

# **Single stranded DNA re-synthesis at uncapped telomeres requires replication polymerases**

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# Abstract

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Telomeres are specialised DNA-protein structures capping or protecting the chromosome ends from shortening, degradation and fusions. Telomere uncapping occurs when some of the proteins associated with telomeres lose their integrity. For example in yeast a point mutation in the gene encoding the telomere binding protein Cdc13 called *cdc13-1*, leads to conditional telomere uncapping at temperatures above 26° C.

In this thesis I have utilised the *cdc13-1* model system to study repair after telomere uncapping. De-protection of the telomere triggers resection of the AC rich strand in 5' to 3' direction and formation of single stranded DNA (ssDNA). Checkpoint proteins are readily recruited to the damage and halt the cell cycle. However no ssDNA re-synthesis has been observed in *cdc13-1* cells with uncapped telomeres. Here I will show that the ssDNA damage in *cdc13-1* cells recruits polymerase  $\alpha$ ,  $\epsilon$  and  $\delta$  and the clamp PCNA but in normal circumstances efficient repair is not observed. Only when telomeres are recapped the ssDNA could be re-synthesised and this depended on the polymerase  $\delta$  subunit Pol32 but did not require the non-essential subunits Dpb3 and Dpb4 from polymerase  $\epsilon$ .

Interestingly, ssDNA re-synthesis at uncapped telomeres could be stimulated through mild osmotic pressure and required both polymerase  $\delta$  and  $\epsilon$ . Furthermore mild osmotic pressure could also rescue cells damaged with methyl methanesulfonate but not with UV light or hydroxyurea.

My data suggests that single stranded DNA re-synthesis may be specifically inhibited or compete with resection when telomeres are uncapped and that osmotic pressure stimulated re-synthesis by regulating polymerases  $\alpha$ ,  $\epsilon$  and/or  $\delta$ .

I would like to dedicate this thesis to the memory of my grandfather, Ivan Genchev Ivanov, who ignited my love for science. He was an exceptionally kind and loving person and is greatly missed by family and friends.

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## Abbreviations:

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ALT	alternative lengthening of telomeres
ATM	ataxia telangiectasia mutated
ATR	ataxia telangiectasia and Rad3-related protein
BrdU	5-bromo-2'-deoxyuridine
BrdU-IP	5-bromo-2'-deoxyuridine immunoprecipitation
BrdUMP	5-bromo-2'-deoxyuridine monophosphate
CDC	cell division cycle replication protein
ChIP	chromatin immunoprecipitation
CST	Cdc13-1/Sten1/Ten1 or Ctc1/Sten1/Ten1
DAPI	4',6-diamidino-2-phenylindole
DBD	DNA binding domain
DIG	digoxigenin
D-MEM	Dulbecco's modified eagle medium
DNA	deoxyribonucleic acid
DPB	DNA polymerase B
DSB	double strand break
dsDNA	double stranded DNA
dTMP	deoxythymidine monophosphate
dUMP	deoxyuridine monophosphate
EDTA	ethylenediaminetetraacetic acid
EST	ever short telomeres
EXO1	exonuclease 1
FEAR	cdc-fourteen early anaphase release pathway
G418	geneticin
HML	hidden mating type cassette - left

HMR	hidden mating type cassette – right
HOG	high osmolarity glycerol response
HR	homologous recombination
HU	hydroxyurea
IP	inositol polyphosphate or immunoprecipitation
MAT	mating type locus
MEN	mitotic exit network
MMS	methyl methanesulfonate
MRX	Mre11, Rad50, Xrs2
MSN	multicopy suppressor of SNF1 mutation
NAD <sup>+</sup>	nicotinamide adenine dinucleotide
NEF	nucleotide excision factor
NER	nucleotide excision repair
NHEJ	non-homologous end joining
OB fold	oligonucleotide/oligosaccharide binding fold
PCNA	proliferating cell nuclear antigen
PCR	polymerase chain reaction
PMSF	phenylmethylsulfonyl fluoride
POL	polymerase
POT1	protection of telomeres 1
PRR	post replication repair
QAOS	quantitative amplification of single stranded DNA
RAD	radiation sensitive
RAP1	repressor activator protein
RD	recruitment domain
rDNA	ribosomal DNA
RIF	Rap1 interacting factor
RNA	ribonucleic acid
RPA	replication protein A

SD	standard deviation
SIR	silence information regulator
ssDNA	single stranded DNA
STN1	suppressor of cdc thirteen 1
TBS	tris buffer saline
TBST	tris buffer saline, tween 20
TCA	trichloroacetic acid
TEN1	telomeric pathways with Stn1
TERRA	telomeric repeat-containing RNA
TLC1	telomerase component 1
TPE	telomere positioning effect
TRF	telomere repeat binding factor
UV	ultra violet
YEP	yeast extract, peptone
YEPD	yeast extract, peptone, dextrose
YKU	yeast KU

# 1 Introduction

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In 1938 Herman Muller first proposed the existence of a structure at the end of chromosomes that he referred to as the “terminal gene” or telomere from the Greek words for “end” (telos) and “part” (meros). He was working on deletions and inversions following irradiations in *Drosophila melanogaster* but he never found such mutations at the very end of chromosomes. Muller suggested that the “terminal gene” must seal the chromosome end and prevent it from alterations. Later on in 1978 Elisabeth Blackburn identified the repetitive telomeric sequence required to maintain linear chromosomes in the protozoa *Tetrahymena* and it soon became apparent that the telomere was conserved in many organisms including human (Greider, 1998).

## 1.1 Telomere function

Telomeres are specialised protein bound DNA repeats situated at eukaryotic chromosome ends that function in chromosome end protection. These structures carry out several essential functions that regulate cell fate. First of all telomeres “hide” the chromosome terminus from the DNA damage response. This became apparent when loss of telomere bound proteins led to checkpoint activation followed by repair through recombination or fusion leading to severe genomic instability (Deng & Chang, 2007). Thus it seems like unprotected telomeres are perceived by the cells as double strand breaks.

The other crucial function of telomeres is to ensure telomere length maintenance. Normal telomere length is crucial for proliferation capacity and prevents chromosome shortening. Short telomeres either attract a special polymerase, called telomerase, to perform telomere elongation or trigger cell cycle arrest, preventing potential loss of genetic information. Severe telomere attrition triggers senescence – a process leading to permanent irreversible growth arrest. This arrest happens after a defined number of cell divisions and is also termed “the Hayflick limit” (Rodier & Campisi, 2011). Therefore telomeres also determine the cellular life span.

Another not well understood function of telomeres is chromosome end anchoring at the nuclear periphery in both mammalian and yeast cells (Hediger et al, 2002; Molenaar et al, 2003). Interestingly it has been shown that short telomeres and irreparable or slow repaired double strand breaks can be shuttled specifically to the nuclear pores, where recombination repair takes place (Lisby et al, 2010).

## 1.2 Telomere organization

Telomeric DNA is composed of tandem TG/AC repeats that can span from 300bp in the yeast *Saccharomyces cerevisiae* to 10kb in human and up to 50kb in some rodents (Gatbonton et al, 2006; Kipling & Cooke, 1990; Wise et al, 2009). The very ends of telomeres have a single stranded stretch called the 3' overhang. Because the overhang is rich in TG repeats, it can also be referred to as G tail or TG strand, while the opposite strand is called 5' strand or AC strand. In mammalian cells the telomeres loop back and the 3' overhang invades the double strand providing a more closed conformation of the telomere. This looping is mediated by the telomere binding protein TRF2 and could be involved in telomere protection and telomere length regulation (Greider, 1999). Yeast telomeres also loop and this is mediated by Sir proteins but it is not clear whether the 3' overhang also invades the DNA double strand (de Bruin et al, 2001).

Telomeres can form another structure, called G quadruplex, when four guanines form a square arrangement, stabilised by ligands such as  $K^+$ . These structures are more stable than dsDNA and their formation at telomeres can inhibit telomerase – the specialised polymerase that extends telomeres using its own RNA moiety as template (Huppert, 2008). G-quadruplexes are also present at promoter regions where they participate in gene transcription regulation (Balasubramanian et al, 2011).

Another set of repetitive regions termed subtelomers is situated adjacent to telomeres. There are two types of subtelomeres: X and Y' elements. The Y' subtelomere is not present on all chromosomes and can be repeated up to four times, while all chromosomes contain the more structurally diverse X subtelomere. Recent studies show that Y' subtelomeres exhibit high nucleosome density and are transcriptionally active, while X subtelomeres have virtually no histones but were bound by two telomere binding proteins Rap1 and Sir3. These proteins confer transcription repression at X



elements.(Zhu & Gustafsson, 2009) The human subtelomere organisation is very diverse and can span from 1kb to 300kb (Riethman et al, 2005).

