i>UPF1</i>	.....	20	0(0%)	20(100%)	0(0%)
<i>SRB2</i>	.....	20	0(0%)	20(100%)	0(0%)
<i>GTR1</i>	.....	20	0(0%)	20(100%)	0(0%)
<i>OGG1</i>	.....	20	0(0%)	20(100%)	0(0%)
<i>RPB4</i>	.....	20	0(0%)	20(100%)	0(0%)
<i>CAX4</i>	.....	20	0(0%)	20(100%)	0(0%)
<i>RPP1A</i>	.....	20	0(0%)	20(100%)	0(0%)
<i>MET7</i>	.....	20	0(0%)	20(100%)	0(0%)

**Table 3.1 Genes that were deleted in type II indicated strains**

The percentages of telomere type after genes deletion at ~ 150 generation is presented in the right three columns for each single gene.

survivor cells were able to maintain their telomere elongation as type II in the absence of these genes. Therefore, it is clear that the MRX-complex genes are not required to sustain type II telomeres at least until  $\sim 150$  generations. However, these genes are required for type II telomere establishment in the absence of telomerase (Lundblad and Blackburn, 1993, Le et al., 1999, Teng and Zakian, 1999, Teng et al., 2000).

The budding yeast RecQ family helicase *SGS1* is required for genome stability (Watt et al., 1996), and is required to establish yeast type II survivors (Huang et al., 2001, Cohen and Sinclair, 2001, Johnson et al., 2001). Interestingly, this gene was not required to maintain the same type of cell survivors after it has been established. However, the MRX complex and *SGS1* displayed telomere shortening in telomerase-positive strains (Askree et al., 2004). According to what has been found in this study, and comparing with the previous work it seems that the type II survivors cells were utilising a different pathway to maintain their telomeres as type II than to establish survival type.

*RAD52* is a crucial gene for all types of recombination (Symington, 2002). *Rad52* $\Delta$  type II strains were not able to survive beyond  $\sim 35$  generations. Some of these cells were grown until  $\sim 25$  generations after deleting this gene, but at a short time later, all the cell lines (20) were dead as a result of *rad52* deletion. This result is consistent with the important role of this gene in telomere elongation (Chen et al., 2001, Le et al., 1999). However, *S. cerevisiae* strains have been found to survive in the absence of telomerase and *RAD52* (Maringele and Lydall, 2004b, Grandin and Charbonneau, 2009,

Lebel et al., 2009). *RAD52*-independent survivor cells are based on different pathways such as removal of *EXO1*, so the key role was for this exonuclease, but not more for the absence of *RAD52*. So the finding in this study and previous work together, can explain that yeast cells cannot survive in the absence of telomerase and *RAD52*, except under some conditions like *EXO1* removal or when cells possess long telomeres at the senescence stage. *EXO1* is responsible for generating single strand DNA (ssDNA) which is essential for cell cycle arrest (Maringele and Lydall, 2004b). However, *RAD52*-independent survivors were also generated in the presence of *EXO1* (Grandin and Charbonneau, 2009).

*KU80* is involved in HR and NHEJ mechanisms, implicated in DNA damage repair and telomere maintenance (Dudasova et al., 2004, Gullo et al., 2006). In this work, a new type of survivor was displayed when *KU80* was deleted in type II survivor strain. These survivor strains exhibited a different pattern to *RAD52* dependent survivors, type I and II. Thus, *KU80* is required for type II maintenance, but not for survivors state. However, previous studies have found that deletion of *KU80* leads to lethality in the absence of telomerase (Gravel et al., 1998, Nugent et al., 1998). Moreover, telomere shortening has been found as result of *KU80* deletion (Boulton and Jackson, 1996). Type II-like survivor strains were established in the absence of *KU80* and telomerase (*ku80* $\Delta$  *est2* $\Delta$ ), but in a triple mutation *ku80* $\Delta$  *est2* $\Delta$  *exo1* $\Delta$ , both type I and type II survivors could be generated (Bertuch and Lundblad, 2004). The member of the *KU* heterodimer, *KU70*, was required to generate type II survivors in the absence and present of *EXO1*, since deletion of this gene lead only to type I survivors (Maringele and Lydall, 2004a). This gene was required

to establish survivors (Liti and Louis, unpublished). The absence of this gene leads to telomere shortening in a telomerase positive strain, this may be due to its role in protecting telomere from degradation (Askree et al., 2004). According to this study and previous work, *KU80* exhibits a key role in telomere maintenance in survivors. It may suggest that KU plays an important role in telomere maintenance in survivor strains, which differs from its role in establishing survivors.

The rest of the candidate genes, listed in table 3.1 displayed type II telomere structures when they were deleted in type II survivor strains. Consequently, they were not required for type II telomere maintenance at this time point (~ 150 generations after deleting the target gene). These genes have various functions; most of them play a direct or indirect role in telomere metabolism (Askree et al., 2004). They displayed telomere shortening in telomerase-positive yeast strains, except these genes *RIF1*, *RIF2*, *ELG1*, and *OGG1*, which exhibited telomere elongation in the same strain background (Askree et al., 2004). These genes were identified to play a role in telomere negative regulation. RIF1p and RIF2p interact with RAP1p, preventing telomerase from accessing the telomeres (Bianchi and Shore, 2008, Hardy et al., 1992), hence *rif1* $\Delta$  and *rif2* $\Delta$  strains were able to generate long telomeres in telomerase positive strain yeast (Askree et al., 2004). *ELG1* is an important gene for genome stability; it may play a role to repress replication of damaged DNA (Ben-Aroya et al., 2003, Bellaoui et al., 2003). It has been found that *ELG1* is essential for DSB repair by HR (Ogiwara et al., 2007). As HR is required for survivor yeast strain establishment, *ELG1* is thought to have a role in telomere elongation in survivor cells. However, this is not consistent with the

findings here where type II telomeres are maintained in the absence of this gene; showing a limited role for this gene in the survivor state. *OGG1* is a central gene for DNA damage repair through BER (Klungland and Bjelland, 2007, Boiteux et al., 2002). However, it has been found to inhibit *RAD52* function, so its deletion can activate HR (de Souza-Pinto et al., 2009) which is consistent with the maintenance of type II survivors in this study. *RPP1A* is a large ribosomal subunit gene. *Rpp1a* $\Delta$  strains in this study displayed slow growth, but they still elongate their telomeres as type II. The slow growth did not affect telomere length. However, *rpp1a* $\Delta$  in telomerase positive strain cells led to short telomere (Askree et al., 2004).

In conclusion, none of the candidate genes was required for telomere maintenance as type II. Therefore, survivor cells displayed the TG repeat telomere pattern of type II at  $\sim$ 150 generations after deleting these genes. The exception was *KU80* which was required for type II maintenance. In *KU80* deletion, strains switched to a new type of survivor, which differs from both type I and type II survivors. According to this study and the previous studies, cells seem to be utilizing a different pathway to maintain their type II telomeres than the pathway which was utilized to establish this type of survivor.

## **Chapter 4: Genetic requirements for type II survivor maintenance in multiple strains**

### **4.1. Introduction**

It is well known that *S. cerevisiae* cells are able to replicate their telomeres in the absence of telomerase by utilizing homologous recombination (HR) (Le et al., 1999, Chen et al., 2001). Two types of survivors following senescence can be easily distinguished by Southern blot hybridisation according to their telomere structure (Lundblad and Blackburn, 1993). Specific genes are required to establish each of these survivor types, I and II (described in Chapter 1) and both types require *RAD52* (Le et al., 1999, Chen et al., 2001, Huang et al., 2001). The genes required for telomere maintenance at post-senescence were discussed in Chapter 3. These genes were deleted individually in one type II survivor strain (YGL2.15). However, it has been found that survivor strains displayed an increased mutation rate suggesting that genetic changes may have taken place (Lundblad and Blackburn, 1993, Hackett et al., 2001). Therefore, two genes (*RAD50* and *RPB9*) were individually deleted in three different type II survivor strains. This experiment was done to see if any mutation changes that might occur at senescence affect gene deletions in different survivor strains.

### **4.2. Comparison between type II survivor strains**

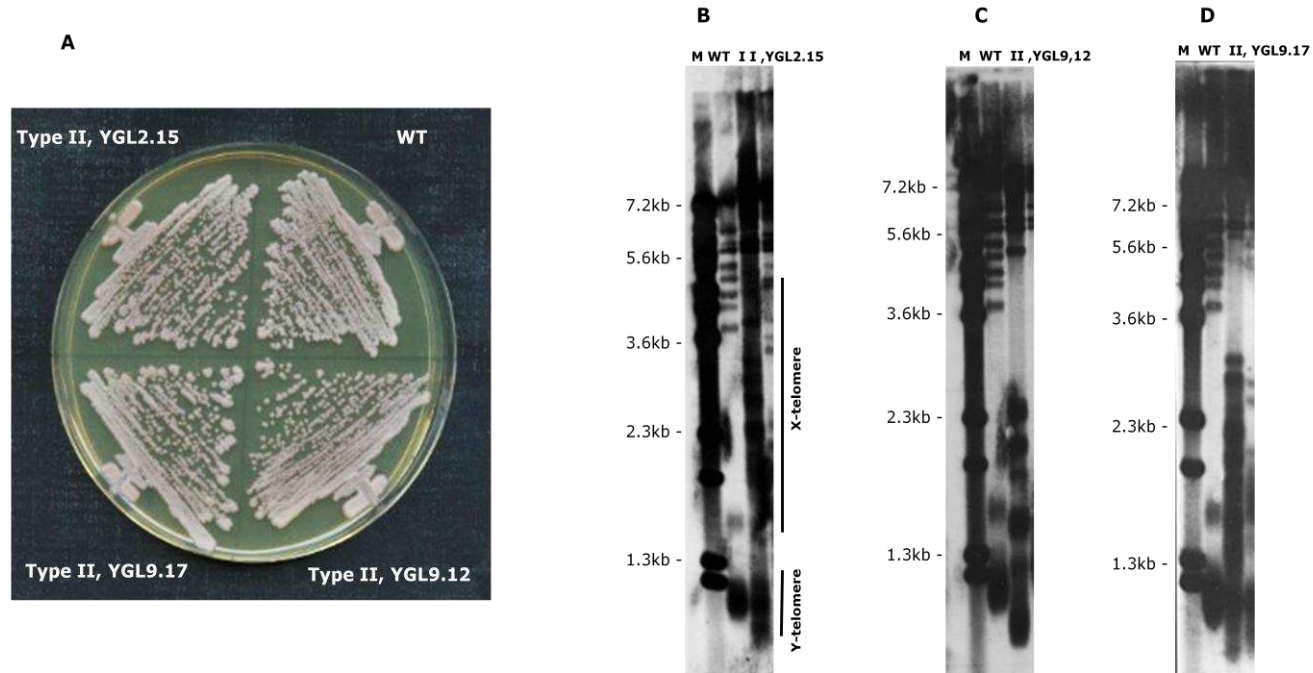
Along with YGL2.15, two more type II survivor strains (YGL9.12 and YGL9.17) were generated from a parental strain (YGL9). All three strains displayed similar growth to a wild-type strain at ~150 generations after telomerase was inactivated (Fig. 4.1 A). The telomere structure for these survivor strains was analysed. It was found that X-Y' ends displayed a similar

pattern to each other, but X-only ends displayed a dissimilar diverged pattern. It is clear that the YGL2.15 strain displays long telomeres that have X only ends (~4.0 kb). In the contrast, YGL9.12 and YGL9.17 display short telomeres that have X only ends. Their X-telomere sizes are ~2.3 kb and ~3.0 kb respectively (Fig 4.1 B, C, and D). Therefore, the survivor strains that were derived from the same parent are likely to have genetic changes that have taken place. To further investigate the changes in these survivor strains, two genes (*RAD50* and *RPB9*) were deleted individually and Southern blot analysis was performed. The absence of these genes in a telomerase-positive strain resulted in shortening of the telomeres (Askree et al., 2004).

#### ***4.3. RAD50 is not required for type II telomere maintenance at post-senescence***

*RAD50* was investigated for telomere maintenance in three independent type II survivor strains. This gene was chosen based on its requirement to establish type II survivors (Chen et al., 2001, Le et al., 1999). *RAD50* was deleted in each one of these survivor strains (YGL2.15, YGL9.12, and YGL9.17). Tetrads were dissected, and spores were incubated on YEPD plates at 30°C for two days. Cells were replica-plated onto YEPD-HYG plates and incubated at 30°C for two days to select *rad50*Δ mutants. Independent cell lines (20–22) for each strain were incubated on a YEPD plate at 30°C for two days. Single colonies were then propagated under the same conditions described above five more times bringing cells to ~150 generations after deleting *RAD50*. The genomic DNA was extracted, digested with *XhoI* and run at 45V for 16 hours on a 0.9% w/v agarose gel as described in Chapter 2. DNA was transferred to





**Figure 4.1 Growth and telomere structure of parental strain (YGL9) compared to three type II survivors (YGL2.15, YGL9.12 and YGL9.17)**

**(A)** Growth comparison of wild-type and three different type II survivor *S. cerevisiae* strains. Survivor cells are at ~150 generations after telomerase inactivation. Wild-type and survivor cells displayed similar growth at 30°C after two days incubation. **(B, C, and D)** the genomic DNA was extracted from wild-type and type II survivors after ~150 generations. DNA was digested with *XhoI*, and probed with a Y'-TG<sub>1-3</sub> specific probe. All type II survivors (YGL2.15, YGL9.12, and YGL9.17) displayed differences in the length of X-only ends, but exhibited similar length of X-Y' ends. Bands between ~1.3 kb and 3.6 kb come from telomeres with X only ends. The prominent band at ~1.0 kb comes from telomeres with X-Y ends.

Nylon membrane. The nylon membrane was probed with a Y'-TG<sub>1-3</sub> specific probe and the film developed to define telomere structure. YGL2.15*rad50*Δ strains (22) displayed a type II telomere pattern (Table 4.1). Whereas, most of the YGL9.12*rad50*Δ and YGL9.17*rad50*Δ strains (95%) displayed telomere shortening, but remained as type II survivors. One independent cell line of YGL9.12*rad50*Δ and YGL9.17*rad50*Δ changed to a type I survivor pattern (Table 4.1 and Fig. 4.2, 4.3). In Figure 4.3, lanes 15-22 have a spot, but these lanes displayed a type II telomere pattern on the original film. Moreover, strains that these lanes came from displayed type II survivor growth behaviour during all streaking. Therefore, it is considered that *RAD50* is not required for type II telomere maintenance at ~150 generations in all strains. Notably, the YGL9.12*rad50*Δ and YGL9.17*rad50*Δ type II strains displayed shortened telomeres at X-only ends. YGL9.12*rad50*Δ and YGL9.17*rad50*Δ survivors that display type I telomere patterns had slower growth compared to wild-type or type II survivor strains (Fig. 4.4 A, B).

#### ***4.4. RPB9 display different requirement for telomere maintenance in different strains***

*RPB9* was examined to determine whether or not it was required for telomere maintenance in three independent type II survivor strains. Firstly, the type II survivor strains (YGL2.15, YGL9.12 and YGL9.17) were generated from a telomerase-positive strain (YGL9) as described previously in Chapter 2. Secondly, *RPB9* was deleted in a YGL2.15 strain, using the *hphMX* cassette (Wach et al., 1994). Spores were dissected, but all (20) that had a deleted copy of this gene died immediately after dissection (Fig. 4.5). Therefore it was concluded that *RPB9* was required for survival maintenance in this strain.

Therefore, *RPB9* was chosen to be investigated in two more type II survivor strains (YGL9.12 and YGL9.17). YGL9.17*rpb9* $\Delta$  strains exhibited the same result as the YGL2.15*rpb9* $\Delta$  strain. All YGL9.17*rad50* $\Delta$  spores died after dissection (Table 4.1, fig. 4.5). Therefore, *RPB9* is required for survival maintenance in these strains. On the other hand, a different result was found in the third strain (YGL9.12) as shown here. In this strain, *RPB9/rpb9::HYG* YGL9.12 tetrads were dissected and incubated at 30°C for two days. Spores were replica-plated onto YEPD-HYG plates incubated at 30°C for two days. *Rpb9* $\Delta$  spores were streaked on YEPD plates and incubated at 30°C for two days. A single colony from each spore was propagated for a further five times, bringing cells to ~150 generations. The genomic DNA was extracted, digested with *XhoI* and Southern blot hybridisation was performed. Nylon membrane was probed with the Y'-TG<sub>1-3</sub> specific probe and developed to define telomere type. All 20 independent cell lines exhibited a type II telomere pattern (table 4.1 and fig. 4.6). Therefore, *RPB9* is not essential for type II telomere maintenance in YGL9.12 survivor strains.