Recent studies show that telomeres are associated with non-coding telomeric repeat-containing RNAs (TERRA) that may function in telomerase regulation. TERRA is more abundant at long telomeres and forced telomere transcription leads to telomere shortening (Luke et al, 2008; Schoeftner & Blasco, 2008). Transcription of this telomere associated RNA starts at the subtelomeres and is present at all chromosomes.

## **1.3 Telomere binding proteins**

Telomere binding proteins confer most of the functions of the telomere. Although there are subtle differences between the proteins associated with telomeric repeats from different organisms, most of them are functionally conserved. Telomeres contain both single stranded and double stranded binding proteins as well as proteins that do not bind DNA directly (Figure1).

### **1.3.1 Cdc13, Stn1 and Ten1 or the CST complex**

Cdc13, Stn1 and Ten1 are essential telomere binding proteins that associate specifically to single stranded telomeric repeats via Cdc13 (Figure 1A). (Gao et al, 2007; Hughes et al, 2000). The main functions of the CST complex are to protect the telomere from degradation and to ensure telomere replication by recruitment of telomerase and polymerase  $\alpha$ .

Based on structural similarities it has been proposed that Cdc13, together with its essential binding partners Stn1 and Ten1 form a RPA-like complex, termed CST complex (Gao et al, 2007; Paschini et al, 2010). However, while RPA has low specificity for DNA and aids initiation of the DNA damage response after it binds ssDNA; CST shows specificity to single stranded TG repeats and is involved in telomere protection and telomerase recruitment. It is believed that the functional differences between RPA and CST are due to the different organisation of the OB-folds in Cdc13 and the additional domains in Stn1, compared to their RPA homologues Rpa1 and Rpa2 respectively.

Although binding of Cdc13 to DNA is essential for cell viability, it is not sufficient for function, because a truncated Cdc13 that contains only the DNA binding domain (DBD) fails to grow above 27°C (Hughes et al, 2000; Wang et al, 2000). Interestingly when Stn1 is fused to DBD<sub>cdc13</sub>, viability is restored but the strain enters senescence. Further fusion of Est1 to the Stn1-DBD<sub>cdc13</sub> rescued the senescence phenotype (Pennock et al, 2001). This shows that Stn1 is sufficient for telomere protection and Est1 recruitment to telomeres is sufficient for telomere maintenance. Furthermore overexpression of Stn1 and Ten1 could completely bypass the requirement for Cdc13, perhaps due to better recruitment of Stn1 and Ten1 to telomeres (Petreaca et al, 2006).

Cdc13 contains four OB-folds and a recruitment domain (RD). The OB3-fold exhibits strong, TG specific ssDNA binding, while the telomerase interacting OB1-fold has lower DNA binding affinity. Disruption of OB1 DNA binding but not OB3-fold led to telomere elongation, suggesting that OB1 association with DNA is required for telomerase dependent telomere maintenance (Mitchell et al, 2010). Furthermore, *in vitro* experiments suggest that Cdc13 forms stable dimers in liquid via its N terminal. Cdc13 dimerization seemed to be important for recruitment of telomerase to telomeres *in vivo*, because mutants that could not form dimers had longer telomeres (Mitchell et al, 2010). Interestingly point mutations in Ten1 and Stn1, led to telomere elongation, linking the CST complex to both positive and negative regulation of telomere length.(Grandin et al, 2001).

Recent studies also show that Cdc13 is phosphorylated *in vivo* probably through Mec1 and/or Ten1 and Cdk1. It was proposed that such phosphorylation events might also be important for telomerase recruitment through Est1.(Gao et al, 2010; Li et al, 2009; Tseng et al, 2006).

The capping function of the CST complex was confirmed in Ten1, Stn1 and Cdc13 temperature sensitive mutants. These mutants accumulate single stranded DNA at elevated temperatures indicating a telomere capping defect (Booth et al, 2001; Petreaca et al, 2007; Xu et al, 2009). Interestingly while Cdc13 mutants generated ssDNA in 5' to 3' direction starting from the end on the telomere, some *TEN1* and *STN1* point mutants had only internal ssDNA.

Apart from recruiting Stn1, Ten1 and telomerase to telomeres, Cdc13 can also interact *in vivo* with the catalytic subunit of polymerase  $\alpha$  Pol1, the silencing regulators Sir4 and Zds2 and the component of U3 snoRNA Imp4, required for pre 18S rRNA processing. (Hsu et al, 2004; Qi & Zakian, 2000).

Mutations in Pol1 or Cdc13 that disrupt the interaction between these two proteins did not compromise cellular growth but led to elongated telomeres, suggesting that this interaction is only required for telomerase regulation but not for viability (Qi & Zakian, 2000). Stn1 also interacts with Polymerase  $\alpha$  through the B subunit Pol12 (Puglisi et al, 2008). Previously it was shown that addition of TG repeats by telomerase requires polymerase  $\alpha$  and  $\delta$ , suggesting that telomerase activity on the TG strand is coupled to AC strand synthesis and that the CST complex could aid telomere elongation by recruiting any of those enzymes (Diede & Gottschling, 1999).

The mammalian homologue of Cdc13 is also a 3' overhang binding protein, called Pot1 (Figure 1B). Pot1 is recruited to telomeres through its DNA binding domain and through an additional interaction with the mammalian telomere binding protein TPP1 and their interaction is essential for telomerase recruitment (Wang et al, 2007).

Mammalian cells also have a CST complex; however it does not contain POT1 but instead is formed by Ctc1, human Stn1 and human Ten1. Interestingly this complex does not bind with high specificity to TG repeats and was associated with only a fraction of the telomeres (Miyake et al, 2009). CST does not play an essential role at telomeres but similarly to yeast Stn1, loss of human Stn1 also lead to single stranded DNA accumulation at telomeres (Miyake et al, 2009).

### **1.3.2 Rap1 and binding partners**

Rap1 is an essential double stranded DNA binding protein that recruits at least two sets of proteins to the telomere: the Rif complex (Rif1 and Rif2) that control telomere length and the Sir complex (Sir3, Sir4 and Sir2), required to establish silencing of the DNA around the telomere (Figure 1A). The protein interactions of Rap1 are mediated through its C terminal while the N-terminal is required for DNA binding (Feeser & Wolberger, 2008). Interestingly, a C-terminal deletion is viable, showing that Rap1 has other essential functions beside the ones conferred by its well known binding partners. Therefore it seems like the N-terminus may contain the essential function of Rap1. Apart for binding telomeres, Rap1 also acts as a transcription activator or silencer for ribosomal genes and mating type loci *HML* and *HMR* (Kurtz & Shore, 1991; Shore & Nasmyth, 1987). Rap1 controls about 5% of all promoters in yeast, including genes involved in glycolysis (Pina et al, 2003). Perhaps the essential func-

tion of Rap1 lies in transcription control, while at telomeres Rap1 functions only as a scaffold for tethering other proteins.

Human Rap1 can not bind DNA directly, instead it interacts with the telomere binding protein TRF2 and inhibits homologous recombination (HR) between sister telomeres (Figure 1B) (Kabir et al, 2010). Interestingly loss of human Rap1 leads to recombination without triggering any checkpoint response. Human Rap1 also plays a role in transcription regulation similar to yeast (Kabir et al, 2010).

### 1.3.2.1 Rif1 and Rif2

Both Rif1 and Rif2 proteins are non essential and function as negative regulators of telomere length because deleting any of them leads to telomere elongation. Moreover it seems like the Rif complex competes for Rap1 binding with the Sir complex, because telomere silencing was enhanced in cells lacking Rif (Wotton & Shore, 1997).

It has been suggested that telomere length regulation is achieved through a protein counting mechanism, where the amount of bound Rap1, Rif1 and Rif2 along the telomere can determine when an elongation event will occur (Barinaga, 1997; Marcand et al, 1997). Namely when a telomere is short, less Rap1 and therefore also less Rif1 and Rif2 would be present, decreasing the negative telomerase regulation effect conferred by those proteins and telomeres will be extended. Furthermore when the telomere reaches a certain length, correlating to a certain amount of bound Rap1/Rif1/Rif2, telomerase is halted allowing precise telomere length regulation.

Recently our group has also shown that Rif1 is specifically recruited at uncapped telomeres that accumulate ssDNA independently from Rap1, where it inhibits checkpoint binding (personal communication with Yuan Xue). In humans, Rif1 is not associated to healthy telomeres, but is recruited only following telomere damage, suggesting that Rif1 may have lost its telomere capping function in evolution, but kept its role at DNA damage sites (Silverman et al, 2004). In contrast Rif2 has no known mammalian homologue. Nevertheless yeast Rif2 is required to inhibit non-homologous end joining (NHEJ) at telomeres, whereas Rif1 is not (Marcand et al, 2008).