#### **4.5. Discussion**

Candidate genes were investigated for type II telomere maintenance in Chapter 3. All genes were deleted individually in a type II strain (YGL2.15), but none of these genes was required for type II telomere maintenance. However, it has been found that telomere shortening at senescence increases mutation rate (Lundblad and Blackburn, 1993, Hackett et al., 2001, Hackett and Greider, 2003), suggesting that genetic changes can take place. The same result was expected to occur for the independent type II survivor strains

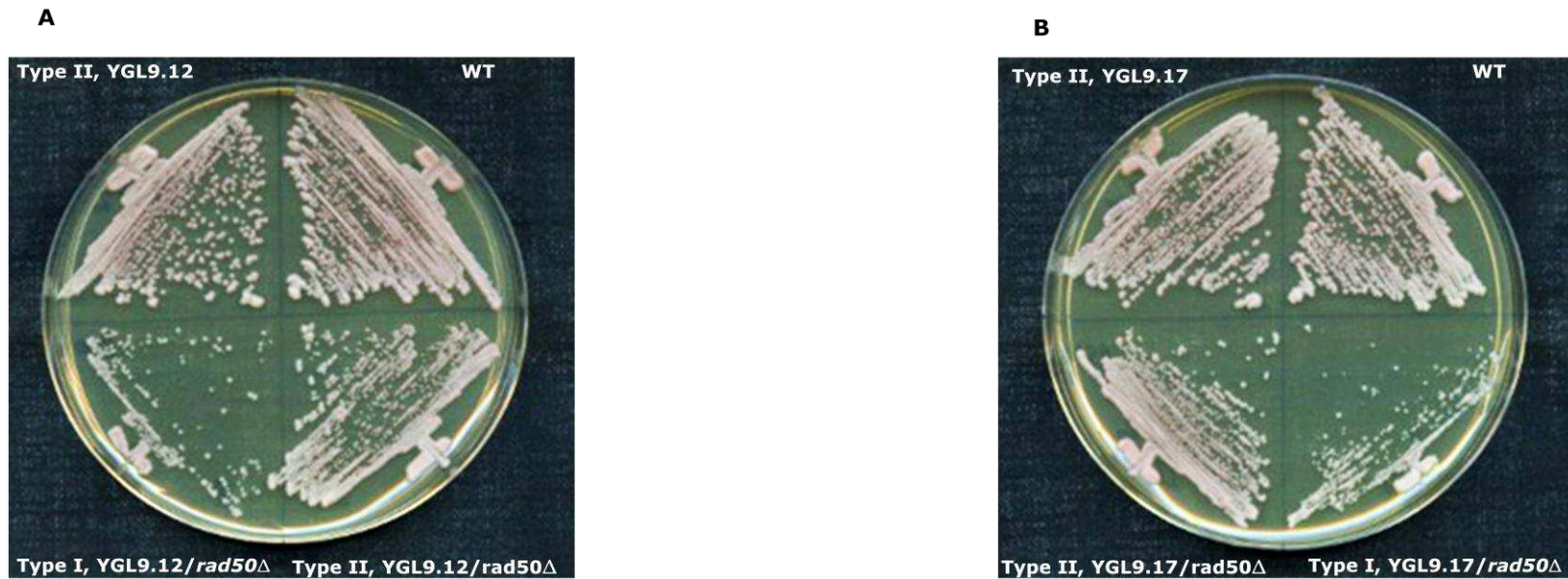
<b>Gene deleted</b>	<b>Yeast strain</b>	<b>Total colonies</b>	<b>No. Colonies (%)</b>	
			<b>I</b>	<b>II</b>
<i>RAD50</i>	YGL2.15	22	0(0%)	22(100%)
<i>RAD50</i>	YGL9.12	20	1(5%)	19(95%)
<i>RAD50</i>	YGL9.17	20	1(5%)	19(95%)
<i>RPB9</i>	YGL2.15	20	Spores died after dissection	
<i>RPB9</i>	YGL9.12	20	0(0%)	20(100%)
<i>RPB9</i>	YGL9.17	20	Spores died after dissection	

**Table 4.1. *RAD50* and *RPB9* were deleted in three different type II survivor strains**

This table shows the percentage and type of survivor strains after *RAD50* and *RPB9* have been deleted. Cells were propagated for ~150 generations. All YGL2.15*rad50* $\Delta$  strains continued as type II. Whereas 5% of each YGL9.12*rad50* $\Delta$  and YGL9.17*rad50* $\Delta$  changed to type I. Spores for YGL2.15*rpb9* $\Delta$  and YGL9.17*rpb9* $\Delta$  died after dissection, but YGL9.12*rpb9* $\Delta$  strains continue as type II.

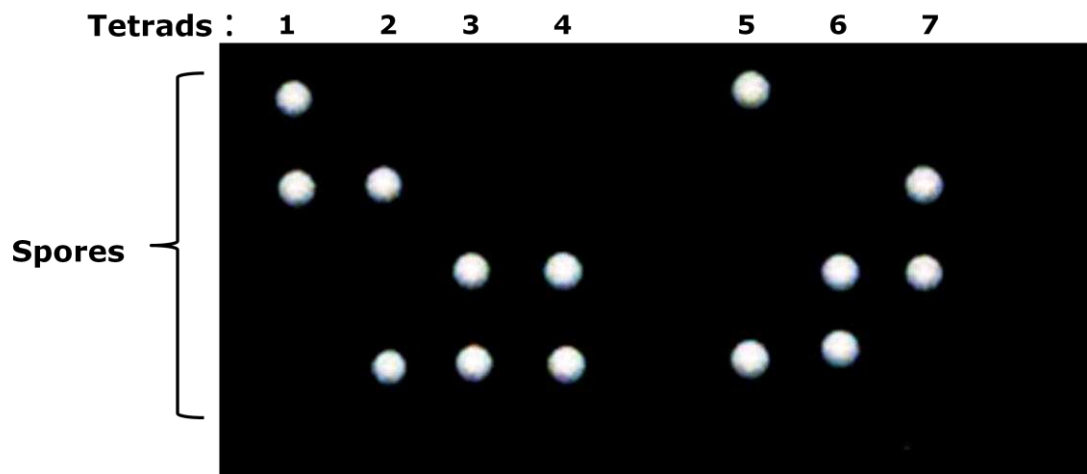






**Figure 4.4. Cell growth of WT, type II (YGL9.12, YGL9.17) and *rad50* mutant**

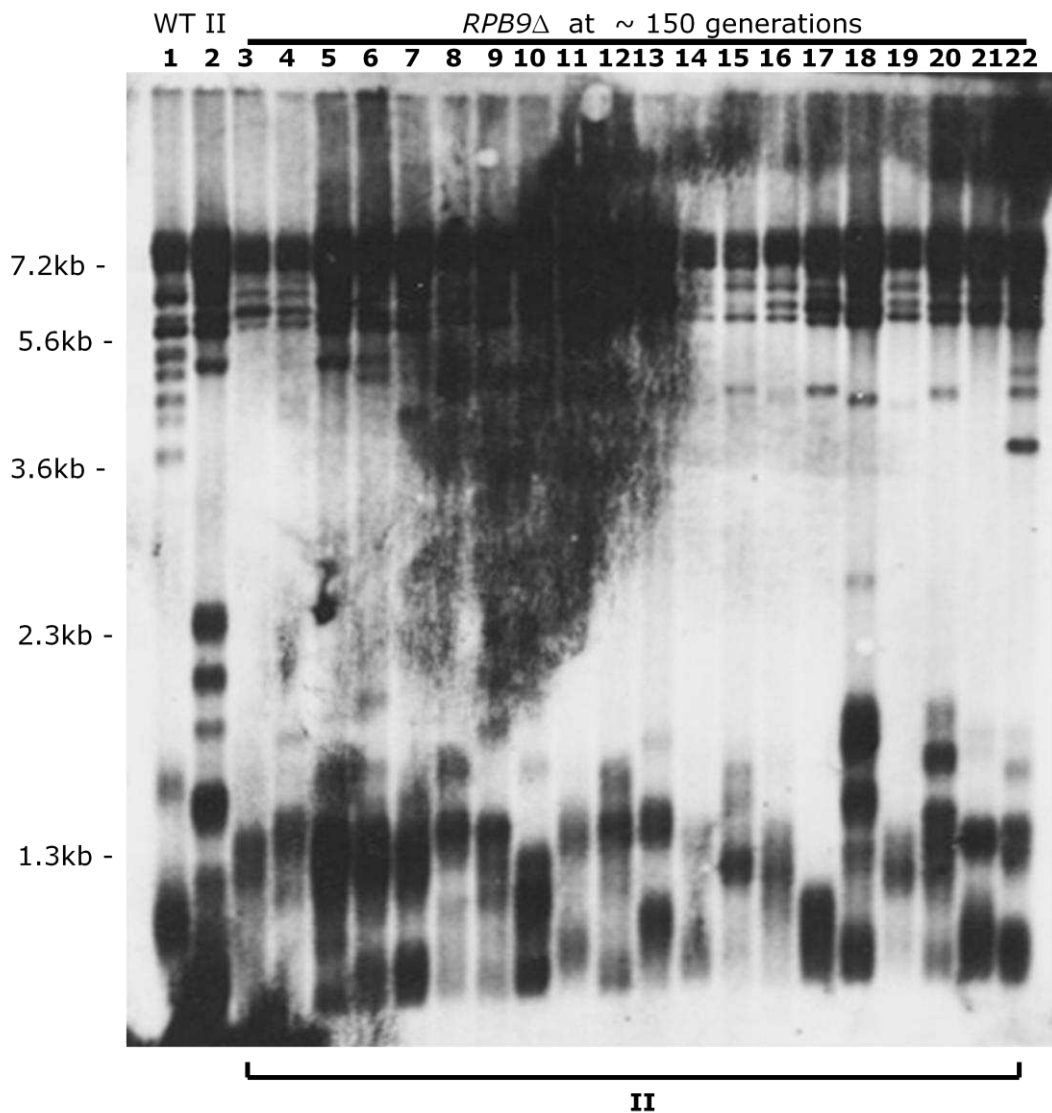
(A) YGL9.12*rad50*Δ type I at ~150 generations displayed slow growth, compared to wild-type, type II and *rad50* type II. Type I cells display fewer colonies of small size. (B) Similar result for YGL9.17*rad50*Δ type I survivor.



**Figure 4.5. Deletion of *RPB9* is lethal to YGL2.15 and YGL9.17 type II survivor strains**

*RPB9* was deleted in two different type II survivor strains (YGL2.15 and YGL9.17) by using *hphMAX* cassette. Only two spores of each tetrad were able to grow on a YEPD plate. Spores were replica-plated on YEPD-HYG plates then they died. Therefore YGL9.17*rpb9* $\Delta$  spores died immediately after dissection. YGL2.15*rpb9* $\Delta$  spores also died after dissection.





**Figure 4.6. Southern blot hybridisation analysis of type II survivor strain YGL9.12 with *rpb9* deleted**

Genomic DNA was purified from these strains and digested with *XhoI* which cuts within the Y' element. The DNA was then run on 0.9% agarose gel at 45V for 16 hours. DNA was transferred to a nylon membrane and probed with a Y'-TG<sub>1-3</sub> specific probe. (Lane 1) Telomerase-positive strain, (Lane 2) Type II survivor strain before *RAD50* has been deleted; (Lanes 3-22) are YGL9.17*rad50*Δ type II survivor strains at ~150 generations, cells displayed TG<sub>1-3</sub> repeat as shown between 1.3 and ~4.0kb. DNA sizes indicated in the left side of the blot.

(YGL2.15, YGL9.12 and YGL9.17) that were generated from the same parent. Therefore, it was worth investigating type II telomere maintenance in these strains. Two genes (*RAD50* and *RPB9*) were chosen to be investigated individually in these strains. Deletion of these genes displayed a dissimilar result.

*RAD50* was not required to maintain type II telomeres in all three strains. Therefore, all *rad50*Δ independent survivor strains displayed the type II telomere structures. Thus, cells were amplifying TG<sub>1-3</sub> repeats to replicate their telomeres. A small fraction of YGL9.12*rad50*Δ and YGL9.17*rad50*Δ strains changed to a type I telomere pattern (Table 4.1; Fig 4.2, 4.3). Moreover, *rad50*Δ type I strains displayed fewer colonies of smaller size than type IIs (Fig 4.4 A, B). This is consistent with the behaviour of type I cells as previously described (Teng and Zakian, 1999). However, *RAD50* was required to establish type II survivors (Chen et al., 2001, Le et al., 1999). Additionally, it displayed a decrease in telomere length in telomerase-positive yeast strains (Askree et al., 2004). These studies showed that *RAD50* exhibited an obvious role in telomere elongation in both telomerase-dependent and independent strains. Consistently, *RAD50* displayed a similar role here. This role was clear when X-only ends displayed a decreased length at ~150 generations. However, most strains continued as type II. This result suggests that *rad50*Δ type II strains may utilise a different pathway of HR to elongate their telomeres. This pathway seems to be different than the pathway which is used to establish type II survivors. Further investigation will reveal what pathway is used in each case.

On the other hand, the *S. cerevisiae* RNA polymerase II subunit gene *RPB9* displayed different results in three different type II survivors. All YGL9.12*rpb9* $\Delta$  strains exhibited TG<sub>1-3</sub> repeat telomere pattern. Consequently, *RPB9* is not required for type II telomere maintenance at least until ~150 generations. All YGL2.15*rpb9* $\Delta$  and YGL9.17*rpb9* $\Delta$  strains died immediately after dissection. Interestingly, these strains were derived from the same yeast strain background (YGL9). Therefore, the changes here may be due to a mutation which occurred at senescence (Lundblad and Blackburn, 1993, Hackett et al., 2001, Hackett and Greider, 2003). This result indicates a direct or indirect role for *RPB9* in telomere elongation. This may be due to *RPB9* transcription functions (Walmacq et al., 2009). It perhaps affects the transcription of some genes that are involved in telomere biology. However, a telomerase positive strain exhibited telomere shortening when *RPB9* was deleted (Askree et al., 2004). Therefore, the result of the Askree group seems to be consistent with what is found here in two strains (YGL2.15 and YGL9.17), but not consistent with the result of the third strain (YGL9.12).

## **Chapter 5: Genetic requirements for type II survivor maintenance at long term after deleting candidate genes**

### ***5.1. Introduction***

Genetic requirements for diploid type II survivor telomere maintenance were discussed in Chapter 3. Generally, strains do not require the candidate genes (20 genes) to maintain their telomeres. Genes were deleted individually as described previously in Chapter 3. This result was obtained at ~150 generations after deleting the candidate genes. Therefore, it is very important to perform further investigation for genetic requirements in long term propagation. Two independent cell lines derived from two spores for some of these genes were propagated for the long term to test the requirement for these genes. Most cells which have the deleted candidate genes were propagated for approximately 500 generations, but some for ~1000 to ~1500 generations. All these genes exhibited a clear decrease in telomere length after ~ 150 generations. The absence of some genes changed type II survivor strains to type I or another survivor.

### ***5.2. Absences of RAD50 switched diploid type II to type I survivor in the long term***

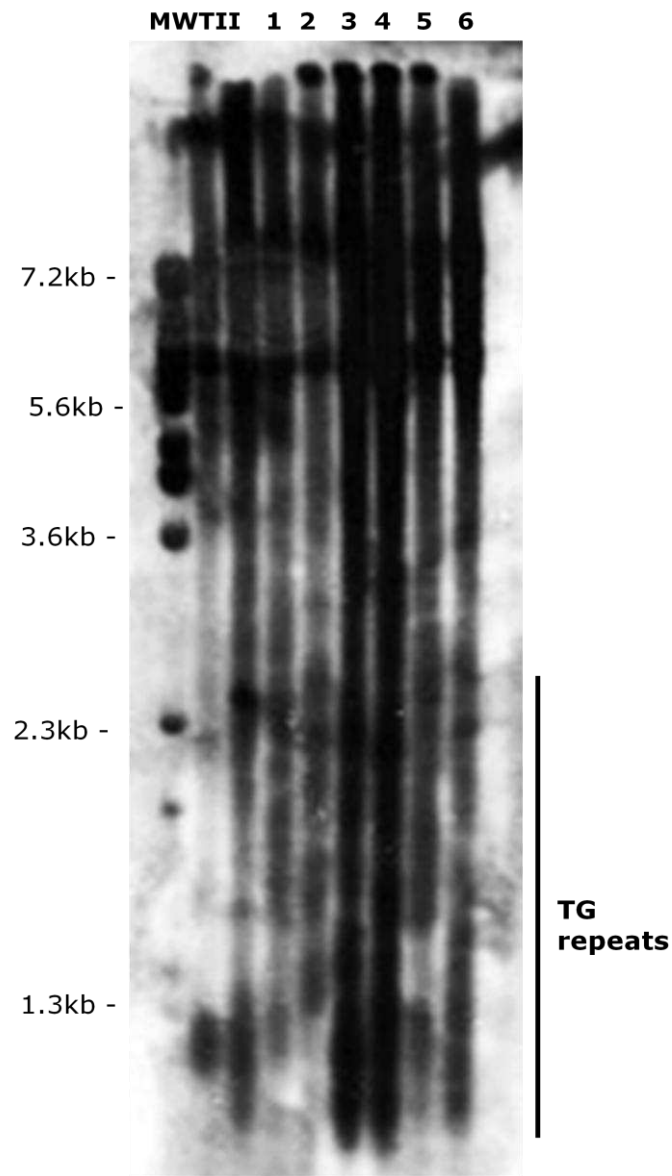
*RAD50* was examined for type II survivor maintenance in the long term. Two independent YGL2.15*rad50*Δ strains were propagated on YEPD at 30°C for the long term. Cells were stored at -80°C in 25% glycerol at different time points starting from ~175 generations, then after each ~100 generations. Cells derived from spore one died at ~1,200 generations, while cells for the second spore were able to grow more than ~1,500 generations. During this long time course, cells exhibited changes regarding their growth. Cells grew slower than

the normal growth of type II survivors at  $\sim 225$ ,  $\sim 275$  and  $\sim 1,000$  generations. YGL2.15*rad50* $\Delta$  along with all type II strains that have the candidate genes deleted were compared to *est2* $\Delta$  strains at the long term. Two independent type II strains (YGL2.15) with only *EST2* deleted were propagated for the long term ( $\sim 550$  generations). These strains do not display telomere shortening (Fig. 5.1). These strains were used as control for all type II strains that have the candidate genes deleted. The genomic DNA was extracted, digested with *XhoI* and run overnight on a 0.9% agarose gel. The nylon membrane was probed with Y'-TG<sub>1-3</sub> specific probe and the film was developed to see the telomere structure. Telomeres for both cell lines (spores one and two) start shortening at  $\sim 300$  generations and continue at each time point. Cells derived from spore one switched to type I then died at  $\sim 1,200$  generations. Whereas, cells from the second spore changed to type I at  $\sim 925$  generations and continued at the same pattern till propagation stopped at  $\sim 1,500$  generations (Fig 5.2 A). However, type I survivors derived from both spores exhibited amplification of Y' element but no TG<sub>1-3</sub> termini can be seen. Therefore, they are slightly different from the *RAD52* dependent type I survivor, which normally displays TG<sub>1-3</sub> termini. To confirm if these strains are type I or not, a CHEF gel was run for cells that were derived from spore two at all time points. Chromosomes were not able to enter the gel starting from  $\sim 925$  generations (Fig 5.2- B), this is a clear sign of type I behaviour as documented previously (Liti and Louis, 2003). However, at 1100 generations, chromosomes enter the gel, but they differ from both type I and type II. Thus it is possibly different behaviour than type Is (fig 5.2- B, lane 9). It is obvious that the last hundreds of generations displayed type I pattern regardless of the shift at  $\sim 1100$  generations time point. For more investigation, *RAD50* was

deleted in two more type II survivor strains (YGL9.12 and YGL9.17) as described in Chapter 4. Two independent cell lines for each strain were propagated for a long time course. DNA was isolated at these time points ~150, ~250, ~350, ~450, and ~550 generations. DNA was digested with *XhoI* and run at 45V for 16 hours. DNA fragments were transferred to a positive charged nylon membrane and probed with a Y'-TG<sub>1-3</sub> specific probe. Telomeres displayed clear shortening for each spore in both strains. Moreover, one of the cell lines for each strain altered to type I telomere pattern at ~ 550 generations (Fig. 5.3 A, B). Finally, it is obvious that *RAD50* was required to maintain telomeres as type II at long term in three independent type II strains. Thus, cells changed to type I survivor pattern.

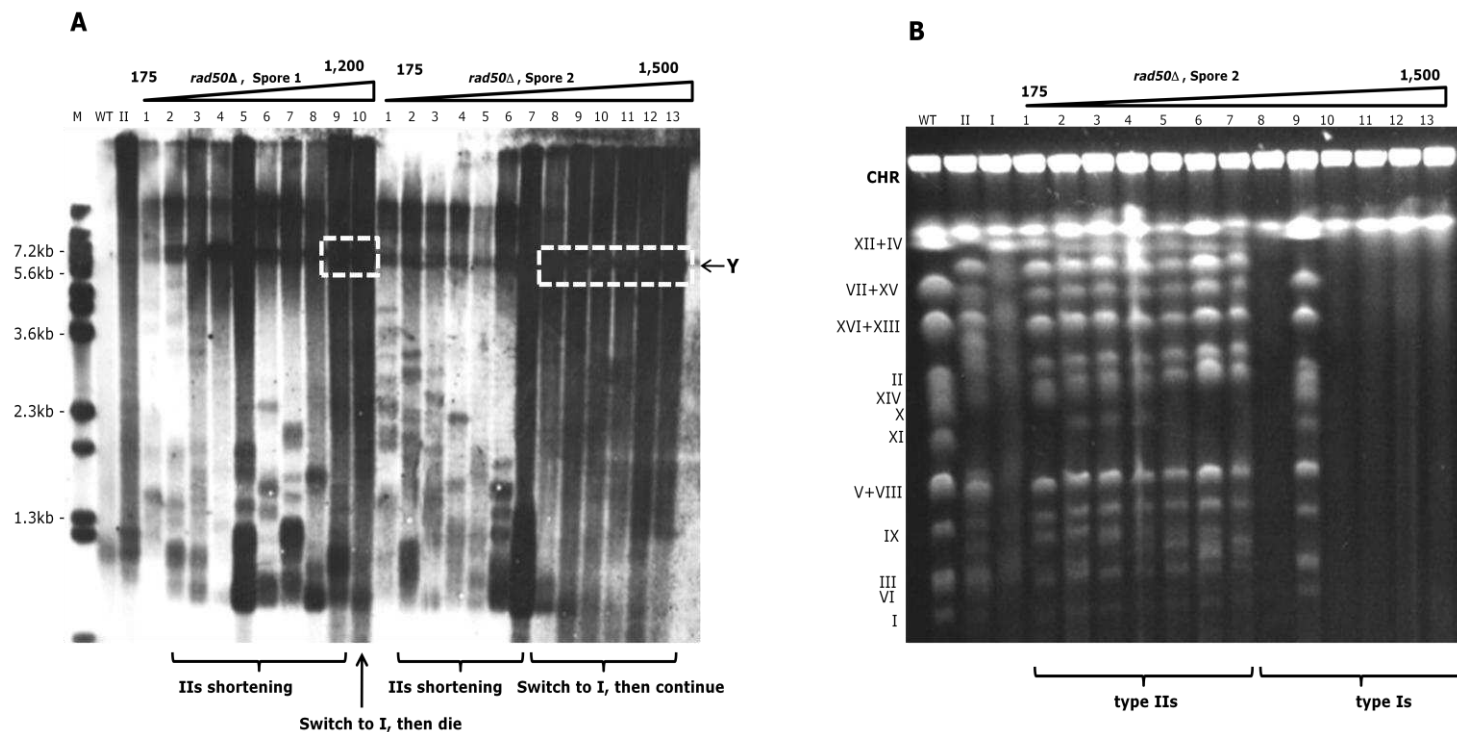
### ***5.3. KU80 deletion altered type II survivor strain to new type***

*Ku80* was investigated for type II maintenance in the long term. *Ku80* was deleted in type II survivors (YGL38) using the KANMX cassette as described in Chapter 2. The *KU80/ku80::KANMX* YGL38 strain was sporulated. Tetrads were dissected to separate spores. They were replica-plated on G418 containing plates to define their genotype. Two *ku80*Δ spores were streaked on YEPD plates, incubated at 30° for two days. Single colonies were restreaked for the long term. Cells were stored in 25% glycerol at -80 °C at frequent time points starting from ~150 then after each 100 generations ending with 1,000 generations. The genomic DNA was extracted, digested with *XhoI* and run overnight on a 0.9% agarose gel as described in Chapter 2. DNA was transferred to Nylon membrane. The Nylon membrane was probed with Y'-TG<sub>1-3</sub> specific probe and the film was developed to examine telomere



**Figure 5.1. Telomere pattern of type II strains at long term propagation**

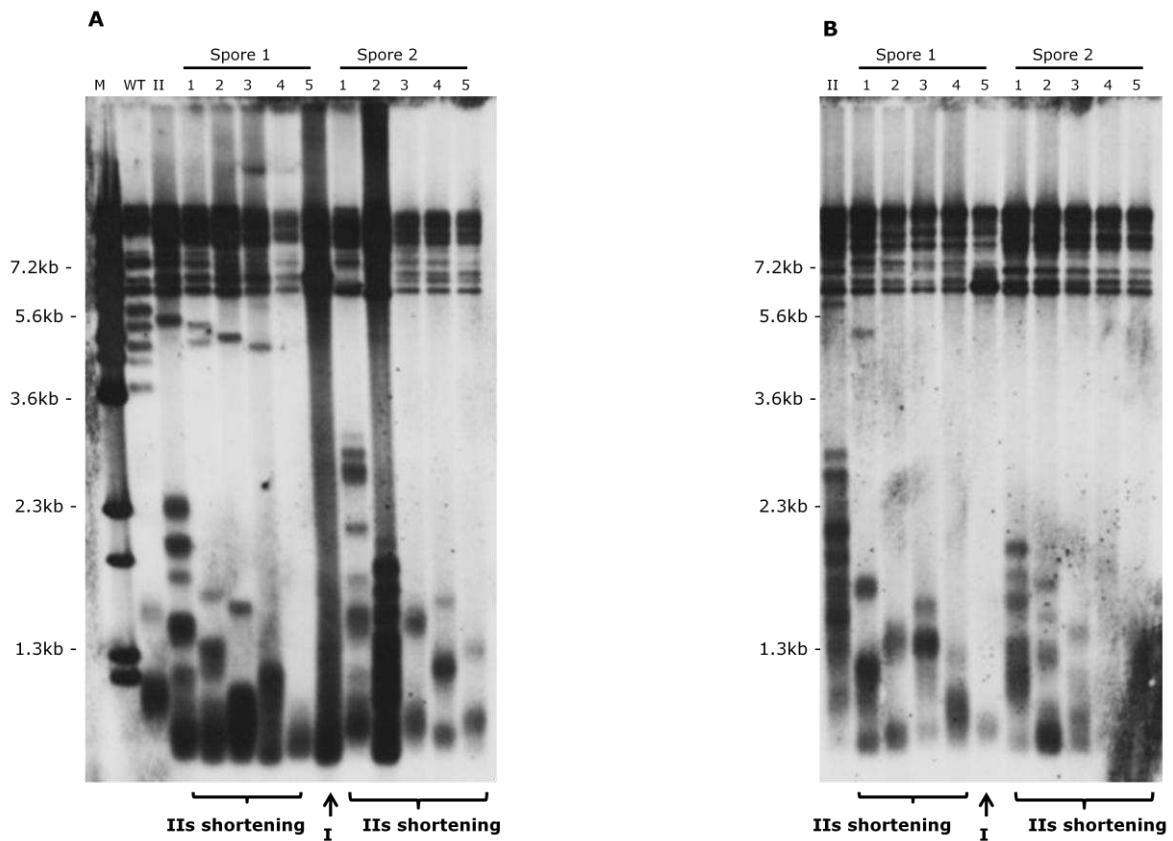
Southern blot hybridisation of YGL2.15 strains (two strains). Single colonies for each strain were propagated for long time starting from  $\sim 150$  generations after *EST2* deletion and end with  $\sim 550$  generations. Genomic DNA was purified from these strains at frequent points. DNA was digested with *XhoI* restriction endonuclease which cut within  $Y'$  element. The DNA was then transferred to a nylon membrane and probed with  $Y'$ -TG<sub>1-3</sub> specific probe. (Lane 1) marked with M DNA marker  $\lambda$  digested with *BstEII* (from NEW England Biolabs). (Lane 2) marked with WT telomerase positive strain, displaying 1.0kb telomere size. (Lane 3) marked with II parental strain YGL2.15 type II survivor. Lanes marked with 1 and 2 are two different strains that derived from YGL2.15 at  $\sim 300$  generations. Lanes 3 and 4 are the same strains in 1 and in the same order at  $\sim 425$  generations. Lanes 5 and 6 are the same strains in 1 and 2 in the same order at  $\sim 550$  generations. Strains don not display telomere shortening during the whole propagation period.



**Figure 5.2. Southern blot hybridisation analysis and CHFF gel of type II survivor strain YGL2.15 with *rad50* deletion at long term**

(A) Southern blot hybridisation of YGL2.15*rad50Δ* two strains. Single colonies for each strain were propagated for long time starting from ~175 generations after gene deletion and end with 1,200 or 1,500 generations. Genomic DNA was purified from these strains and digested with *XhoI* restriction endonuclease which cut within Y' element. The DNA was then transferred to a nylon membrane and probed with Y'-TG<sub>1-3</sub> specific probe. (Lane 1) marked with M DNA marker  $\lambda$  digested with *BstEII* (from NEW England Biolabs). (Lane 2) marked with WT telomerase positive strain, displaying 1.0kb telomere size. (Lane 3) marked with II parental strain YGL2.15 type II survivor. Lanes (1-10) in spore YGL2.15*rad50Δ* survivor strain at many generations starting with 175 and end with 1,200 generation. Telomeres in YGL2.15*rad50Δ* strain (derived from spore one) start shortening then changed to type I and died at 1,200 generations. Lanes (1-13) in spore two YGL2.15*rad50Δ* survivor strain start at 175 generations and end with 1,500 generations. Telomeres start shortening then changed to type I at ~925 and continue until ~1,500 generations. (B) Chef gel for Telomerase-positive strain, type II, I survivors and cells derived from spore two at the same order in A.





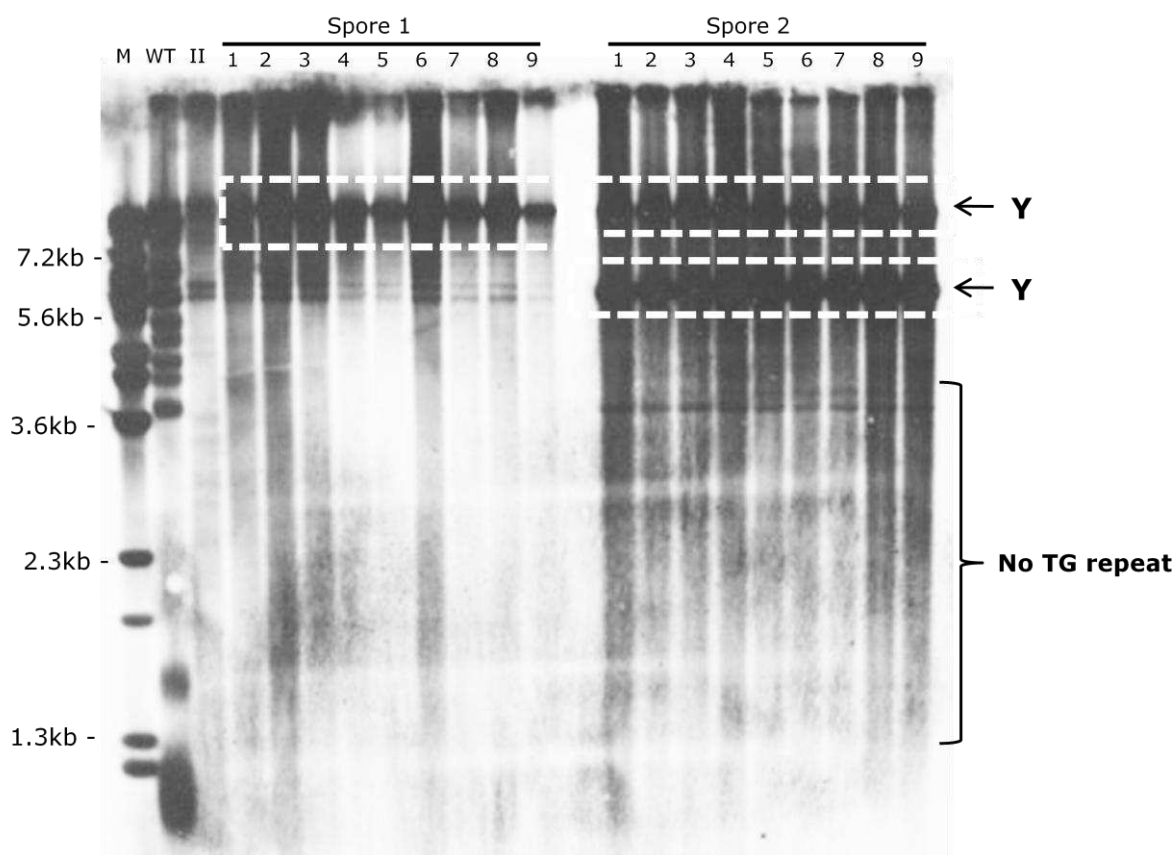
**Figure 5.3. Southern blot hybridisation analysis of type II survivor strains YGL9.12 and YGL9.17 with *rad50* deletion at long term**

(A) Southern blot hybridisation of YGL9.12*rad50*Δ strains (two spores). Single colonies for each spore were propagated for long time starting from ~150 generations after gene deletion and end with ~550 generations. Genomic DNA was purified from these strains after each 100 generations. DNA was digested with *Xho*I restriction endonuclease which cut within Y' element. The DNA was then transferred to a nylon membrane and probed with Y'-TG<sub>1-3</sub> specific probe. (Lane 1) marked with M DNA marker λ digested with *BstEII* (from NEW England Biolabs). (Lane 2) marked with WT telomerase positive strain, displaying 1.0kb telomere size. (Lane 3) marked with II parental strain YGL9.12 type II survivor. Lanes (1-5) in spore one YGL9.12*rad50* survivor strain at different points. Telomeres start shortening then changed to type I at 550 generations. Cells derived from spore two as indicated in top displayed telomere shortening, but maintain as type II. (B) The same experiment for YGL9.17*rad50*Δ (two spores). Both cell lines displayed telomere shortening, but cells derived from spore one changed to type I at 550 generations

structure. Cells derived from spore one amplify Y' elements at one position larger than the two classes (6.7 and 5.2 kb) which type I's normally have. While, cells derived from spore two amplify two different sizes of Y' element, one of them the same as which spore one had and the second one is close to 5.2 kb. Cells derived from both spores displayed no TG<sub>1-3</sub> repeat termini (Fig. 5.4). *Ku80*Δ deleted cells derived from the same transformant exhibited the same pattern of telomere structure at ~150 generations and chromosomes enter the CHEF gel as discussed in Chapter 3. Consequently, cells from both spores continued this new type of telomere structure for many cell generations. Finally, it is obvious to consider that *KU80* is required for type II survivor telomere maintenance, but not for survivor state.