### 1.3.2.2 Sir proteins

The Sir proteins associated to telomeres and subtelomeres are Sir2, Sir3 and Sir4 (Figure 1A). Sir2 is a NAD<sup>+</sup> dependent deacetylase required for deacetylation of histones 3 and 4 (Landry et al, 2000). This reaction leads to recruitment of Sir3 and Sir4 to chromatin. As a result the chromatin is condensed and prohibits transcription. Therefore the role of the Sir complex is to mediate chromatin silencing at certain regions. In yeast these are the mating type loci, rDNA repeats and regions close to the telomere. The ability of the Sir proteins to repress the transcription of genes near the telomeres is referred to as telomere positioning effect (TPE) (Sandell & Zakian, 1992).

Deregulation of the Sir complex leads to increased recombination, chromosome instability and decrease in the life span of yeast (Gottlieb & Esposito, 1989; Kaeberlein et al, 1999; Palladino et al, 1993). The Sir proteins or sirtuins, as they are known in higher eukaryotes, are structurally and functionally conserved throughout evolution (McGuinness et al, 2011).

### 1.3.3 yKu70/yKu80 heterodimer

The single stranded-double stranded junction at telomeres is bound by the heterodimer yKu70/yKu80, which is required for telomerase recruitment, telomere silencing, anchoring and protection from degradation (Figure 1A).

yKu deleted cells have short telomeres but long 3' overhangs can be observed throughout the cell cycle. In contrast wild type cells have detectable overhangs only during S phase (Gravel et al, 1998). Furthermore an interaction between the RNA component of telomerase and yKu has been confirmed and distortion of this interaction was responsible for telomere shortening (Stellwagen et al, 2003). All together these findings infer that yKu contributes to telomere maintenance by mediating telomerase recruitment similar to Cdc13.

yKu also inhibits Exo1 dependent shortening of the telomere because *yku80Δ exo1Δ* mutants have longer telomeres than *yku80Δ* alone and a substantial reduction in overhang length was observed in the absence of *EXO1* (Bertuch & Lundblad, 2004). The telomere silencing function of yKu is mediated through yKu dependent loading of the

silencing protein Sir4 onto Rap1. This is required presumably because the Rif and Sir complexes compete for Rap1 binding sites (Mishra & Shore, 1999). Furthermore yKu can also mediate telomere anchoring independent of Sir, although the functional consequences of this anchoring are not well understood (Hediger et al, 2002).

Apart from its function at the telomere the yKu heterodimer is also involved in double strand break (DSB) repair, where it binds the broken end and mediates NHEJ (Bertuch & Lundblad, 2003). Furthermore the functions of yKu, or Ku as referred in mammalian systems, seem to be conserved in different species (de Lange, 2002). Mammalian Ku binds directly to TG repeats or via interaction with the dsDNA binding proteins TRF1 and TRF2 (Figure 1B) (Bianchi & de Lange, 1999; Hsu et al, 2000; Song et al, 2000).



## 1.4 Telomerase and telomere replication

During linear chromosome replication a RNA primer is required to initiate synthesis at both strands. The RNA primer is then removed from the very end of the chromosomes, leading to a short gap or 3' overhang that cannot be filled by the replication machinery. This problem is known as “the end replication problem” and as a consequence chromosomes shorten after each round of replication (Levy et al, 1992). Cells have developed strategies for coping with this issue by evolving a specialised chromosome end or telomere.

As mentioned before telomeres aid chromosome stability in two ways. First, they ensure that no important genetic information can be lost by providing a buffer of non coding repeats that can be lost during each cell division without affecting the rest of the genome. Second, when the telomeres reach a critically short length they can signal the cells to either halt the cell cycle or initiate a round of telomere elongation, ensuring continuous genomic stability. Telomere elongation is carried out by a reverse transcriptase, called telomerase, which utilises a RNA template to add TG repeats to the 3' overhang. This way telomeres can be extended and maintain constant length. Yeast telomerase is composed of four subunits coded by the following genes: *TLC1*, *EST1*, *EST2* and *EST3* (Lendvay et al, 1996; Lundblad & Szostak, 1989).

*EST2* encodes the catalytic subunit of yeast telomerase, known as TERT (telomerase reverse transcriptase) in mammalian cells and together with its RNA component TLC1 is required for *in vitro* enzymatic activity of telomerase (Counter et al, 1997). Est1 aids telomerase recruitment to telomeres by providing a physical link between Cdc13 and telomerase (Evans & Lundblad, 2002; Qi & Zakian, 2000; Tuzon et al, 2011). Est3 is a GTPase essential for *in vivo* telomerase activity but its exact function remains elusive (Shubernetskaya et al, 2011).

Interestingly telomerase only extends short telomeres during late S phase (Bianchi & Shore, 2007). This is probably due to Rif1 and Rif2 dependent regulation of telomere accessibility or frequency of telomerase association to telomere (Teixeira et al, 2004). When the telomere is long Rif1 and Rif2 confer a non-extendable telomere structure and telomere replication starts in late S phase but Est1 and Est2 are not recruited, although Cdc13 and the yKu complex are present (Bianchi & Shore, 2007).



The current model for telomere replication suggests that when the telomere is short, Tel1 is associated to it throughout the cell cycle, presumably “marking” it for elongation. Firing of the origin of replication closest to the telomere happens earlier in S phase, possibly providing more time for telomerase action (Bianchi & Shore, 2007). Then the 3' overhang is resected by MRX (Mre11, Rad50, Xrs2) which may promote increased Cdc13 association to the telomere end (Tsukamoto et al, 2001). Telomerase is recruited through an interaction with Cdc13 and the yKu complex. Furthermore Tel1 might facilitate telomerase recruitment by phosphorylating Cdc13 (Tseng et al, 2006). This leads to addition of TG repeats to the 3' overhang. The CST complex recruits Polymerase  $\alpha$ , triggering dissociation of telomerase and synthesis of the RNA primer for lagging AC strand synthesis (Grossi et al, 2004; Puglisi et al, 2008; Qi & Zakian, 2000).

Interestingly *in vivo* telomerase activity depends on DNA polymerase  $\alpha$ /primase and DNA polymerase  $\delta$  and no repeats can be added to telomeres in the absence of these enzymes, suggesting that lagging strand synthesis is coupled to telomere extension and that it requires at least DNA polymerase  $\alpha$ /primase and  $\delta$  (Diede & Gottschling, 1999).

#### **1.4.1 Type I and type II survivors**

In the absence of telomerase (when either *TLC1*, *EST1*, *EST2* or *EST3* are deleted) yeast cells exhibit a senescence phenotype – decline in proliferation leading to cell cycle arrest, increased size and telomere shortening. However rarely some cells manage to escape the cell cycle arrest and continue to proliferate. These cells are called survivors and utilise homologous recombination (HR) to amplify telomeres or subtelomeres, thus providing telomere capping and preventing chromosome degradation (Lundblad & Blackburn, 1993). All survivors require Rad52 but depending on the amplified region they can be classified as type I or type II survivors. In type I survivors the Y' subtelomere is amplified, while the telomere remains short. These survivors proliferate slowly and require the following HR genes for survival: *RAD51*, *RAD54*, *RAD55* and *RAD57* (Chen et al, 2001; Teng & Zakian, 1999). In contrast type II survivors amplify the G-rich telomeric repeats, acquiring a very long heterogeneous telomere. These cells grow as fast as wild type and require the MRX complex along with *RAD59*, *SRS2*, *SGS1* and *TID1* for survival. Type II survivors are *RAD51* inde-

pendent and constitute only 10% of survivors in yeast (Chen et al, 2001; Teng & Zakian, 1999).

Human stem cells express telomerase and can proliferate indefinitely. However telomerase is down regulated in somatic cells leading to cellular senescence after a limited amount of cell divisions (Rodier & Campisi, 2011). In 90% of human cancer telomerase is upregulated, allowing proliferation of the malignant cells (Shay & Bacchetti, 1997). Interestingly, 10% of human cancers maintain their telomeres through a telomerase-independent mechanism called alternative lengthening of telomeres (ALT). Similar to yeast survivors in these cancers telomeres are maintained by homologous recombination (Cesare & Reddel, 2010).

## **1.5 Telomere uncapping**

Telomere uncapping happens when telomeres lose their ability to protect the chromosome end either due to telomere shortening or loss of telomere binding proteins. This leads to either NHEJ dependent telomere fusions or to generation of ssDNA due to nucleolytic degradation of the AC strand. Telomere uncapping has been used to study the function of the telomeres and provides insights into telomere homeostasis as a whole unit as well as the specific functions of telomere binding proteins

Some hallmarks of uncapped telomeres can also be seen in cancers. For example telomere fusions are known to be responsible for chromosomal loss and large deletions in a number of different tumours (Lin et al, 2010; Sawyer et al, 1994; Sawyer et al, 2003). Decreased levels of telomere protecting proteins such as TRF1, TRF2 and POT1 have been found in lung cancer (Lin et al, 2005). Hence, telomere dysfunction could be one of the events leading to carcinogenesis.