#### **5.4. *RPB9* deletion changed type II to type I survivors at long term**

*RPB9* was investigated for type II telomere maintenance at long term. *RPB9* was deleted in type II strain (YGL9.12) by *hphMX* cassette as described in Chapter 2. Cells were patched on KAc plate and incubated at room temperature for at least three days. Spores were dissected on YEPD plate, incubated at 30°C for two days. They were replica-plated to YEPD-HYG plates to define their genotype. Two *rpb9*Δ spores were streaked on YEPD plate, incubated at 30°C for two days. Single colonies were restreaked at the same conditions for long time. Cells were stored at -80 °C in 25% glycerol at these time points 150, 250, 350, 450, and 550 generations. DNA was isolated, digested with *XhoI*. DNA fragments were transferred to a positive charged nylon and probed with Y'- TG<sub>1-3</sub> specific probe. A film was developed to analyse telomere structure. Cells derived from both spores displayed telomere shortening before switching to type I pattern. However, spore one



**Figure 5.4. Southern blot hybridisation analysis of type II survivor strains YGL38 with *ku80* deletion at long term**

Southern blot hybridisation of YGL38*ku80* $\Delta$  strains (two spores). Single colonies for each spore were propagated for long time starting from  $\sim$ 150 generations after gene deletion and end with  $\sim$ 1,000 generations. Genomic DNA was purified from these strains after each 100 generations. DNA was digested with *Xho*I restriction endonuclease which cut within Y' element. The DNA was then transferred to a nylon membrane and probed with Y'-TG<sub>1-3</sub> specific probe. (Lane 1) marked with M DNA marker  $\lambda$  digested with *Bst*EII (from NEW England Biolabs). (Lane 2) marked with WT telomerase positive strain, displaying 1.0kb telomere size. (Lane 3) marked with II parental strain YGL38 type II survivor. Lanes (1-9) in spore one YGL38*ku80* $\Delta$  survivor strain at different points. Cells displayed Y' element amplification as indicated by the white box, but no TG<sub>1-3</sub> repeat can be seen. Cells derived from spore two changed type II to new type. This type strain can be distinguished by the amplification of Y' element and the absence to TG<sub>1-3</sub> repeat. Two sizes of Y element were amplified (indicated by white boxes).

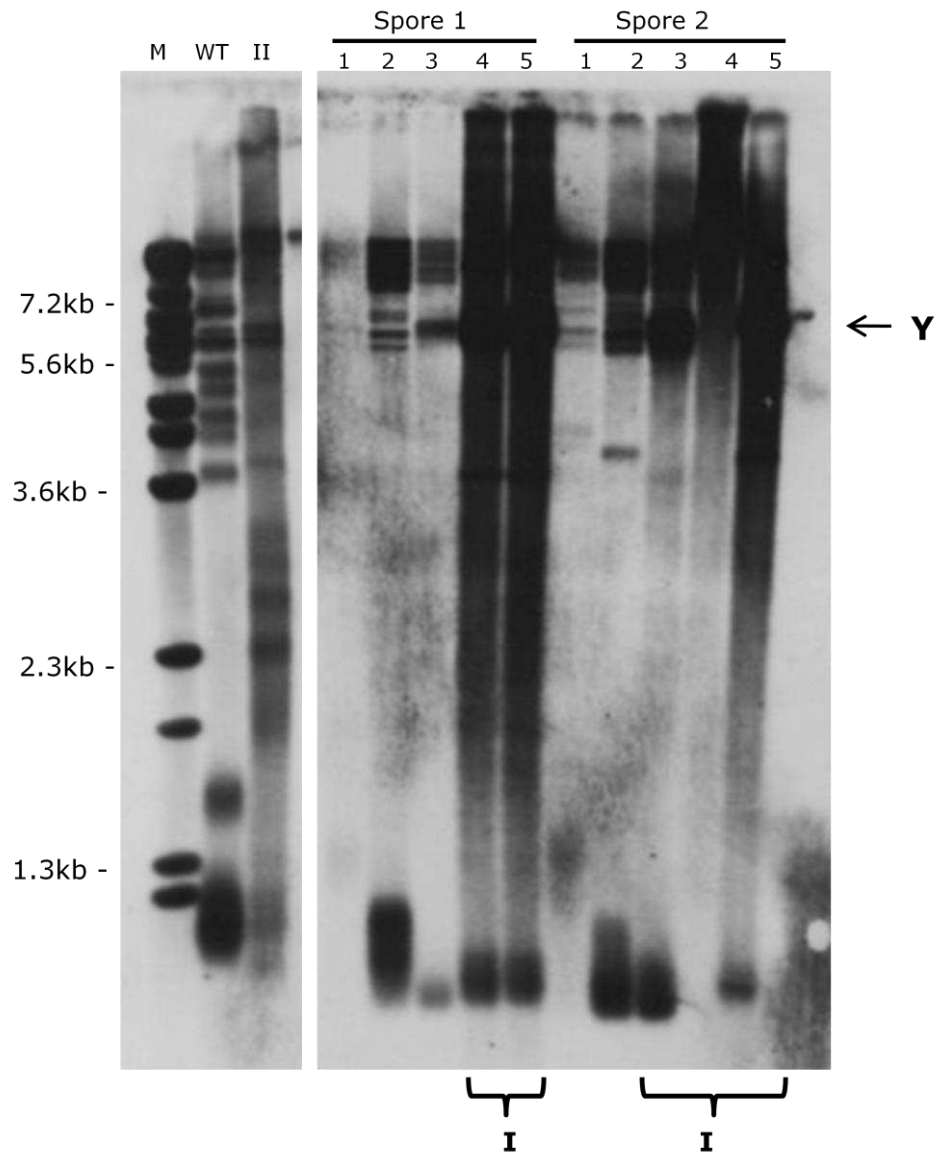
was changed to type I at ~ 450, while spore two switched at 350 generations (Fig 5.5).

### ***5.5. RIF1 Deletion reduced telomere length in type II survivors in the long term***

*RIF1* was examined for telomere maintenance at long term. Gene was deleted individually by *hphMX* cassette in a type II survivor strain. Cells were grown on KAc plates for three days at room temperature to sporulate. Spores were dissected on YEPD plates; incubated at 30°C for two days and replica-plated to YEPD-HYG plates. *Rif1*Δ strains (two independent strains) were streaked on YEPD plates, incubated at 30°C for two days. Single colonies derived from these spores were restreaked for long term. Cells were stored at -80°C at deferent time points (~150, ~275, ~400, and ~500 generations) for further analyses. DNA was extracted, digested with *XhoI*, run and probed with Y'-TG<sub>1-3</sub> specific probe. A film was developed to identify telomere structure. Both *rif1*Δ strains displayed telomere shortening. Cells derived from strain one were less affected than cells from spore two (Fig. 5.6).

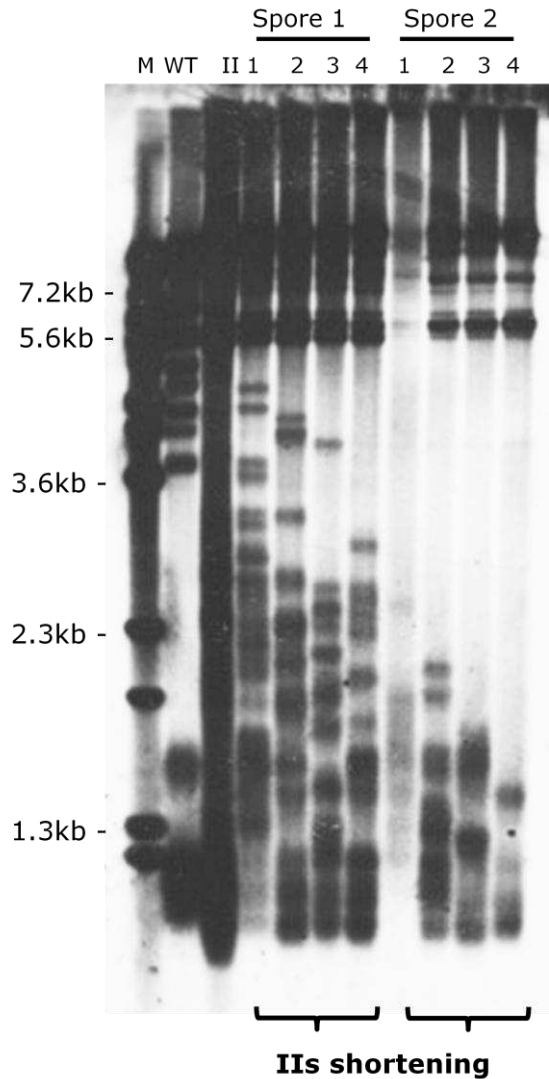
### ***5.6. Absence of SGS1 in type II survivors affected telomere length at long term***

*SGS1* was investigated for telomere maintenance as type II. The gene was deleted by the *hphMX* cassette in diploid type II survivor strain (YGL2.15). As a result of that a heterozygous strain of the gene was obtained (*SGS1 / sgs1::YHGMX*). Cells were patched on the KAc plate to grow in room



**Figure 5.5. Southern blot hybridisation analysis of type II survivor strains YGL9.12 with *rpb9* deletion at long term**

Southern blot hybridisation of YGL9.12*rpb9*Δ strains (two strains). Single colonies for each strain were propagated for long time starting from ~150 generations after gene deletion and end with ~550 generations. Genomic DNA was purified from these strains after each 100 generations. DNA was digested with *XhoI* restriction endonuclease which cut within Y' element. The DNA was then transferred to a nylon membrane and probed with Y'-TG<sub>1-3</sub> specific probe. (Lane 1) marked with M DNA marker λ digested with *BstEII* (from NEW England Biolabs). (Lane 2) marked with WT telomerase positive strain, displaying 1.0kb telomere size. (Lane 3) marked with II type II survivor. Lanes (1-5) in spore one YGL9.12*rpb9*Δ survivor strain at different points. Cells displayed telomere shorting then amplify Y' element to change to type I at ~450 generations as indicated in the blot. Cells derived from spore two changed to type I survivor at ~350 generations.



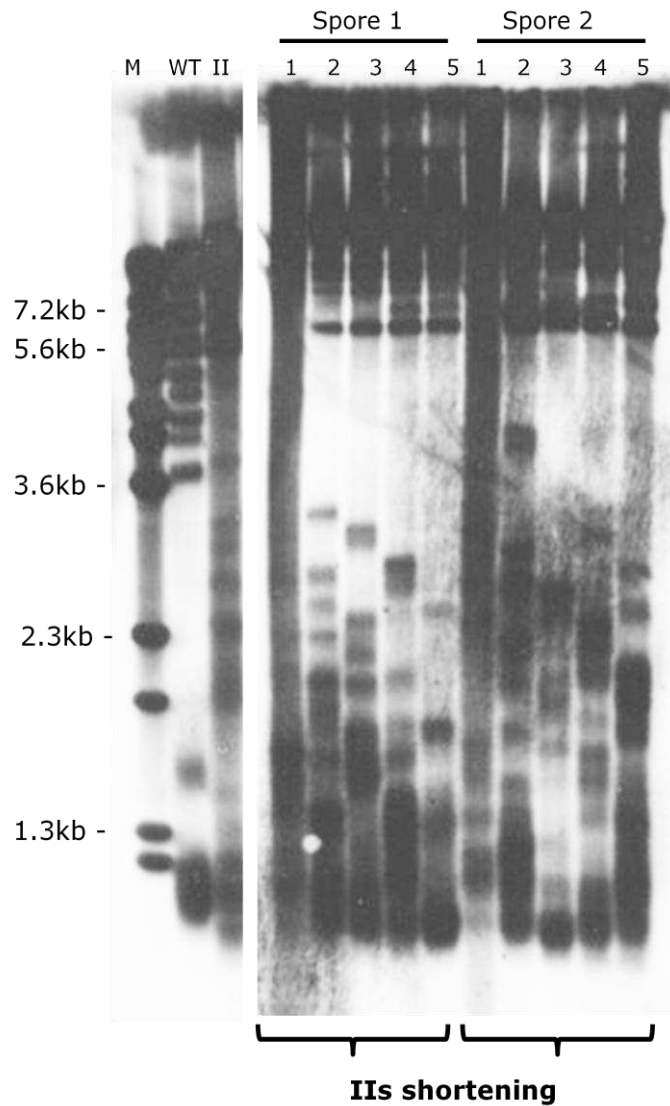
**Figure 5.6. Southern blot hybridisation analysis of type II survivor strains YGL2.15 with *rif1* deletion at long term**

Southern blot hybridisation of YGL2.15*rif1*Δ strains (two spores). Single colonies for each spore were propagated for long time starting from ~150 generations after gene deletion and end with ~500 generations. Genomic DNA was purified from these strains after each ~100 generations. DNA was digested with *XhoI* restriction endonuclease which cut within Y' element. The DNA was then transferred to a nylon membrane and probed with Y'-TG<sub>1-3</sub> specific probe. (Lane 1) marked with M DNA marker λ digested with *BstEII* (from NEW England Biolabs). (Lane 2) marked with WT telomerase positive strain, displaying 1.0kb telomere size. (Lane 3) marked with II type II survivor. Lanes (1-4) in spore one YGL2.15*rif1*Δ survivor strain at different points. Cells displayed telomere shortening but they carry on as type II. Cells derived from spore two also displayed telomere shortening as indicated in blot.

temperature for three days. Tetrads were dissected and spores were incubated on a YEPD plate at 30°C for two days. Cells were replica-plated onto YEPD-HYG media to select *SGS1* mutants. Cells derived from two independent *sgs1Δ* spores were streaked on a YEPD plate, incubated at 30°C for two days. Single colonies for each spore were restreaked on a YEPD plate at the same conditions for a long time course. Cells were stored in 25% glycerol at -80°C at different points during the time course (~150, ~250, ~350, ~450, and ~550 generations). DNA was isolated, digested with *XhoI*, run for 16 hours at 45 V and probed with a Y'-TG<sub>1-3</sub> specific probe. Film was developed to analyse telomere structure. Both cell lines were displayed a decrease in telomere length at ~250 generations (Fig 5.7). Cells continued as type II survivors until the streaking was stopped at ~550 generations.

### ***5.7. Absence of RPP1A increased telomere length in type II survivors in the long time course***

*RPP1A* was investigated at long term for type II survivor telomere maintenance. The gene was deleted in the type II survivor strain (YGL2.15) using a *hphMX* cassette (see methods). Thus, heterozygous (*RPP1A* /*rpp1a::HYG*) cells were obtained. Cells were grown in KAc media (plate) at room temperature for three days to sporulate. Tetrads were dissected on a YEPD plate, then incubated at 30°C for two days. Cells were replica-plated onto YEPD-HYG media to select mutated gene. Two *rpp1aΔ* strains were streaked on a YEPD plate, incubated at 30°C for three days. Cells displayed a slow growth, because of that the incubation time was extended. Cells were restreaked for long term and stored at -80 °C at different time points (~150,



**Figure 5.7. Southern blot hybridisation analysis of type II survivor strains YGL2.15 with *sgs1* deletion at long term**

Southern blot hybridisation of YGL2.15*sgs1*Δ strains (two spores). Single colonies for each spore were propagated for long time starting from ~150 generations after gene deletion and end with ~550 generations. Genomic DNA was purified from these strains after each ~100 generations. DNA was digested with *XhoI* restriction endonuclease which cut within Y' element. The DNA was then transferred to a nylon membrane and probed with Y'-TG<sub>1-3</sub> specific probe. (Lane 1) marked with M DNA marker λ digested with *BstEII* (from NEW England Biolabs). (Lane 2) marked with WT telomerase positive strain, displaying 1.0kb telomere size. (Lane 3) marked with II type II survivor. Lanes (1-5) in spore one YGL2.15*sgs1*Δ survivor strain at different points. Cells displayed telomere shortening but they carry on as type II. Cells derived from spore two also displayed telomere shortening as indicated in blot.



~250, ~350, ~450 and ~550 generations). The genomic DNA was isolated, digested with *XhoI*, probed with Y'-TG<sub>1-3</sub> specific probe. Film was developed to define telomere pattern. Surprisingly, *rpp1a*Δ strain (spore one) displayed telomere lengthening at ~550 generations. However, *rpp1a*Δ strain (spore two) exhibited telomere lengthening at ~350 generations and then shortening until ~550 generations (Fig. 5.8). Similar to what is found in this study, *tlc1*Δ type II strains displayed telomere shorting and then lengthening (Teng et al., 2000).

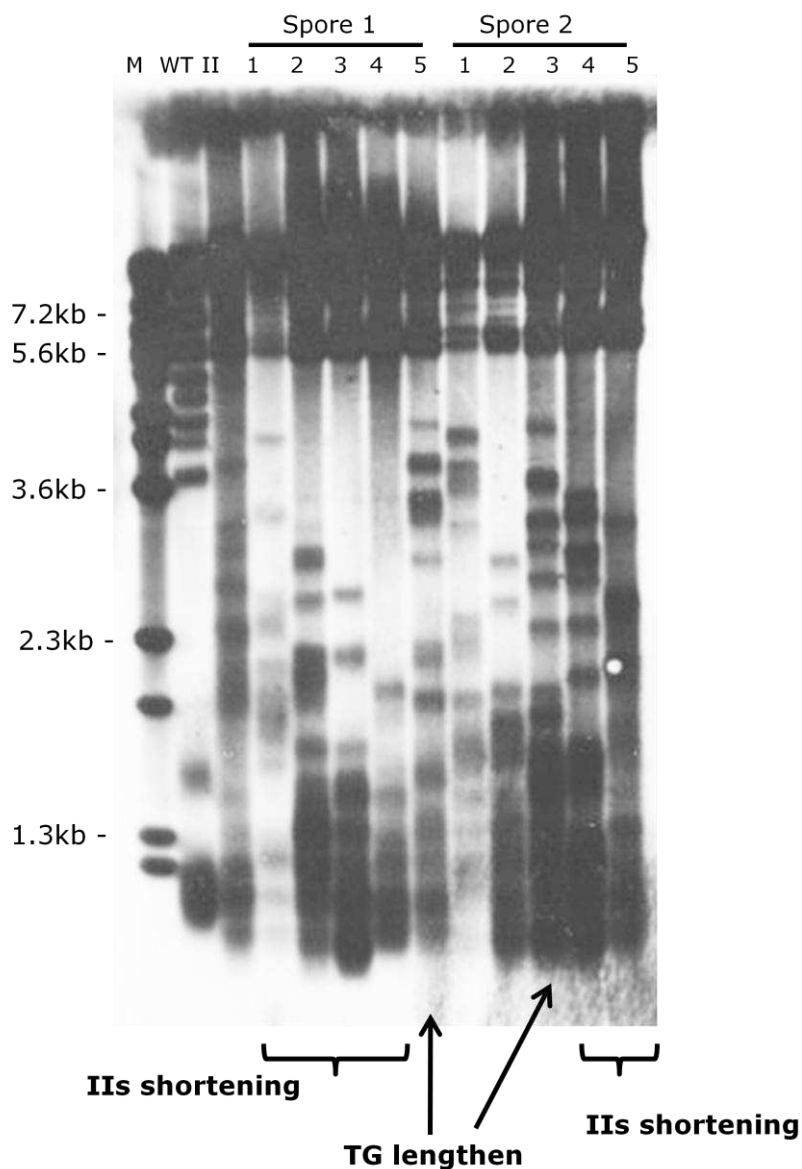
### **5.8. Absence of candidate genes decreased telomere length in type II survivors at long term**

Additional candidate genes were investigated for type II telomere maintenance at long term. The candidate genes are *GTR1*, *OGG1*, *UHR1*, *UPF1* and *RPB4*. Genes were deleted individually in type II survivor strain (YGL2.15), using the hphMX cassette as described in Chapter 2. Thus, a heterozygous strain was obtained (for each gene). Strains were grown in KAc plates, left at room temperature for three days. Tetrads were dissected on YEPD plates and incubated at 30°C for two days. Spores were replica-plated onto YEPD-YHG plates to select mutants. Two spores that have the deleted copy for each gene were streaked on YEPD plates, incubated at 30°C for two days. Single colonies were restreaked for long time course at the same conditions above. Cells were stored in 25% glycerol in -80 °C at frequent times (normally after each ~100 generations starting from ~150 and end with ~550). The genomic DNA was extracted, digested with *XhoI* and run at 45V for 16 hours. The membrane was probed with Y'-TG<sub>1-3</sub> specific probe and the film was developed to define telomere structure. All strains that have deleted

gene displayed telomere shortening, but they remained as type II. This result can be seen clearly through *gtr1* $\Delta$  and *ogg1* $\Delta$  strains Southern blots (Fig. 5.9). Moreover, two independent YGL2.15*hur1* $\Delta$  strains displayed telomere shortening after long term propagation. This shortening can be seen clearly in strain 2 more than strain 1 as indicated by white arrows (Fig. 5.10). Also, type II survivor strains that have these genes deleted *upf1*, *rpb4* displayed telomere shortening after long term propagation as presented respectively in (Fig. 5.11 and 5.12). YGL2.15*rpb4* $\Delta$  strains displayed no bands at ~150 generations for both examined strains. This is due to the low amount of DNA in this Southern blot. These two strains are clearly type II survivors as indicated in their Southern in Chapter 3.

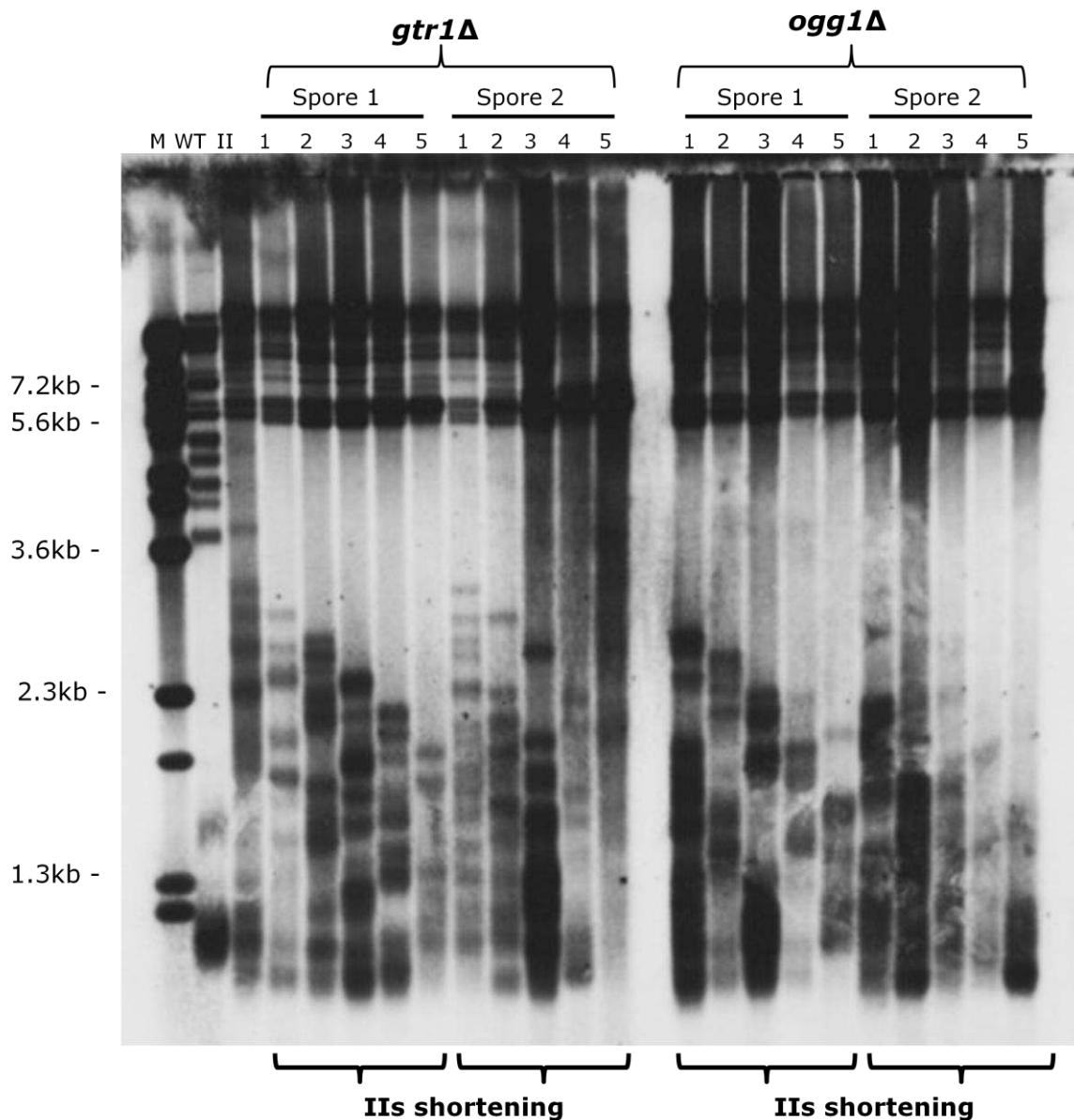
## **5.9. Discussion**

Candidate genes were investigated for type II telomere maintenance at ~150 generations in Chapter 3. Most of these genes were not required for type II survivor maintenance. Therefore, cells displayed TG<sub>1-3</sub> repeat heterogeneity after deleting the genes individually. Only *KU80* $\Delta$  strains displayed a different type of survivor that is similar to type I, but these cells did not have the terminal Y' element that is followed by very short tracts of TG<sub>1-3</sub> repeat which normally appear in type I survivors. For further investigation, type II survivor strains that have some of the candidate genes deleted were propagated for long term. For most of the genes, cells were streaked until ~500-550 generations. *Ku80* $\Delta$  and *rad50* $\Delta$  strains were propagated until 1,000 and 1,500 generations respectively. Survivor strains that were propagated for long



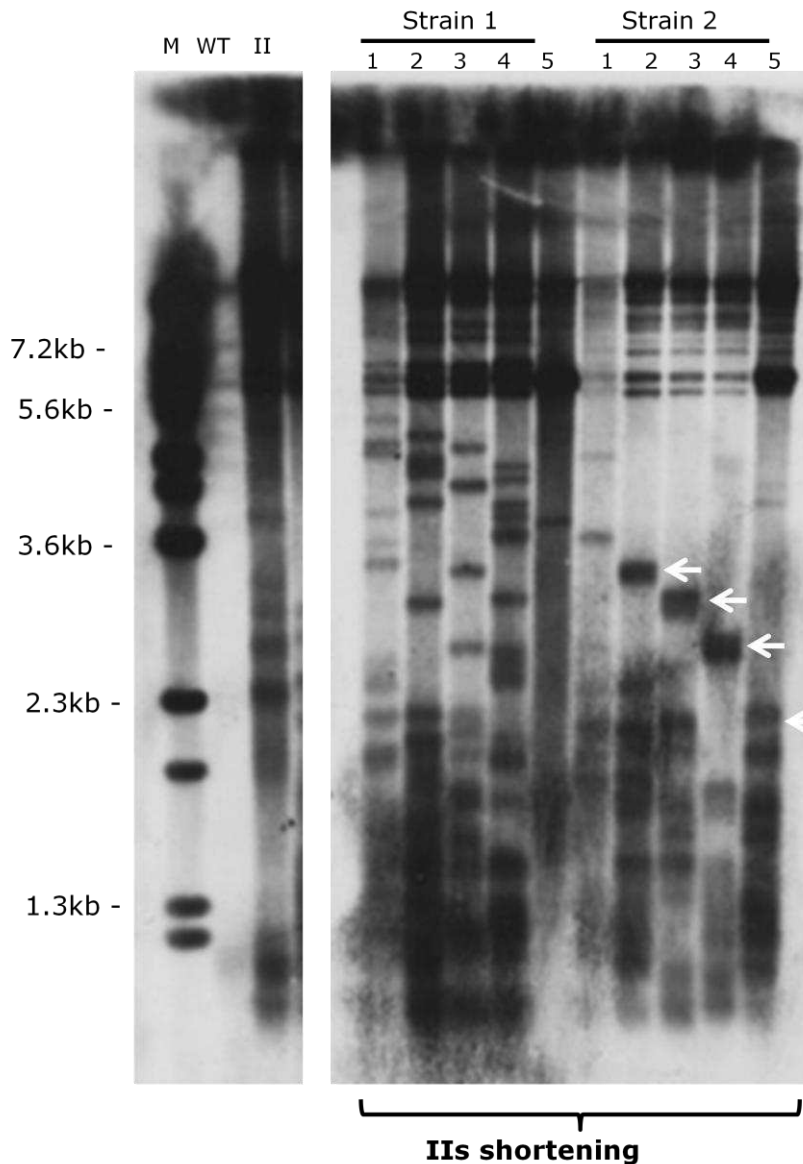
**Figure 5.8. Southern blot hybridisation analysis of type II survivor strains YGL2.15 with *rpp1a* deletion at long term**

Southern blot hybridisation of YGL2.15*rpp1a*Δ strains (two spores). Single colonies for each spore were propagated for long time starting from ~150 generations after gene deletion and end with ~550 generations. Genomic DNA was purified from these strains after each ~100 generations. DNA was digested with *XhoI* restriction endonuclease which cut within Y' element. The DNA was then transferred to a nylon membrane and probed with Y'-TG<sub>1-3</sub> specific probe. (Lane 1) marked with M DNA marker λ digested with *BstEII* (from NEW England Biolabs). (Lane 2) marked with WT telomerase positive strain, displaying 1.0kb telomere size. (Lane 3) marked with II type II survivor. Lanes (1-5) in spore one YGL2.15*rpp1a*Δ survivor strain at different points. Cells displayed telomere shortening then changed to lengthening at ~550 generations as indicated in the blot. Cells derived from spore two also displayed telomere shortening as indicated in blot.



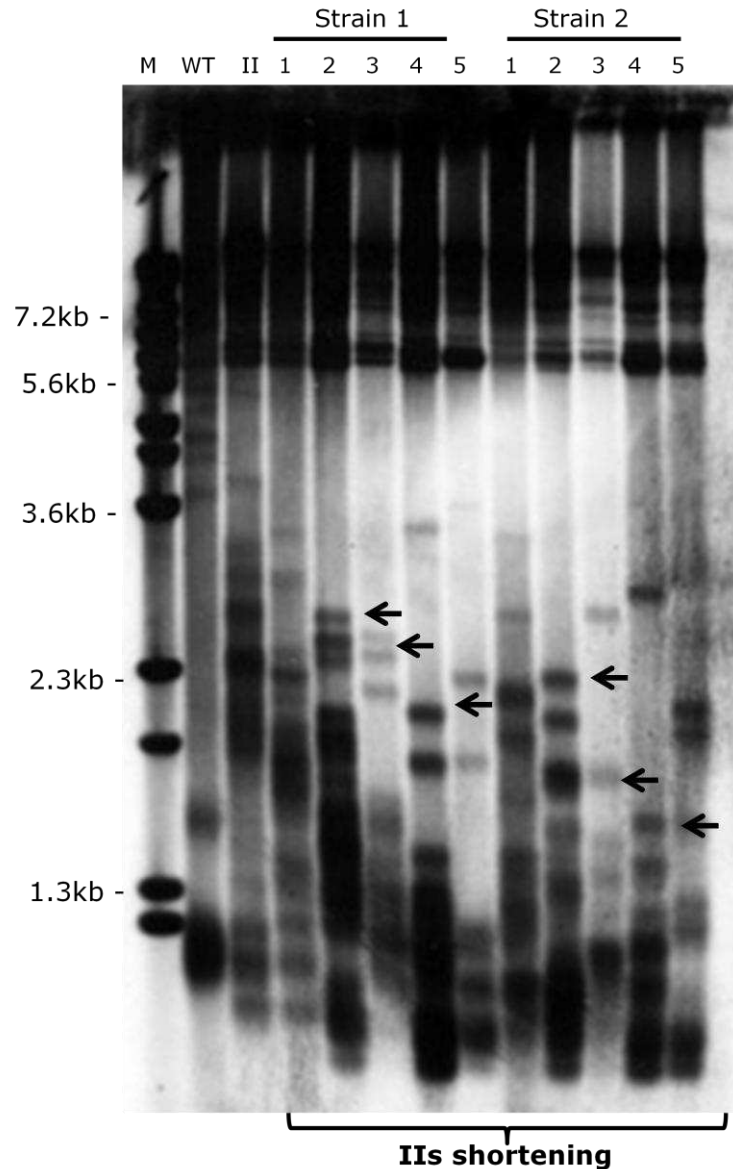
**Figure 5.9. Southern blot hybridisation analysis of type II survivor strains YGL2.15 with *gtr1* and *ogg1* individually deletion at long term**

Southern blot hybridisation of YGL2.15*sgs1Δ* strains (two spores for each gene). Single colonies for each spore were propagated for long time starting from ~150 generations after genes deletion and end with ~550 generations. Genomic DNA was purified from these strains after each ~100 generations. DNA was digested with *XhoI* restriction endonuclease which cut within Y' element. The DNA was then transferred to a nylon membrane and probed with Y'-TG<sub>1-3</sub> specific probe. (Lane 1) marked with M DNA marker λ digested with *BstEII* (from NEW England Biolabs). (Lane 2) marked with WT telomerase positive strain, displaying 1.0kb telomere size. (Lane 3) marked with II type II survivor. Lanes (1-5) in spore one YGL2.15*gtr1Δ* survivor strain at different points. Cells displayed telomere shortening but they carry on as type II. Cells derived from spore two also displayed telomere shortening as indicated in blot. Cells derived from both spores of YGL2.15*ogg1Δ* displayed telomere shortening, but carry on as type II until ~500 generations.