Two approaches can be used to achieve telomere uncapping in yeast – deletion of telomerase components or inactivation of a telomere binding protein such as Cdc13.

### **1.5.1 The *cdc13-1* model system**

One of the well studied telomere uncapping model systems in yeast is the *cdc13-1* mutant. In this system a P371S point mutation in Cdc13 triggers 5' to 3' resection of

the telomere, checkpoint activation and cell cycle arrest in G2/M phase (Garvik et al, 1995). More importantly the *cdc13-1* mutation is temperature sensitive, providing an excellent opportunity for studying telomere uncapping under controlled experimental conditions. When *cdc13-1* cells are grown below 26° C no telomere capping phenotype is observed. These temperatures are called permissive because the cells proliferate as well as wild type. Above 26° C the mutants fail to grow, therefore such temperatures are non-permissive or restrictive for *cdc13-1*. This way conditional telomere uncapping could be achieved depending on the temperature at which the cells are grown.

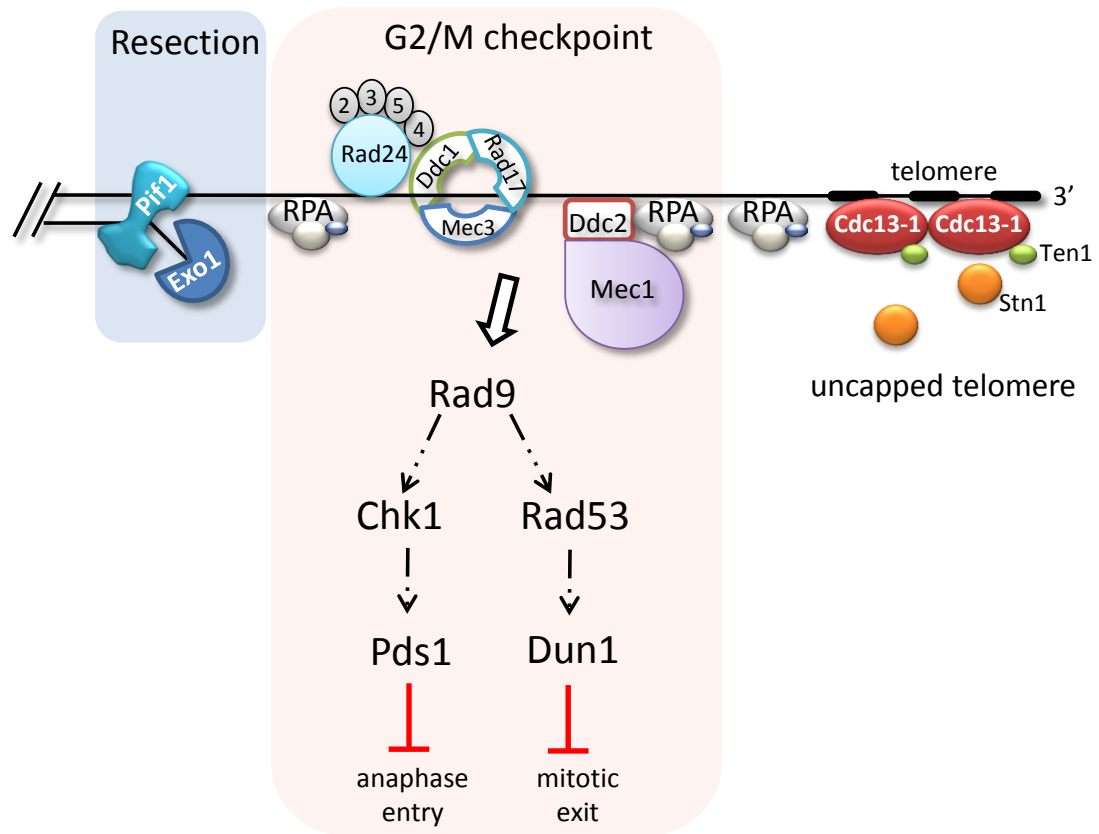
When *cdc13-1* cells are grown at restrictive temperatures, telomere capping is compromised leading to an exonucleolytic attack on the chromosome ends (Figure 2). Degradation or resection happens in 5' to 3' direction on the AC rich strand and is mainly dependent on Exo1 because almost no ssDNA was generated in *cdc13-1 exo1Δ* cells (Booth et al, 2001; Zubko et al, 2004). However there are other yet unidentified exonucleases contributing to resection (Zubko et al, 2004). During telomere replication and DSB repair the MRX complex (Mre11, Rad50, Xrs2) is responsible for 5' to 3' resection, while during telomere uncapping in *cdc13-1*, MRX confers a protective function at the telomere by inhibiting generation of ssDNA (Foster et al, 2006; Tsukamoto et al, 2001). AC strand resection is not limited to telomeres but continues in subtelomeres and single gene loci up to 14,500bp from the chromosome end at 36° C (Zubko et al, 2004). Recent studies show that the resection in *cdc13-1* cells is controlled by the helicase activity of Pif1 because *pif1Δ exo1Δ cdc13-1* and *pif1Δ cdc13-1* mutants accumulate less ssDNA and have reduced temperature sensitivity (Dewar & Lydall, 2010).

Interestingly ssDNA accumulation at uncapped telomeres can only occur, at the G2/M phase after completion of DNA replication and requires cyclin dependent kinase Cdk1 activity (Vodenicharov & Wellinger, 2006).

The single stranded DNA produced at uncapped telomeres triggers a strong checkpoint response (Garvik et al, 1995). This happens when RPA coated ssDNA is recognised by two checkpoint sensor branches. One is composed of the kinase Mec1 (yeast homologues of human ATR) and the other involves a PCNA-like clamp composed of Rad17, Mec3 and Ddc1 (Figure 2). This clamp is homologous to the mammalian 9-1-1 complex (Rad9, Rad1, Hus1). The yeast ATM kinase Tel1 plays very little role in Cdc13 dependent telomere uncapping because *cdc13-1 tell1Δ* cells exhibit normal

checkpoint activation (Morin et al, 2008). The checkpoint sensors are loaded onto the ssDNA independent of each other. Mec1 is recruited through the RPA binding protein Ddc2, while the clamp is assembled at the single stranded double stranded junction by a clamp loader composed of Rad24, Rfc2, Rfc3, Rfc4, and Rfc5 or briefly Rad24 Rfc complex (Kondo et al, 2001; Majka et al, 2006). After the sensors have recognised the ssDNA damage, a phosphorylation cascade is triggered and the signal is transduced downstream to the mediator Rad9 (Figure 2) (Vialard et al, 1998). Deletion of proteins from the two sensing branches leads to partial downstream kinase phosphorylation, showing that both are required for full checkpoint activity (Jia et al, 2004; Morin et al, 2008). Two pathways downstream of Rad9 regulate G2/M cell cycle arrest in *cdc13-1* cells (Figure 2). These are Chk1/Pds1 and Rad53/Dun1. Chk1 phosphorylates the anaphase inhibitor Pds1, which leads to Pds1 stabilisation and blockage of the cdc-fourteen early anaphase release pathway (FEAR), responsible for anaphase entry (Blankley & Lydall, 2004; Liang & Wang, 2007). On the other hand Rad53 inhibits the mitotic exit network (MEN) as shown by complete arrest in *cdc13-1 rad53Δ*, when the cells were MEN but not FEAR deficient (Liang & Wang, 2007). Thus the checkpoint provides two blocks, one in anaphase entry and one in mitotic exit (Figure 2).

Apart from triggering cell cycle arrest, the *cdc13-1* checkpoint is also important for ssDNA modulation. The Rad24 Rfc clamp loader and the yeast 9-1-1 clamp stimulate resection at uncapped telomeres while Rad9 inhibits exonucleases. This became apparent from ssDNA measurements showing that *cdc13-1 rad9Δ* cells accumulate 30kb ssDNA, while deletion of clamp loader or clamp subunits in *cdc13-1* dramatically reduced ssDNA at 36° C (Jia et al, 2004; Zubko et al, 2004).



**Figure 2** Schematic representation of the checkpoint response following resection at uncapped telomeres in *cdc13-1*

## 1.5.2 Telomere uncapping in POT1 deficient cells

Loss of POT1 in mammalian cells also leads to telomere uncapping. Knockdown of POT1 with siRNA in human cancer cell lines triggers apoptosis or senescence and chromosome fusions (Veldman et al, 2004). Complete POT1 knock out in chicken cells leads to long 3' overhangs, ATR/ATM dependent checkpoint activation and G2 arrest. The long 3' overhangs observed probably result from AC strand degradation because the overall telomere length did not increase after POT1 removal. No increase in chromosome fusions was observed in POT1 deficient cells but instead some survivors exhibited dramatic chromosomal instability due to defects in chromosomal segregation. (Churikov et al, 2006).

Mice harbour two POT1 genes called POT1a and POT1b. Interestingly POT1a is essential, while POT1b is not, suggesting that both genes may have different functions at telomeres (He et al, 2009; Wu et al, 2006). Deactivation of POT1a in mouse embryonic fibroblasts leads to increased 3' overhang length, DNA damage response at the telomeres and early onset of cellular senescence. When p53 was deleted simultaneously with POT1a, increased homologous recombination at telomeres was observed together with chromosomal instability due to chromosomal fusions and breaks (Wu et al, 2006).

POT1b deficient mice exhibited an ATR dependent checkpoint response in highly proliferative tissues, which caused increased apoptosis, whereas non-proliferating tissues were not affected. Liver and kidney cells from POT1b deficient mice had long 3' overhangs but telomere length was constant. However after passaging POT1b<sup>-/-</sup> mouse embryonic fibroblasts exhibited telomere shortening and telomere-telomere fusions (He et al, 2009)

All these findings confirm that yeast Cdc13 and mammalian POT1 exhibit striking functional similarities and that the cellular responses in *cdc13-1* and POT1 deficient cells are very similar.

## 1.6. DNA repair mechanisms

Damage to the DNA can have deleterious consequences for the cells because it threatens the integrity of the genome. Hence throughout evolution cells have developed robust DNA repair mechanisms that ensure genomic stability throughout the cell cycle. Furthermore different types of damage are specifically recognised and repaired by different DNA repair machineries. For the purpose of this thesis only double strand break repair, post replication repair and nucleotide excision repair will be briefly reviewed.

### 1.6.1 Double strand break repair

DNA double strand breaks (DSB) generated by ionising radiation, chemicals and reactive oxygen species pose a particularly immense threat to cells because they can cause chromosome loss, translocations and malignancy. Two pathways are responsible for DSB repair: homologous recombination (HR) and non-homologous end joining (NHEJ). During NHEJ the break is simply aligned and ligated, thus this pathway can introduce errors, while in HR the break site is first processed to create single stranded overhangs that can search for regions of homology in the genome (Figure 3 and 4). After homology is established the healthy region is used as a template for error free DSB repair. The choice of repair pathway at a particular DSB depends on the phase of the cell cycle and the type of cell (Shrivastav et al, 2008). During G1 and early S phase, NHEJ is the more predominant choice for repair, while HR is utilised in late S and G2. Furthermore DSB resection generating 3' overhangs at the break is essential for HR but not required in NHEJ (Lamarque et al, 2010). The DSB repair machinery is very well conserved from yeast to human and utilises almost identical factors.

In yeast NHEJ is aided by the yKu70/yKu80 complex and MRX complex that bind to the DSB ends and the DNA ligase IV required for re-joining of the broken ends as reviewed in Figure 3 and (Daley et al, 2005). YKu forms a ring structure that can slide onto the DNA end, while MRX seems to encircle yKu. MRX is presumably responsible for bringing the two broken ends close to each other through its Rad50 subunit. However NHEJ occurs also in the absence of MRX, suggesting that there might be another mechanism for aligning.

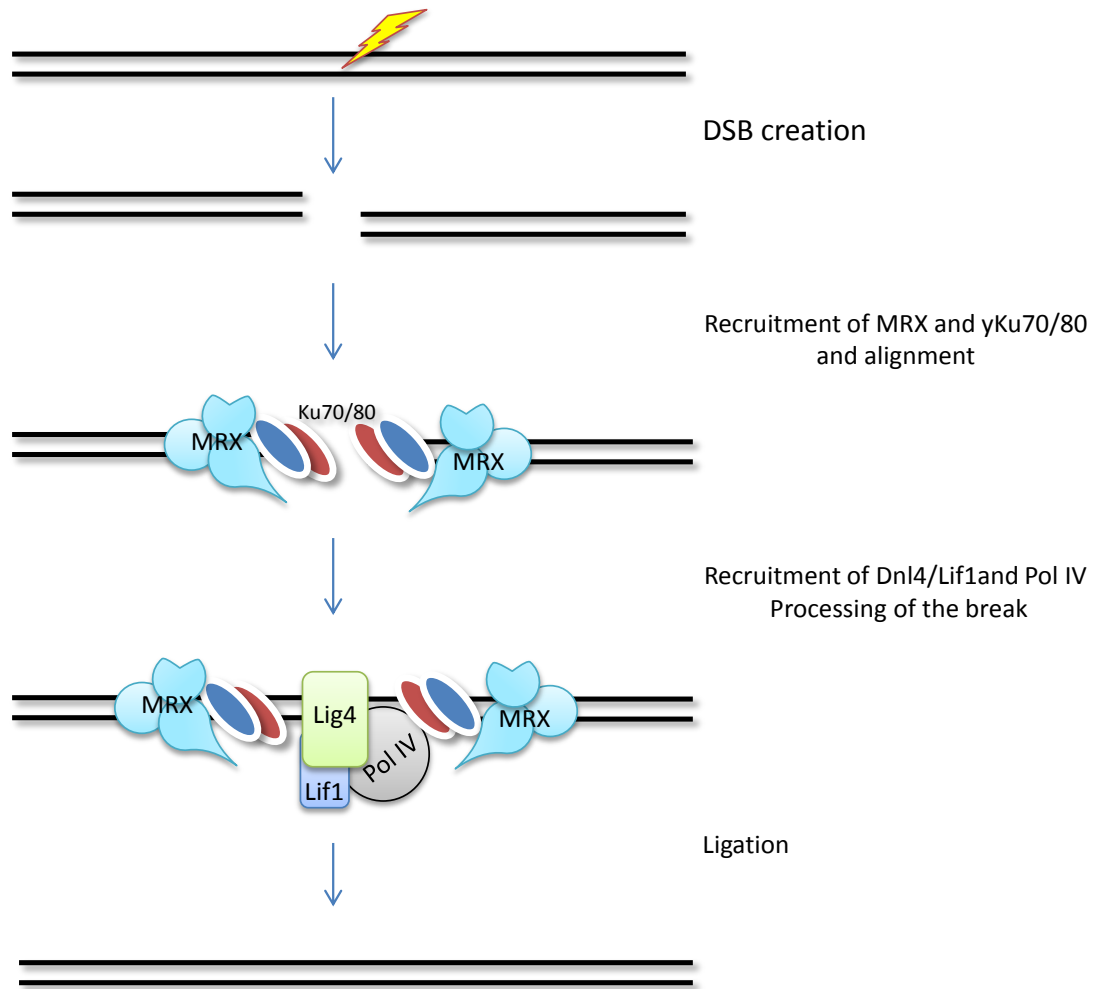
Once bound to the broken ends MRX and yKu recruit the NHEJ specific DNA ligase VI together with its binding partners Lif1 and Nej1. It is known that Lif1 is required for ligase IV stability but the function of Nej1 is not well understood. After ligase VI is recruited a ligation reaction might be attempted one at a time at each DNA strand. If this fails the broken ends need to be processed further to create a suitable substrate for ligation. This is achieved with the help of the NHEJ specific DNA polymerase Pol4 and the flap endonuclease Rad27.

In the current model of yeast HR the DSB has to be processed to form 3' overhangs that are later coated with proteins from the *RAD52* epistasis group required for homology search and strand invasion (Aylon & Kupiec, 2004). Then the homologous region is utilised as template for repair by polymerase  $\alpha$ /primase,  $\delta$  and  $\epsilon$ , followed by ligation (Figure 4). The DSB is processed in 5' to 3' direction by different nucleases. MRX and Sae2 initiate resection but Exo1, Dna2 and the helicase Sgs1 are required for generating a suitably long ssDNA overhang for loading of the HR machinery (Mimitou & Symington, 2008; Zhu et al, 2008). Then Rad52 aids the loading and nucleation of Rad51. This is required because RPA coated ssDNA inhibits Rad51 binding. The Rad51 nucleofilaments are stabilised by Rad55 and Rad57 and a search for homology throughout the genome is initiated.