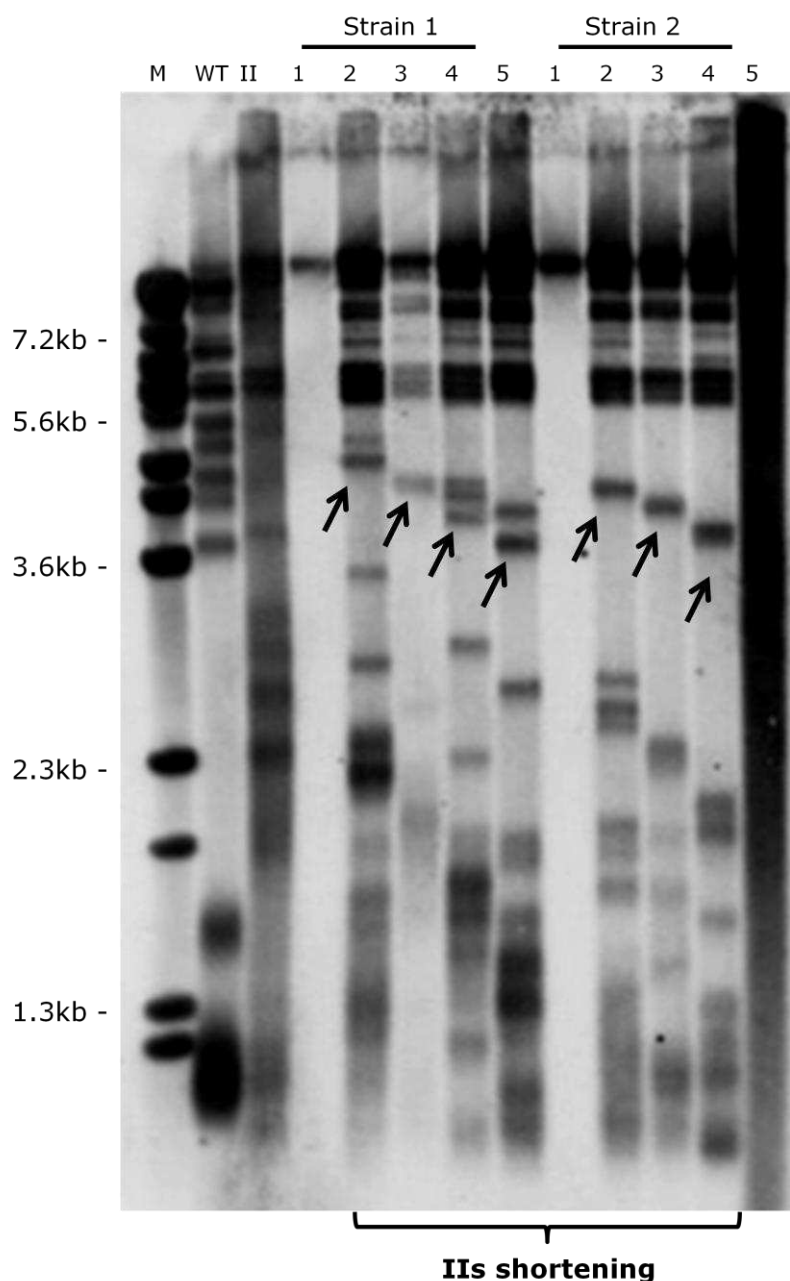
**Figure 5.10. Southern blot hybridisation analysis of type II survivor strains YGL2.15 with *hur1* deletion at long term**

Southern blot hybridisation of YGL2.15*hur1*Δ strains (two strains). Single colonies for each strain were propagated for long time starting from ~150 generations after gene deletion and end with ~550 generations. Genomic DNA was purified from these strains after each ~100 generations. DNA was digested with *XhoI* restriction endonuclease which cut within Y' element. The DNA was then transferred to a nylon membrane and probed with Y'-TG<sub>1-3</sub> specific probe. (Lane 1) marked with M DNA marker λ digested with *BstEII* (from NEW England Biolabs). (Lane 2) marked with WT telomerase positive strain, displaying 1.0kb telomere size. (Lane 3) marked with II type II survivor. Lanes (1-5) in strain one YGL2.15*hur1*Δ survivor strain at different points. Cells displayed telomere shortening but they carry on as type II. Cells derived from strain two also displayed telomere shortening as indicated in blot. White arrows showed telomere shortening in type II survivor strains.



**Figure 5.11. Southern blot hybridisation analysis of type II survivor strains YGL2.15 with *upf1* deletion at long term**

Southern blot hybridisation of YGL2.15*upf1*Δ strains (two strains). Single colonies for each strain were propagated for long time starting from ~150 generations after gene deletion and end with ~550 generations. Genomic DNA was purified from these strains after each ~100 generations. DNA was digested with *XhoI* restriction endonuclease which cut within Y' element. The DNA was then transferred to a nylon membrane and probed with Y'-TG<sub>1-3</sub> specific probe. (Lane 1) marked with M DNA marker λ digested with *BstEII* (from NEW England Biolabs). (Lane 2) marked with WT telomerase positive strain, displaying 1.0kb telomere size. (Lane 3) marked with II type II survivor. Lanes (1-5) in strain one YGL2.15*upf1*Δ survivor strain at different points. Cells displayed telomere shortening but they carry on as type II. Cells derived from strain two also displayed telomere shortening as indicated in blot. Arrows showed telomere shortening in type II survivor strains.



**Figure 5.12. Southern blot hybridisation analysis of type II survivor strains YGL2.15 with *rpb4* deletion at long term**

Southern blot hybridisation of YGL2.15*rpb4*Δ strains (two strains). Single colonies for each strain were propagated for long time starting from ~150 generations after gene deletion and end with ~550 generations. Genomic DNA was purified from these strains after each ~100 generations. DNA was digested with *XhoI* restriction endonuclease which cut within Y' element. The DNA was then transferred to a nylon membrane and probed with Y'-TG<sub>1-3</sub> specific probe. (Lane 1) marked with M DNA marker λ digested with *BstEII* (from NEW England Biolabs). (Lane 2) marked with WT telomerase positive strain, displaying 1.0kb telomere size. (Lane 3) marked with II type II survivor. Lanes (1-5) in strain one YGL2.15*rpb4*Δ survivor strain at different points. Cells displayed telomere shortening but they carry on as type II. Cells derived from strain two also displayed telomere shortening as indicated in blot. Arrows showed telomere shortening in type II survivor strains.

term displayed different changes in their telomeres depending on which gene was deleted. Therefore, most of these genes seem to be essential for type II survivor strain maintenance at long term, but not absolutely required for telomere maintenance at regular propagation (150 generations).

YGL2.15*rad50* $\Delta$ , YGL9.12*rad50* $\Delta$  and YGL9.17*rad50* $\Delta$  survivor strains displayed a type II telomere structure at  $\sim$ 150-175 generations, but switched to type I pattern at long term ( $\sim$ 550 or 1,500 generations). YGL2.15 type II survivor strain displayed long X-only end compared to YGL9.12 and YGL9.17 survivors as discussed in Chapter 4. YGL2.15*rad50* $\Delta$  strains displayed telomere shortening then they changed to type I at  $\sim$ 1,200 and 1,500 generations for both cell lines (Fig.5.2 A, B). Whereas, YGL9.12*rad50* $\Delta$  and YGL9.17*rad50* $\Delta$  strains displayed telomere shortening then changed to type I telomere pattern at  $\sim$ 550 generations (one cell line for each strain) (Fig. 5.3 A, B). This result suggests that the telomere length of survivor strains could effect telomere changes after gene deletions. However, *RAD50* is essential to generate type II survivors (Chen et al., 2001, Le et al., 1999), since the deletion of *RAD50* at pre-senescence generate type I survivors. It seems that *RAD50* has a significant affect when it is deleted at pre-senescence stage, thus type II survivors cannot be generated. On the other hand the gene appears to have a minor role once type II survivors are already generated, hence strains maintain as type II for long time then telomeres start shortening before they switched to type I. It is known that most *S. cerevisiae* chromosome ends have the Y' element (Louis and Haber, 1992). *Rad50* $\Delta$  survivor strains displayed telomere shortening and the Y' element become terminus. Therefore,



recombination is then initiated between Y' elements and type I survivors generated.

YGL38*ku80*Δ survivor strains displayed a Y' element amplification at ~150 generations, but they had lost the terminal Y' fragment and its TG repeat tracts. However, two independent cell lines retained this telomere pattern for ~1,000 generations (Fig.5.4). Cells displayed type I telomere structure in the Southern blot hybridisation. However, Chromosomes of these strains entered the CHEF gel at ~150 generations as discussed in Chapter 3. Therefore these strains did not follow the type I survivor pathway. KU proteins plays a very important role to protect telomeres from recombination and nucleolytic degradation (reviewed in (Fisher and Zakian, 2005)). Therefore, the absence of *KU80* in YGL38 survivor strains may lead to telomere degradation. The Y' element is highly conserved with approximately 1% variation (Louis and Haber, 1992). Therefore, YGL38*ku80*Δ strains may take advantage of that and amplify Y' elements to elongate their telomeres. However, *KU80*Δ strains died in the absence of telomerase (Gravel et al., 1998, Nugent et al., 1998). Moreover; *KU80*Δ displayed short telomeres in telomerase-positive strains (Askree et al., 2004).

YGL2.15*rpb9*Δ and YGL9.17*rpb9*Δ strains died immediately after dissection. Whereas, YGL9.12*rpb9*Δ strains (two independent cell lines) switched to type I at ~450 and ~350 generations (Fig. 5.5). This result is consistent with previous work, once *rpb9*Δ strains displayed a telomere shortening in a telomerase-positive strain (Askree et al., 2004). *RPB9* is a subunit of RNA polymerase II that plays an important role in transcription fidelity (Walmacq

et al., 2009). Therefore, deletion of this gene may affect the transcription mechanism of genes that are directly or indirectly involved in telomere biology.

YGL2.15 survivor strains displayed telomere shortening at long term when the candidate genes were deleted. However, deletion of these genes reduced telomere length, while the deletion of some, *RIF1*, and *OGG1* lead to telomere lengthening in telomerase-positive strains (Askree et al., 2004). *SGS1* is required to establish type II survivor strains (Huang et al., 2001, Johnson et al., 2001, Cohen and Sinclair, 2001). Consistently, it has been found that *SGS1* deletion reduced recombination leading to slow senescence (Lee et al., 2007). *SGS1* seems to be playing a significant role at pre-senescence, but this role is impaired in type II survivors. Thus, telomere shortening occurred only at long term and strains continued as type II survivors (Fig 5.7). Unexpectedly, YGL2.5*rif1* $\Delta$  strains displayed telomere shortening at long term (Fig. 5.6). This gene is known as a telomerase negative regulator (Bianchi and Shore, 2008, Hardy et al., 1992). It may play a role in telomere protection. Thus, when it is deleted, exonucleases maybe able to shorten telomeres. YGL2.15*rpp1a* $\Delta$  strains displayed type II telomere pattern and slow growth at short term (~150 generations) as discussed in Chapter 3. Surprisingly, strains exhibited telomere shortening at long term followed by lengthening in one cell line (Fig 5.8). YGL2.15*ogg1* $\Delta$  and YGL2.15*gtr1* $\Delta$  survivor strains displayed telomere shortening (Fig 5.9 A, B). However, the deletion of *OGG1* lengthens telomeres. This may be due its role in reducing the binding sites of telomerase negative regulators, RAP1p and RIF2p (Lu and Liu, 2010). Type II survivor strains with only *est2* deleted, did not display telomere shortening after long

term propagation. These strains continued as type II survivors and there is no sign of telomere erosion (Fig 5.1). Thus, it is clear that telomere shortening occurred as result of the candidate genes absence.

In conclusion, deletion of the candidate genes in type II survivor strains clearly affects telomere length in the long term. It is obvious that all strains displayed telomere shortening at long term. Whereas strains continued type II telomere structure at ~150 generations after gene deletions. Deletion of *RAD50* changed type II survivor strains to type I at long term. Moreover, *RPB9* deletion strains displayed type I telomere pattern. It is notable that *ku80* $\Delta$  strains displayed a new survivor type (similar to type I). These results indicate a mild effect of these genes in type II survivor strains. Therefore, telomere shortening appeared only at long term.

## **Chapter 6: Phenotypic analysis of survivor cells after deleting candidate genes**

### ***6.1. Introduction***

Telomeres play an important role to protect chromosomes ends from end to end fusion and degradation. They prevent cells from recognising chromosomes ends as double strand breaks (DSBs) (Sandell and Zakian, 1993, Lundblad, 2000). Uncapped or short telomeres activate checkpoint machineries (AS and Greider, 2003), which leads to slow growth and cell cycle arrest. The absence of each of the candidate genes changed telomere length in telomerase-positive strains, some with telomere shortening, others with telomere lengthening (Askree et al., 2004). However, deletion of most these genes in type II survivor strains did not clearly affect telomere length at least at ~150 generations as discussed in Chapter 3. Temperature and ultra-violet (UV) sensitivity analysis were performed to check DNA damage response (DDR) in the absence of the candidate genes in type II survivor strains. This was done at ~150 generation after deleting the candidate gene. In general, deletion of the candidate genes did not affect cell growth compared to wild-type and type II survivors at 30°C and 37°C. However, some mutated genes resulted in more or less sensitivity to UV as explained below.

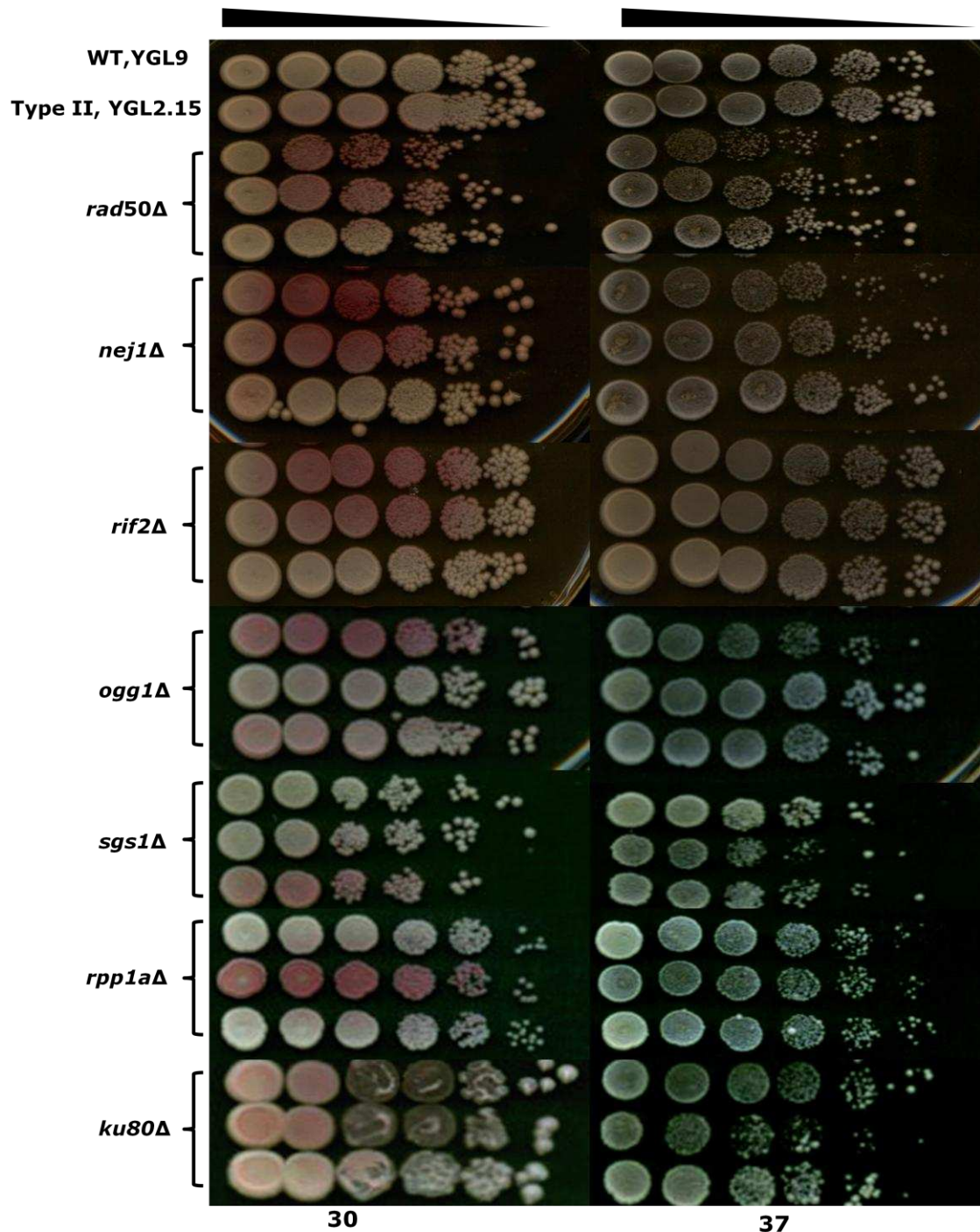
### ***6.2. General method for temperature and UV sensitivity***

Temperature and ultra-violet (UV) sensitivity were measured for wild-type, type I and type II survivor strains that had deleted candidate genes. First, cells that had the deleted gene were propagated for ~150 generations as discussed in Chapter 3. Three independent cell lines for each gene were analysed for both temperature and UV experiments. Second, a single colony of

wild type, type II, mutant type II (after each gene has been deleted, ~150 generations) was re-suspended in 45 µl of distilled water. Three independent cell lines were examined for each gene. Six ten-fold serial dilutions were made. 5 µl of each dilution was spotted on YEPD plates. For the temperature experiment, cells were incubated at 30°C and 37°C for two days. For the UV experiment, cells were exposed with 20J/ m<sup>2</sup> and 80J/ m<sup>2</sup> and incubated at 30°C for two days. UV irradiation was performed in the dark room then the plates were wrapped with foil against the light. A set of the same three cell lines for each gene with no UV irradiation (control) were incubated on YEPD plates at 30°C for two days (Fig. 6.1).