Once homology is established resection of the DSB is terminated and Rad54 mediates strand invasion of the Rad51 nucleofilaments presumably through chromatin remodeling. Then at least polymerase  $\delta$  and  $\epsilon$  are required to copy the invaded template. In some cases HR might also require polymerase  $\alpha$ /primase (Lydeard et al, 2007). Recently it has also been shown that almost all replication factors are assembled at an induced DSB showing that DNA repair is remarkably similar to DNA replication (Lydeard et al, 2010b). Furthermore Rad51 filament removal by Srs2 is crucial because repair can not be completed without filament removal. When only one of the broken strands shares homology with the genome, only one strand is able to search for homology. This type of repair is called break induced replication and is one of the possible mechanisms for telomere replication in the absence of telomerase.

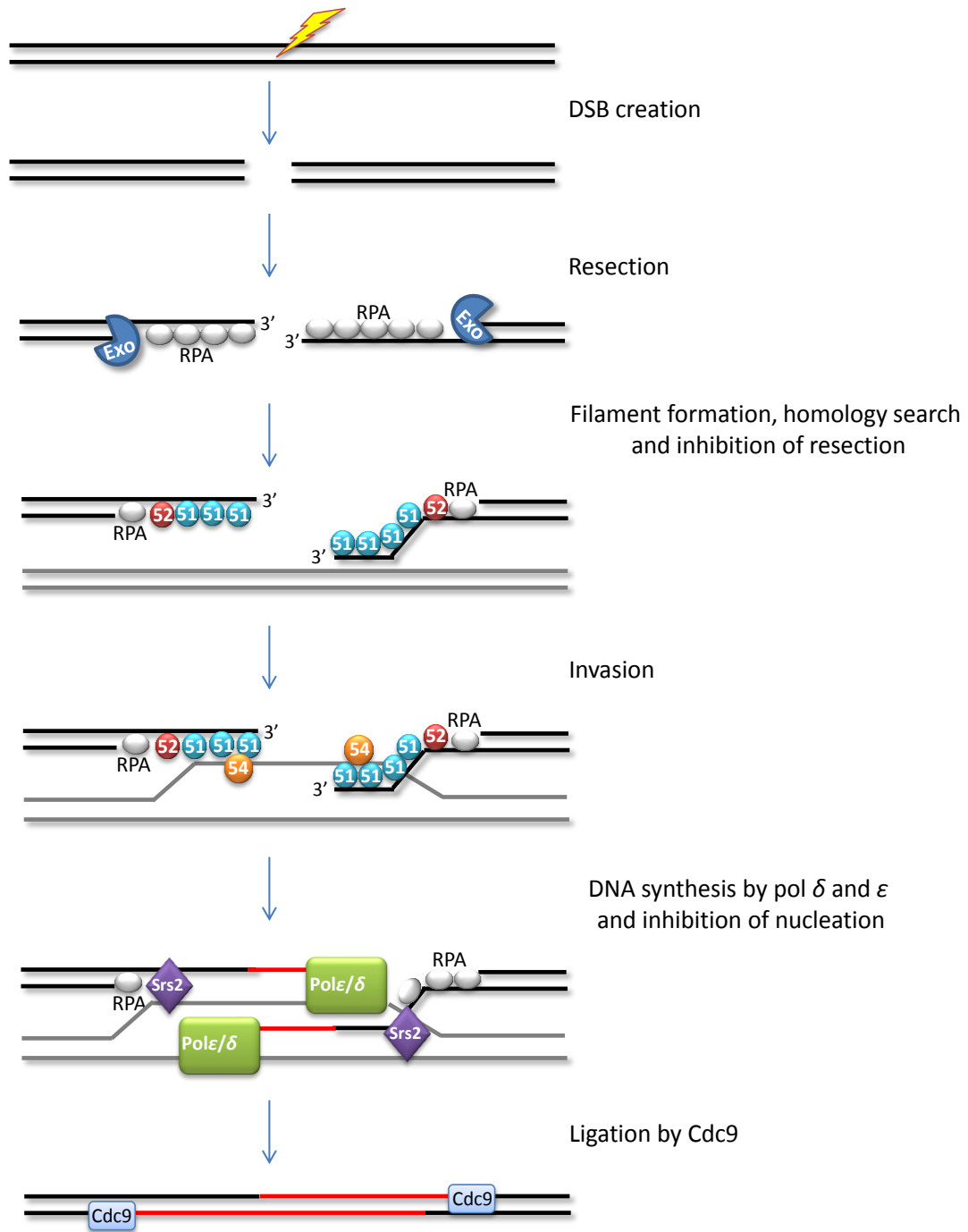


## Non Homologous End Joining



**Figure 3** Basic model of non-homologous end joining (NHEJ)

# Homologous Recombination



**Figure 4** Model of homologous recombination (HR) based on (Aylon & Kupiec, 2004)

## 1.6.2 Post replication repair

Post replication repair (PRR) is required to fill up single stranded gaps in the DNA immediately after DNA synthesis. Such gaps arise when the DNA replication forks stall due to naturally occurring barriers in the genome or due to irresolvable DNA lesions, such as abasic sites and UV induced pyrimidine dimers present at the DNA template (Labib & Hodgson, 2007). Replication fork stalling is also observed upon hydroxyurea poisoning that causes depletion of nucleotides. Hence hydroxyurea has been used extensively to study replication fork stalling under experimental conditions.

Post replication repair involves polymerases that can fill up the ssDNA, bypassing the DNA lesions either in an error prone or error free manner (Andersen et al, 2008; Broomfield et al, 2001). Regulation of PRR depends on ubiquitination of the sliding clamp PCNA required for processivity of replicative polymerases. The E2 ubiquitin conjugating enzyme Rad6 and E3 ubiquitin ligase Rad18 are responsible for monoubiquitination of PCNA on Lysine 164. This reaction requires PCNA to be bound to the stalled replication fork and stimulates error prone translesion synthesis that can bypass pyrimidine dimers and abasic sites.

The main polymerases involved in translesion synthesis are polymerases  $\zeta$  and  $\eta$  encoded by *REV1* and *REV3/REV7* respectively. Polymerase  $\zeta$  can insert dCMPs opposite abasic sites and contributes to 60-80% of abasic site bypass. Interestingly *rev1* $\Delta$  cells display no mutagenesis activity, showing that error prone translesion synthesis in these mutants is impeded. Since Rev1 interacts with all other translesion polymerases it may function as scaffold for assembly of the translesion repair machinery. Polymerase  $\eta$  can correctly insert AA opposite some thymine-thymine dimers, thus it is the only error free translesion polymerase. Polymerase  $\kappa$  and  $\iota$  are the other known translesion polymerases but their function is less well understood.

Monoubiquitinated PCNA could be further polyubiquitinated by Mms2/Ubc13/Rad5, presumably stimulating the error free branch of PRR. Although the mechanism of error free PRR has not yet been established, it has been suggested that this branch may use the newly synthesised sister chromosome as template.

### 1.6.3 Nucleotide excision repair

Nucleotide excision repair (NER) is responsible for repairing UV induced pyrimidine dimers and photoproducts as well as chemical adducts and DNA crosslinks. Highly transcribed genes are repaired faster than the rest of the genome mainly due to a difference in damage recognition (Waters et al, 2009). Damage at these genes is recognised by the stalling of the transcription RNA polymerase II which signals NER. Repair in the rest of genome the requires Rad16, Rad7 and Abf1 (Liu et al, 2010). Binding and cleavage around the region is accomplished by several nucleotide excision factors, indicated as NEF1-4 (Prakash & Prakash, 2000).

NEF1 is composed of the DNA damage recognising protein Rad14 and the ssDNA endonuclease Rad1/Rad10. Upon damage detection the Rad1/Rad10 complex cleaves the modified strand on both sites around the lesion. NEF2 is composed of Rad4 and Rad23, NEF3 comprises Rad2 and the transcription factor TFIIH. The last factor is NEF4 and it contains Rad7 and Rad16 but is not required for incision. Instead NEF4 co-operates with NEF2 in recognition, binding and cleavage of lesions.

After the damage strand is cleaved a ~20 oligonucleotide ssDNA gap is created which is then filled up by polymerase  $\epsilon$  and  $\delta$  with the aid of the clamp PCNA. Consequently the leftover nicks are ligated by DNA ligase I, encoded in yeast by the gene *CDC9* (Liu et al, 2010).

Some unique regions like the centromere and the telomere are more difficult to repair. In G1 and G2/M arrested cells centromeric thymidine dimers are not repaired, however there is disruption to the centromere binding proteins. It appeared as if these dimers could only be repaired during DNA replication (Capiaghi et al, 2004). Similarly silenced regions around the telomere also inhibited repair of UV induced lesions, while other heterochromatic regions in the DNA did not (Rochette & Brash, 2010; Waters et al, 2009).

## 1.7 Aims

As described above controlled telomere uncapping can be induced in *cdc13-1* and this is followed by generation of single stranded DNA damage and a well defined checkpoint response (Lydall, 2009). Similarly, loss of POT1 (the mammalian homologue of Cdc13) also leads to increased ssDNA levels and checkpoint activation in mouse and chicken (Churikov & Price, 2008; Hockemeyer et al, 2005; Wu et al, 2006). Telomere uncapping provides insights into how cells deal with a potential dysfunctional telomere.

Although checkpoint activation following DNA damage is usually required to induce repair, no study focused on any potential ssDNA re-synthesis at uncapped telomeres has been conducted so far. Interestingly, *cdc13-1* cells maintain high viability for a long period of time at restrictive temperatures and if subjected to rounds of telomere damage, followed by periods of rest, the cells continue to form colonies (Blankley & Lydall, 2004). Similarly even when POT1 was removed, chicken cells could maintain viability for at least 24h and reintroduction of POT1 also rescued some of the cells (Churikov et al, 2006). Therefore cells lacking telomere protection may have an intrinsic re-synthesis mechanism that becomes apparent after capping is restored. This proposed mechanism would then fill up the single stranded gaps, allowing cells to re-enter the cell cycle.

The aim of this thesis is to identify any potential ssDNA re-synthesis mechanism present at uncapped telomeres by utilising *cdc13-1* as a model system. This will be achieved through measurements of ssDNA dynamics and BrdU incorporation at sites of damage close to the telomere. Proteins involved in this process will be identified through their interaction with the DNA damage.

Although this ssDNA re-synthesis might be normally only observed after capping is restored, specific conditions may stimulate it at dysfunctional telomeres. The existence of such factors will be investigated and discussed in a global perspective. The specific questions that I would like to address are:

Can natural ssDNA re-synthesis happen in *cdc13-1* cells after telomere recapping and what factors govern this process?

What factors can further stimulate re-synthesis at dysfunctional telomeres in yeast and what genetic pathways are required?

Are factors controlling re-synthesis at uncapped telomeres also responsible for other types of DNA damage?

## 2 Materials and methods

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### 2.1 Yeast strains and plasmids

All yeast strains used in this thesis were in the W303 background (*ade2-1 trp1-1 can1-100 leu2-3,112 his3-11,15 ura3 GAL+ psi+ ssd1-d2 RAD5*). To generate deletion mutants, genes were knocked out using a pFA6a-kanMX6 vector carrying the *E.coli kan<sup>r</sup>* gene for G418 resistance as described below. Tagging was performed with pFA6a-3HA-natMX6 vector and overexpression with pFA6a-kanMX6-PGAL1 or pFA6a-kanMX6-PGAL1-GST (Hentges et al, 2005; Longtine et al, 1998). Strains LMY746, 493, 784 and 785 resulted from crossing. LMY746 was created from crossing CLY152 (*MATa 3xHA-CDC17 HIS3*) with LMY205 (Lee et al, 2010). LMY493 was created by mating TAY73(*MATa ubr1Δ::GAL::UBR1::LEU2 DPB2::6xMYC::kanMX6*) with LMY205 and then swapping the kanMX6 cassette with *URA3* (Kesti et al, 2004). LMY784 and LMY785 were created by mating E3368 (*MATalpha URA3::GPD-TK (7x)*) with LMY43 or LMY769 respectively (Lengronne et al, 2001). Strains are listed in Table 1 and can be found at Dr Laura Maringele yeast collection website under [http://minch-moor.ncl.ac.uk/fmi/iwp/res/iwp\\_home.html](http://minch-moor.ncl.ac.uk/fmi/iwp/res/iwp_home.html)

### 2.2 Media for culturing cells

Yeast media contained:

10g BD Difco™ Yeast Extract

20g BD Bacto™ Peptone

20g BD Difco™ Bacto™ Agar

Liquid media did not contain agar. After autoclaving the following chemicals were added:

2% final concentration Dextrose or Raffinose

0.005% final concentration adenine

Raffinose was used to repress the expression of genes placed under the *GALI* promoter.

Selective media contained:

1.7g BD Difco™ Yeast Nitrogen Base

5g ammonium sulphate (Sigma)

20g BD Difco™ Bacto™ Agar

1.3g amino acids, lacking URA, HIS, TRIP or LEU

After autoclaving the following chemicals were added:

2% final concentration Dextrose

0.005% final concentration adenine

For G418 resistance plates, 400µg/ml G418 (Formedium) was added to the yeast media.



Strain	Genotype	Reference
DLY204	<i>MATa cdc13-1int</i>	David Lydall
LMY114	<i>MATa cdc13-1 int kcs1::kanMX6 cdc15-2 bar1::hisG</i>	This study
LMY129	<i>MATa cdc13-1 int ipk2::kanMX6 cdc15-2 bar1::hisG</i>	This study
LMY132	<i>MATa cdc13-1 int ipk1::kanMX6 cdc15-2 bar1::hisG</i>	This study
LMY135	<i>MATa cdc13-1 int ddp1::kanMX6 cdc15-2 bar1::hisG</i>	This study
LMY138	<i>MATa cdc13-1 int plc1::kanMX6 cdc15-2 bar1::hisG</i>	This study
LMY142	<i>MATa cdc13-1 int vip1::kanMX6 cdc15-2 bar1::hisG</i>	This study
LMY180/305	<i>MATa cdc13-1int exo1::LEU2</i>	David Lydall
LMY198/199	<i>MATa yku80::HIS3 yku70::LEU2</i>	David Lydall
LMY202	<i>MATa ade2-1 trp1-1 can1-100 leu2-3,112 his3-11,15 ura3 GAL+ psi+ ssd1-d2 RAD5</i>	David Lydall
LMY205	<i>MATalpha cdc13-1 int</i>	David Lydall
LMY206	<i>MATa cdc13-1 int cdc15-2 bar1::hisG</i>	David Lydall
LMY207	<i>MATalpha yku70::HIS3</i>	David Lydall
LMY208	<i>MATalpha yku80::kanMX6</i>	David Lydall
LMY31	<i>MATa cdc13-1 int rad52::kanMX6 cdc15-2 bar1::hisG</i>	This study
LMY315	<i>MATa vip1::kanMX6</i>	This study
LMY316	<i>MATa ddp1::kanMX6</i>	This study
LMY319/318	<i>MATa kcs1::kanMX6</i>	This study
LMY321	<i>MATa ipk2::kanMX6</i>	This study
LMY323	<i>MATa ipk1::kanMX6</i>	This study
LMY324	<i>MATa dpb4::kanMX6</i>	This study
LMY326	<i>MATa pol32::kanMX6</i>	This study
LMY328	<i>MATa dpb3::kanMX6</i>	This study
LMY330	<i>MATa plc1::kanMX6</i>	This study
LMY336	<i>MATa cdc13-1int rad9::HIS3</i>	David Lydall
LMY420	<i>Mat alpha cdc13-1 rif1::URA3</i>	Yuan Xue
LMY43	<i>MATa cdc13-1 int dpb3::kanMX6 cdc15-2 bar1::hisG</i>	This study
LMY45	<i>MATa cdc13-1 int dpb4::kanMX6 cdc15-2 bar1::hisG</i>	This study
LMY488	<i>MATa msn2::kanMX6</i>	David Lydall
LMY489	<i>MATa msn4::kanMX6</i>	David Lydall
LMY49	<i>MATa cdc13-1 int pol32::kanMX6 cdc15-2 bar1::hisG</i>	This study
LMY490	<i>MATalpha cdc13-1int msn4::kanMX6</i>	David Lydall
LMY491	<i>MATa msn2::HIS3 msn4::URA3 cdc13-1int</i>	David Lydall
LMY492	<i>MATalpha msn4::kanMX6 cdc13-1int</i>	David Lydall
LMY493	<i>MATa cdc13-1int DPB2::6xMYC::URA3</i>	This study
LMY746	<i>MATa cdc13-1 HIS3::HAx3::CDC17</i>	This study
LMY755	<i>MATa cdc13-1 int rev3::kanMX6</i>	This study
LMY765	<i>MATa cdc13-1int GAL1::PLC1</i>	This study
LMY768	<i>MATa cdc13-1int GAL1::GST::IPK1</i>	This study
LMY769	<i>MATa cdc13-1 int cdc15-2 bar1::hisG GAL1::GST::KCS1</i>	This study
LMY772	<i>MATa cdc13-1 int POL3::HA::kanMX6</i>	This study
LMY780	<i>MATa cdc13-1 int pol4::kanMX6</i>	This study
LMY784	<i>MATa cdc13-1 int URA3::GPD-TK (7x) bar1::hisG</i>	This study
LMY785	<i>MATalpha cdc13-1 int URA3::GPD-TK (7x) GAL1::GST::KCS1</i>	This study
LMY800	<i>MATa cdc13-1 int rad18::LEU2</i>	David Lydall

**Table 1** Yeast strains used in this thesis

## 2.3 One step PCR based gene deletions

Genes were knocked-out in one step by substitution of the wild type gene with a PCR fragment through homologous recombination. The PCR fragment contained the selective marker *kan<sup>r</sup>*, flanked by short sequences (40bp) homologous to the regions before and after the gene of interest.