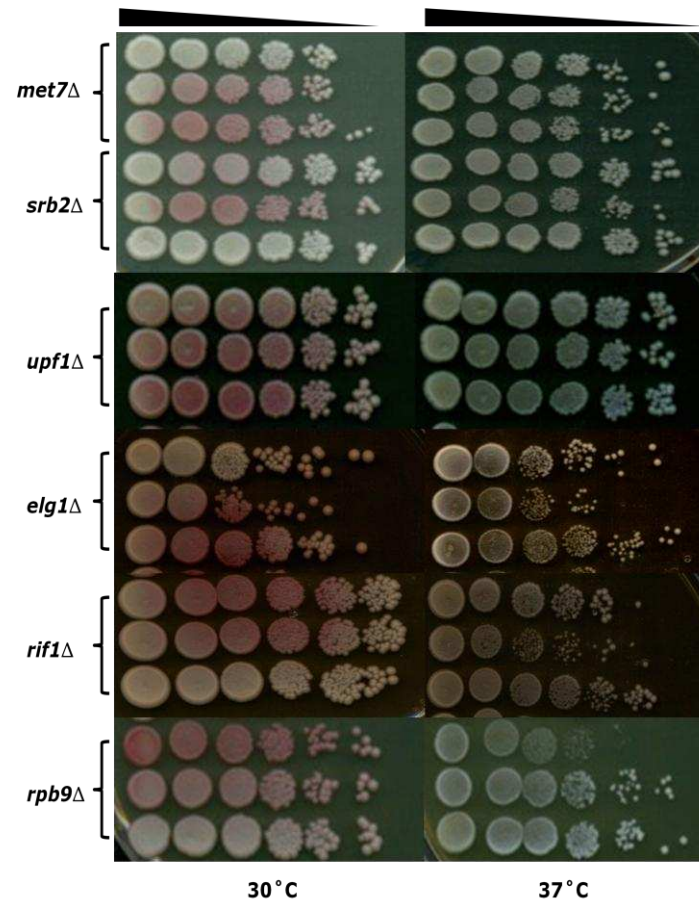
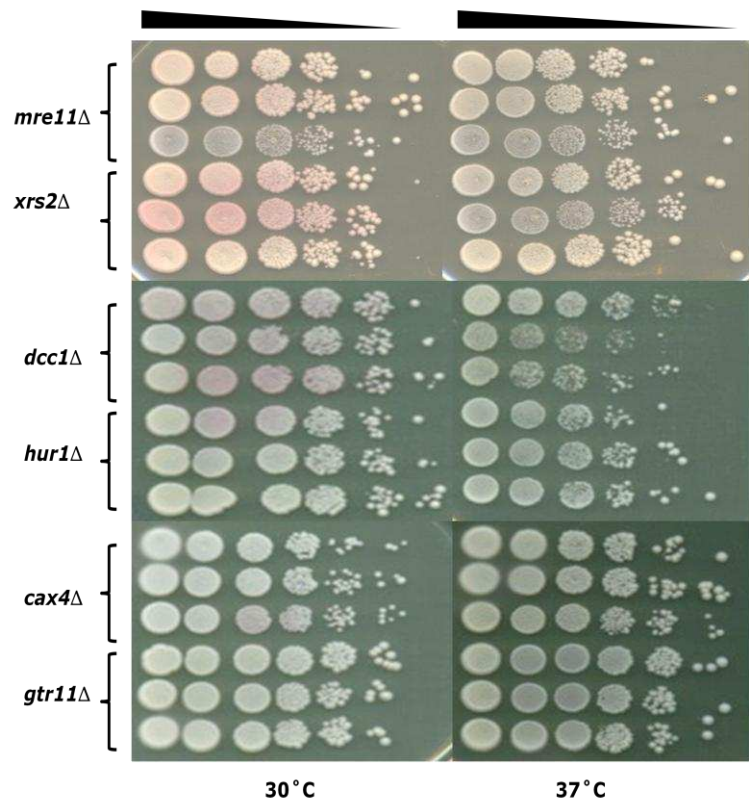
### **6.3. Temperature sensitivity**

Some of the survivor strains that had candidate genes deleted displayed a slight different in cell viability compared to wild-type at both 30°C and 37°C. YGL2.15*rad50*Δ strains propagated for ~175 generations displayed a minor affect in their viability at 30°C and 37°C. Cells displayed ten-fold less viability than wild-type (Fig.6.1). YGL2.15*sgs1*Δ strains displayed similar result as *rad50*Δ strains (see Fig.6.1). This result also found for *mre11*, *xrs2*, *dcc1* and *hur1*. However, deletion of some genes did not affect cell viability. For instance, YGL2.15*nej1*Δ and YGL2.15*ogg1*Δ strains exhibited the same result as wild-type at 30°C and 37°C (Fig6.1). Moreover, YGL2.15 strains that had *rpb4*, *upf1*, *gtr1*, *cax4*, *met7*, *srb2* deleted displayed same result as wild-type, also YGL9.12*rpb9*Δ strains displayed similar result as wild-type. YGL2.15*rpp1a*Δ strains displayed similar growth as wild-type, but they were incubated for three days instead of two



**Figure 6.1. Temperature sensitivity of wild-type, type II survivor strain, type II strains with candidate genes deleted**

Wild type, type II and type II at ~150 generations after the candidate gene have been deleted. Cells were re-suspended in 45 $\mu$ l of distilled water then they were 10 fold diluted six times. Cells were spotted in YEPD plates and incubated at 30 $^{\circ}$ C and 37 $^{\circ}$ C for two days. Wild-type, type II survivor strain (YGL2.15) and type II survivor strains that had deleted genes at ~150 generations are indicated in the left. Three independent mutants for each gene were analysed.



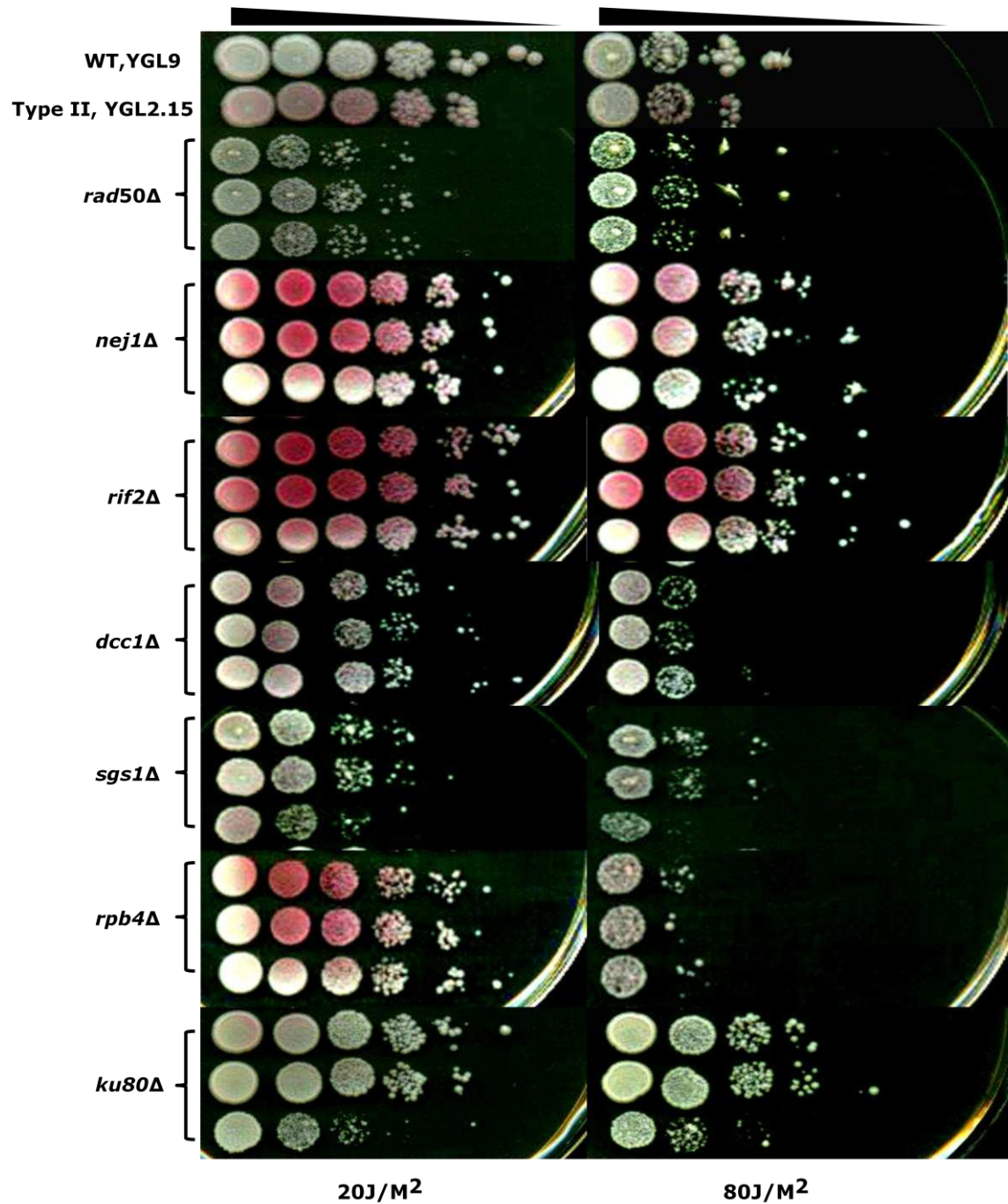
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(Fig6.1). *Rpp1a* $\Delta$  strains were incubated for three days, because of the slow growth of the cells at two days. Notably, YGL2.15*rif1* $\Delta$  and *rif2* $\Delta$  strains displayed better growth than wild-type at 30°C and 37°C (Fig6.1). YGL38*ku80* strains displayed similar result as wild-type strain at 30°C, but they displayed less cell viability for ten-fold at 37°C (Fig6.1).

#### **6.4. UV sensitivity**

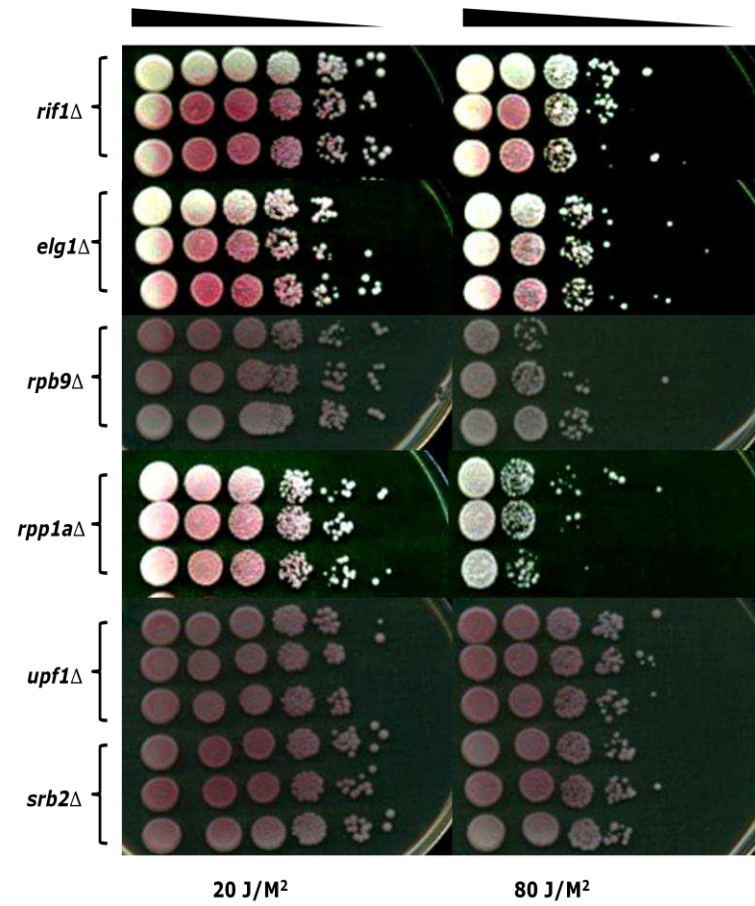
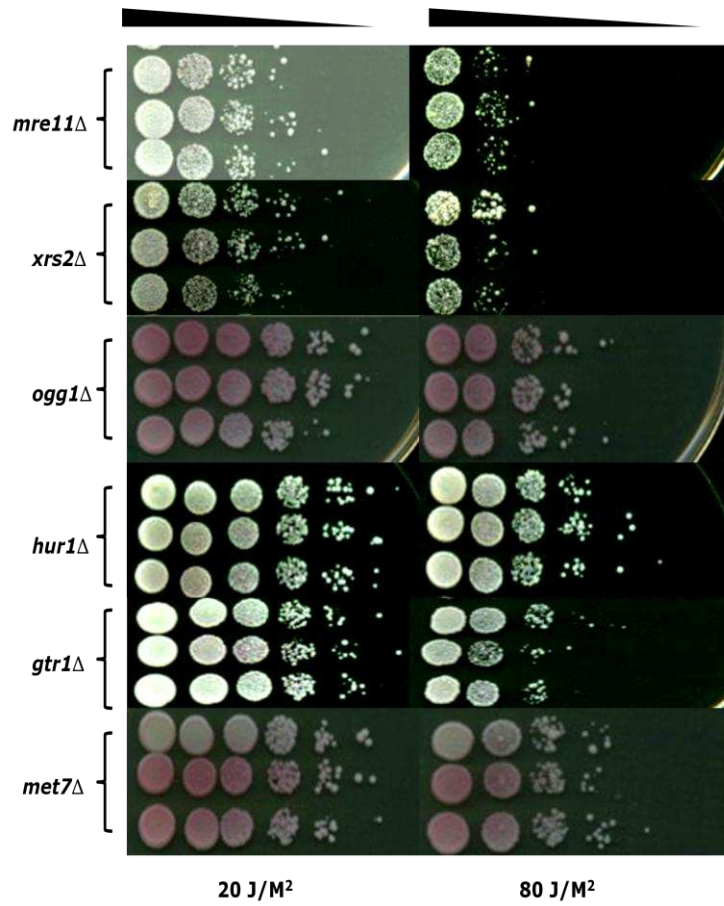
Strains that had candidate genes deleted displayed different sensitivities to UV compared to wild-type. YGL2.15*rad50* $\Delta$  strains displayed sensitivity to 20J/ m<sup>2</sup> and 80J/ m<sup>2</sup> of UV compared to wild-type and type II before the gene has been deleted. *Rad50* $\Delta$  strains (three independent cell lines) displayed ten-fold less cell viability than wild-type (Fig 6.2). Moreover, YGL2.15 type II survivor strains with (*sgs1*, *dcc1*, and *hur1*) deleted displayed similar results as *rad50* $\Delta$  strains. Therefore, they are more sensitive to both doses of UV than the wild-type strain (Fig.9.2). YGL2.5*rpb4* $\Delta$  strains displayed UV sensitivity at 20J/ m<sup>2</sup> similar to wild-type, but they showed more sensitivity than wild-type at 80J/m<sup>2</sup> (Fig.6.2). However, some type II survivor strains with genes individually deleted displayed the same sensitivity as wild-type at both UV doses. YGL2.15*nej1* $\Delta$  survivor strains displayed same sensitivity as wild-type after both UV doses irradiations (Fig 6.2). Type II survivor strains with deletion of *cax4*, *ogg1*, *met7*, *rpb9*, *rpp1a*, and *gtr1* displayed the same sensitivity as wild-type at both UV doses. It is clear that YGL2.15*rif1* $\Delta$  and YGL2.15*rif2* $\Delta$  strains displayed less sensitivity to UV compared to wild-type strain (Fig. 6.2). Two YGL38*ku80* $\Delta$  strains displayed sensitivity to UV that was similar to the wild-type sensitivity, while the third strain appeared more sensitive than wild-type (Fig 6.2).





**Figure 6.2. UV sensitivity of wild-type, type II survivor strain, type II strains with candidate genes deleted**

Wild-type, type II and type II at ~150 generations after the candidate gene have been deleted. Cells were re-suspended in 45 $\mu$ l of distilled water then they were 10 fold diluted for six time. Cells were spotted in YEPD plates, exposed with the indicated UV dosage in the dark room. They were wrapped with a foil and incubated at 30 $^{\circ}$ C for two days. Wild-type, type II survivor strain (YGL2.15) and type II survivor strains that had deleted genes at ~150 generations are indicated in the left. Three independent mutants for each gene were analysed.



See previous page for legend.

## **6.5. Discussion**

Temperature and UV sensitivity of strains that have deleted candidate genes were performed. Experiments were done to reveal how type II survivor strains can be affected by temperature and UV after deletion of the candidate genes. The temperature and UV sensitivity experiments were done at ~ 150 generation after deleting candidate genes in type II survivor strains. The candidate genes were chosen as they are implicated directly or indirectly in telomere length metabolism (Askree et al., 2004). This was discussed in more detail in Chapter 3. It is obvious that short telomeres activate DNA damage response (AS and Greider, 2003). Thus, cells with short telomeres may display slow growth or enter cell cycle arrest. Therefore deletion of any one of the candidate genes may lead to a similar result. Three individual cell lines for each deleted gene were examined for both temperature and UV sensitivity. Strains with deletion of most candidate genes did not display clear length changes in their telomeres as presented in Chapter 3. Consistent with this, strains that have most genes individually deleted did not display temperature sensitivity compared to wild-type and type II survivor. However, strains with some genes deleted displayed UV sensitivity while other strains displayed less UV sensitivity than wild type and type II survivor.

Type II survivor strains with deleted candidate genes displayed a similar cell viability to wild-type at both 30°C and 37°C temperature for most genes. These strains displayed the type II telomere pattern at ~150 generations after deleting the candidate genes without telomere shortening at this time point. Therefore, the absence of each gene did not affect telomere length. The exception was *ku80Δ* strains that displayed amplification of Y' elements

without the terminal sequences of TG repeat. Thus, it is clear that in most cases the strains still have long telomeres. Consequently, proteins that bind telomeres should still be able to interact with telomeres and provide protection. This is consistent with temperature sensitivity and DNA damage checkpoint activation being related to short telomeres (Gravel and Wellinger, 2002).

UV irradiation causes DNA damage. This DNA damage alters the DNA structure. Two thymine (TT) or two cytosine (CC) nucleotides from the same strand can bind to each other (Rastogi et al., 2010). As result of that, checkpoint mechanisms are normally activated to inhibit DNA replication and cells enter cycle arrest. DNA damage that occurs by UV can be repaired by different DNA damage repair mechanisms. Theses mechanisms are photoreactivation, base excision repair (BER), nucleotide excision repair (NER) and mismatch repair (MMR). Additional DNA damage repair mechanisms such as homologous recombination (HR) and non-homologous end joining (NHEJ) can be used (Rastogi et al., 2010).

The absence of some genes in type II survivor strains slightly increased the cells sensitivity to UV. Cells were clearly affected at high dose (80J/m<sup>2</sup>). Some of these genes are *RAD50*, *SGS1*, *DCC1*, and *RPB4* (Fig 6.2). *RAD50* with *MRE11* and *XRS2* are involved in telomere maintenance, HR and NHEJ (Borde, 2007), *SGS1* is essential for genome stability (Watt et al., 1996). Therefore the absences of these genes affect DNA repair mechanisms. In another words cells were not able to recover after UV irradiation compared to wild-type. However, the absence of additional genes such as *NEJ1*, *RIF1* and *KU80* do

not display UV sensitivity compared to wild type. *NEJ1* and *KU80* are involved in NHEJ which is used for DNA repair (Tam et al., 2007, Dudasova et al., 2004). The absence of these genes did not affect NHEJ, therefore DNA damage can be repaired by this mechanism. If the absence of these genes affects NHEJ, the DNA damage can be repaired by different machinery. *Rif1Δ* and *rif2Δ* strains were less sensitive to UV than wild-type. It is known that *RIF1/2* genes involved in telomere protection by the interaction with *RAP1* (Marcand et al., 1997) and they may inhibit HR to occur. Therefore the absence of these genes will allow HR to occur and repair DNA damage.

## Chapter 7: General discussion

The main aim of this study was to test a number of genes to see if they were required for telomere maintenance in type II survivor strains. To address this, several candidate genes (20) were deleted individually in type II survivor strains. Type II survivor strains that have the deleted genes were propagated for ~150 generations. Telomere structure was analysed to determine the survivor type pattern. Some of these candidate genes were required to establish type II survivor strains (Huang et al., 2001, Le et al., 1999, Chen et al., 2001, Johnson et al., 2001). All of these genes affected telomere length in telomerase-positive yeast strains (Askree et al., 2004). The results presented here show that most of these genes are not required for type II survivor telomere maintenance at least at ~150 generations.

The MRX-complex (*RAD50*, *MER11* and *XRS2*) genes are not required for telomere maintenance as type II despite being required for establishment. YGL2.15*rad50* $\Delta$ , YGL2.15*mre11* $\Delta$  and YGL2.15*xrs2* $\Delta$  strains maintain their telomere elongation as type II survivors for at least ~150 generations. At this stage telomere length is not changed as discussed in chapter 3. The cells do not display telomere shortening or lengthening. Type II survivor strains displayed telomere shortening in the absence of these genes after long term propagation (~175 – 550 generations). *Rad50* $\Delta$  strains were propagated even longer and one strain switched to a type I survivor after 1,000 generations as discussed in chapter 4. Strains with these genes deleted do not display temperature sensitivity at 30°C and 37°C compared to wild-type. Strains also do not show clear sensitivity to UV at both doses. All these findings may

indicate the minor role these genes play in type II survivor strain maintenance. Telomere shortening and survival changing can be seen only after long term propagation. YGL9.12*rad50* $\Delta$  and YGL9.17*rad50* $\Delta$  type II strains switched to type I earlier than YGL2.15*rad50* $\Delta$ . This maybe due to short telomeres in YGL9.12 and YGL9.17 type II strains compared to the YGL2.15 type II strain as discussed in chapter 4. The telomere length may have a role in survival changes. The MRX-complex genes are involved in telomere maintenance, DSB repair by HR, non-homologous end joining (NHEJ), and cell cycle response to DNA damage (Taylor et al., 2010, Dinkelmann et al., 2009, Borde, 2007). These genes were required to establish type II survivors (Le et al., 1999, Chen et al., 2001), and their deletion leads to telomere shortening in telomerase-positive strains (Askree et al., 2004). Therefore, it seems that type II survivor strains might utilise a different pathway to maintain their telomeres than that used to establish the same type of survivors. An additional interpretation for this result can be that these genes play a minor role in telomere maintenance in type II survivors. Therefore telomere shortening can be seen only after long term propagation.

The budding yeast RecQ family helicase *SGS1*, was not required to maintain telomeres in type II survivor strains. Hence YGL2.15*sgs1* $\Delta$  displayed the type II telomere pattern at least until  $\sim$ 150 generations. However, type II survivor strains displayed telomere shortening in the absence of *SGS1* at long term propagation ( $\sim$ 500-550 generations). YGL2.15*sgs1* $\Delta$  type II strains displayed slight UV sensitivity compared to wild-type. Thus the absence of this gene seems to impair DNA repair in type II strains. However, this gene was required to establish type II survivor strains (Huang et al., 2001, Cohen and

Sinclair, 2001, Johnson et al., 2001). It seems that *SGS1* has a weak role in type II survivors, but plays an essential role during the senescence stage to generate type II survivors. *SGS1* is essential for maintenance of genome stability and telomere maintenance (Watt et al., 1996, Azam et al., 2006). *SGS1* is suggested to play an important role in telomere recombination during senescence. Thus *tlc1Δ sgs1Δ* mutants enter senescence more rapidly than *tlc1Δ* with active *SGS1p* (Azam et al., 2006). A different role of *SGS1* is inhibition of survival in the absence of telomerase and HR. The *tlc1Δ rad52Δ* double mutation does not generate survivors, while *tlc1Δ rad52Δ sgs1Δ* triple mutation generate survivor strains (Lee et al., 2008). Therefore *SGS1* seems to play a critical role during senescence but not after survival has been established.

*KU80* was required to maintain type II survivors, but it was required to maintain survival state. *YGL38ku80Δ* strains were able to grow for at least 1,000 generations but they displayed type I-like telomere patterns. Strains amplify Y' elements but no terminal TG repeats can be seen. Chromosomes of these strains were able to enter CHEF gels, therefore they were considered as a new type of survivors as type I chromosomes cannot enter the gels. *Ku80Δ* strains did not display temperature sensitivity at either 30°C or 37°C compared to wild-type and type II survivors. Therefore telomeres appear long enough to protect chromosome ends in the absence of *KU80*. However, *ku80Δ* telomerase-positive strains are sensitive to temperature (Boulton and Jackson, 1996). Other proteins that are involved in telomere protection may provide enough protection in the absence of *KU80*. Regarding UV sensitivity, two *Ku80Δ* strains displayed similar sensitivity at both doses as wild-type and type



II survivors, while the third strain was more sensitive than wild-type and type II survivors. This strain maybe has shorter telomere than the other two. It is well known that *KU80* is involved in HR and NHEJ mechanisms, and has an important role in DNA damage repair and telomere maintenance (Dudasova et al., 2004, Gullo et al., 2006). KU proteins also provide telomere protection against nucleolytic degradation and end to end fusion (Polotnianka et al., 1998, Hsu et al., 2000, Fisher and Zakian, 2005). Moreover the absence of this gene leads to telomere shortening in telomerase positive strains (Askree et al., 2004). The result here does not seem to be consistent with *KU* functions, thus there might be other proteins that provide telomere protection in the absence of *KU*.

The absence of *RPB9* in two type II survivor strains leads to death immediately after tetrad dissection. The third type II survivor strain switched to type I ~250 generations after deleting this gene. Thus it appears essential for type II survivor maintenance. This maybe due to its transcription functions (Walmacq et al., 2009). It may affect the transcription of some genes that are directly involved in telomere biology. The absence of this gene causes telomere shortening in a telomerase-positive strain (Askree et al., 2004). The absence of *RPB9* in type II survivor strains did not display temperature or UV sensitivity. Thus it is clearly not affecting the DNA damage repair machinery.

*RIF1* and *RIF2* were not required for type II survivor telomere maintenance at ~150 generations. This result was expected, since these genes were thought to inhibit HR (Marcand et al., 1997), which is used to replicate telomeres in survivors. After long term propagation (~500 generations), strains displayed

telomere shortening. Rif1p and Rif2p interact with Rap1p providing telomere protection. Therefore the absences of these genes may allow some exonucleases to access the telomeres reducing their length in the long term propagation. YGL2.15*rif1* $\Delta$  and YGL2.15*rif2* $\Delta$  strains did not display temperature sensitivity at  $\sim$ 150 generations. Strains also did not display UV sensitivity at  $\sim$ 150 generations. Thus the absence of these genes may facilitate some DNA damage repair machinery such as HR.

The rest of the candidate genes discussed in Chapter 3 and 4, are not required for type II telomere maintenance at least until  $\sim$ 150 generations. Type II survivor strains displayed telomere shortening in the long term ( $\sim$ 550 generations) in the absence of these genes. These strains do not display temperature sensitivity compared to wild-type and type II survivors. This indicates that strains still have long telomeres which keep telomere protection at  $\sim$ 150 generations.

One exception is that *Rpb4* $\Delta$  type II strains displayed UV sensitivity compared to wild-type and type II survivor strains. Therefore this gene seems to be affecting the DNA damage repair machinery. This is consistent with the involvement of *RPB4* in a DNA damage repair pathway that is called transcription-coupled repair (TCR) (Li and Smerdon, 2002).

It is clear to note that the deletion of genes did not affect telomere length at  $\sim$ 150 generations. However, type II strains with deleted genes decreased their telomere length in the longer term ( $\sim$ 250 generations). Some of these strains switched to type I. *Rad50* $\Delta$  type II survivor strains changed to type I

at different points during propagation course. The YGL2.15*rad50*Δ strain changed to type I after ~1,000 generations. Whereas, YGL9.12*rad50*Δ and YGL9.17*rad50*Δ type II survivor strains changed to type I pattern soon after ~500 generations, as discussed in Chapter 5. It is apparent that an YGL2.15 type II survivor strain has longer telomeres compared to YGL9.12 and YGL9.17 type II strains. Therefore, YGL9.12*rad50*Δ and YGL9.17*rad50*Δ type II strains switched to type I earlier than YGL2.15*rad50*Δ type II, this might be due to telomere length differences. Telomere length appears to be linked to survival type changing. Two independent type II survivors with *EST2* deleted displayed a continuous type II telomere pattern for many, (~550) generations (Fig. 5.1). Consequently, telomere shortening after long term propagation in type II survivor strains that have candidate genes additionally deleted, as presented in Chapter 5, is a result of their absence. On the other hand, some type II survivor strains with only *EST2* deleted switched to type I survivors (K. Jarvis, PhD thesis, 2010). Therefore some type II strains maybe can change to type I with or without the candidate genes. However, it is not common behaviour for yeast to change from type II to type I survivors (Teng and Zakian, 1999). Some of the candidate genes such as *RAD50*, *MRE11*, *XRS2*, and *SGS1* are required to generate type II survivors (Chen et al., 2001, Le et al., 1999, Huang et al., 2001, Johnson et al., 2001). *KU80* was required for some strains to establish type II survivors (Liti and Louis, unpublished data). According to the result of this study and previous studies different pathways can exist to maintain telomeres of type II survivors in the absence of candidate genes. One suggestion can be that type II survivor strains use a pathway which differs from the pathway that was needed to establish this type of survivors. This idea is supported by the fact that *tlc1*Δ *rad50*Δ or *tlc1*Δ

*mre11*Δ strains were able to generate type I survivors only ~100 generations after germination (Le et al., 1999). *rad50*Δ type II survivor strains displayed type II telomere pattern at ~150 generations and then started shortening after ~250 generations. The other suggestion is that the candidate genes still have a role in type II survivor maintenance which is the same or similar to their role in generating the survivor state. *RAD52* is required for telomere maintenance of type II survivors, since *rad52*Δ type II strains were not able to grow more than ~20-25 generations. Therefore, it is clear that *RAD52* is essential to generate and maintain type II survivors, but type II establishment and maintenance maybe use different pathways.

Finally, it is clear that most of the candidate genes were not required to maintain type II survivors at least at ~ 150 generations after deleting these genes. The exception was *KU80* and *RPB9* as discussed above. *Ku80*Δ survivor strains displayed a new type of survivors starting from ~150 - 1,000 generations. This type of survivor is different from type I and type II survivors, but it is similar to type I survivors. *RPB9* was required to maintain two independent type II survivor strains and the third type II strain was changed to type I after long term (~250 generations). Thus it seems to be playing an important role in telomere maintenance of survivor strains. This gene can be essential to generate survivors.

Some human cancer cells maintain their telomeres utilizing a homologous recombination pathway that is termed alternative lengthening of telomeres (ALT) (Bryan et al., 1997, Dunham et al., 2000). Cells maintain their telomeres amplifying the TG repeat (Bryan et al., 1995), they are analogous

to the *S. cerevisiae* type II survivors. It is obvious that most of the candidate genes in this study affect telomere length (telomere shortening) in type II survivor strains after long term propagation. Therefore it might be worth looking at double mutations for some of these genes that involved directly or indirectly in HR. If these are required for type II survivors, then targeting these genes may be a possibility for ALT cancer treatment. *KU80* is clearly affecting type II survival maintenance, since strains changed to a type I-like pattern. Thus, this gene may be required for the maintenance of these human cancer cells.

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