### 2.3.1 Designing primers and producing PCR fragments for gene knockout, tagging and overexpression

Plasmid pFA6a-kanMX6 (or any other in case of tagging or overexpression) was used as a template to create a PCR fragment that contains the gene for G418 resistance. The forward primer was designed to anneal to the first 20 bp of *kan<sup>r</sup>*. It also contained 40bp upstream of the start codon of the gene of interest. The reverse primer annealed to the last 20bp of the *kan<sup>r</sup>* and had homology to 40 bp downstream the stop codon of the gene of interest.

For tagging the forward primer was complementary to 40bp before the stop codon and the reverse was designed to have homology with 40bp downstream of the stop codon.

In the case of gene overexpression 300bp in front of the start codon were replaced by the overexpression cassette, thus 40bp from the primers were homologous with the region 340bp upstream of the gene and 40bp upstream of the start codon. All primers were purchased from WMG | Operon. The PCR reaction was set up on ice and run as follows:

Master Mix:

39µl dH<sub>2</sub>O

5µl 10 x Ex Taq Buffer - Takara Bio Inc.

5µl dNTPs (2.5mM) – Takara Bio Inc.

2µl plasmid (1.5ng/µl stock)

0.5µl Primer Mix (4µl dH<sub>2</sub>O + 1µl forward primer (200µM stock) + 1µl reverse primer (200µM stock))

0.5µl Ex-Taq Polymerase (250Units) – Takara Bio Inc.

PCR conditions:

94°C: 4 min (1 cycle)

DNA melting:	95°C: 30 sec.	}	35 cycles
Primer annealing:	55°C: 1 min.		
Elongation:	72°C: 1.5 min.		

Afterwards, the 2µl PCR product was checked on 1% agarose gel for the expected size.

### **2.3.2 Lithium acetate yeast cell transformation**

Yeast cells were pre-grown overnight and diluted in the morning to a final concentration of  $5 \times 10^6$  cells/ml in 50ml of liquid YEPD. Afterwards, the culture was left to shake in the water bath at 21-23°C until it reached a density of  $2 \cdot 10^7$  cells/ml. This amount of cells was sufficient for 10 transformations.

The cells were harvested by centrifugation for 3 min. at 3000g and the supernatant was discarded. The pellet was vortexed in 25ml sterile water in order to wash excess YEPD and spun again for 3 min at 3000g. After that the water was discarded, the cells were resuspended in 1 ml of 1% lithium acetate and transferred to a 1.5ml eppendorf tube. The cells were harvested by centrifugation for 15 sec. at 16000g and the lithium acetate was aspirated. Then, the pellet was resuspended with 400µl 1% lithium acetate to a final volume of 500µl cell suspension and kept on ice until transformation.

50µl of yeast were transferred into separate eppendorf tubes and spun for 15 sec. at 16000g to pellet the cells. The supernatant was aspirated. Consequently, the

transformation mix was added to each tube containing yeast pellets in the following strict order:

240µl 50% polyethylene glycol (PEG)

36µl 10% lithium acetate

50µl 2mg/ml sonicated salmon sperm DNA

10µl PCR product

The cells were mixed carefully with a pipette and incubated for 30 min. at room temperature. During this incubation the yeast cell wall would be degraded in order to allow DNA uptake. After that the samples were heat shocked at 42°C for 20 min. to allow formation of pores in the plasma membrane of the cells. Then the cells were harvested for 15 sec. at 4000g and the supernatant was aspirated. The cells were resuspend in 200µl sterile water and left to recover overnight on solid YEPD plates. In the morning the plates were replica-plated on YEPD plates containing 200µg/ml active concentration of G418 and incubated at 21°C. This way only the cells which integrated the *kan<sup>r</sup>* formed colonies.

### **2.3.3 PCR-based testing for gene deletion**

After colonies were formed they were tested for the presence of the wild type gene or *kan<sup>r</sup>* to eliminate false positives. Testing was performed by PCR which could produce either a band correlating to the wild type gene or a band correlating to the *kan<sup>r</sup>* gene. The forward primer was designed to anneal 75 bases upstream of the disrupted gene and to have approximately 60°C melting temperature. Then two reverse primers were designed in order to distinguish between a *kan<sup>r</sup>* and wild type gene. The *kan<sup>r</sup>* reverse primer annealed 300bp downstream of the start codon and produced a 790bp fragment. The sequence of this primer was 5'TCGCAGTGGTGAGTAACCATGCA3'. The wild type reverse primer was designed to anneal 500bp after the start codon of the wild type gene, yielding a 575bp fragment. Similarly primers were also designed to test tagging and overexpression.

Whole yeast cells were used as genomic DNA templates in a Hot Start PCR as follows:

Master Mix:

15 $\mu$ l dH<sub>2</sub>O

2.5 $\mu$ l Hot Start Taq Buffer (Qiagen)

2.5 $\mu$ l dNTPs (2.5mM) (Takara Bio Inc.)

0.25 $\mu$ l MgCl<sub>2</sub> (25mM stock) (Qiagen)

0.5 $\mu$ l Primer Mix (4 $\mu$ l dH<sub>2</sub>O + 2 $\mu$ l Forward primer (200 $\mu$ M stock) + 1 $\mu$ l Wild type reverse primer (200 $\mu$ M stock) + 1 $\mu$ l *Kan<sup>r</sup>* reverse primer (200 $\mu$ M stock))

0.25 $\mu$ l Hot Start Taq Polymerase (1000units) (Qiagen)

1 $\mu$ l whole yeast cells diluted in dH<sub>2</sub>O

PCR conditions:

94°C, 15 min (1 cycle)

35 cycles	{	DNA melting:	94°C: 30 sec.
		Primer annealing:	56°C: 20 sec.
		Elongation:	72°C: 30 sec.

72°C, 1min (1 cycle)

Consequently, 6 $\mu$ l PCR product was checked on 1% agarose gel for the wild type or *kan<sup>r</sup>* corresponding band.

## 2.4 Generation of yeast strains by mating and random spore analysis

Haploid yeast cells with different sexes (MAT $\alpha$  and MAT $\alpha$ ) were mated on solid YEPD plates by spreading the cells on top of each other. After two days at 23°C, the

cells were transferred to selective plates for diploid screening. The diploid should contain markers from both parents. Afterwards, the diploids were incubated on the wheel at 23°C in 2ml 1% potassium acetate, pH 7.6 supplemented with amino acids for 2-3 days, until spores in asci were seen under the microscope. Then the cells were washed twice with sterile water by centrifugation for 15 sec. at 16000g and finally combined with 0.5ml 1mg/ml zymolyase and 10µl of 98% 2-β mercaptoethanol (Sigma). The cells were incubated on the wheel at 30°C overnight to lyse the haploid cells and free the spores from the ascus.

On the next morning 5ml 1.5% igepal CA-630 detergent was added to the lysed spores followed by incubation on ice for 15min. Afterwards the spores were sonicated on a Sanyo MSE Soniprep 150 sonicator for 30 sec. at amplitude 5 microns for a total of three times with 2 min incubation periods on ice-water between cycles. The cells were spun at 1200g for 10min, resuspended in 5ml 1.5% igepal CA-630 detergent and again sonicated for three cycles as described above.

Finally the spores were spun at 1200g for 10min and resuspended in sterile water. The spores were counted by a hemocytometer, diluted to 1000 spores/ml and 200µl were inoculated on selective plate. After the spores gave rise to single colonies, the colonies were inoculated and replica plated on relevant selective plates. The plates were scored after 2-3 days to select for haploid colonies with the desired genetic background. The mating type of the haploids could be further identified by replica plating onto solid YEPD plates pre-coated with LMY192 (*MATa ade1 arg4 aro2 his7 lys5 met4 ura2*) and LMY193 (*MATalpha ade1 arg4 aro2 his7 lys5 met4 ura2*). On the next day the mating plates were replica plated on selective media. Only strains that have mated form colonies in these conditions.

## 2.5 Spot test

Strains were tested for growth at different temperatures in order to evaluate temperature sensitivity. Cell cultures were picked from plates and diluted to  $3 \cdot 10^7$  cells/ml in a 96-well plate. Then a 5 fold serial dilution was performed and the cells were inoculated on plates with a metal replicator. The plates were imaged on the Fujifilm LAS-3000 imaging system 2 to 4 days after incubation.

## 2.6 UP-DOWN assay

Cells containing the *cdc13-1* mutation were diluted to  $4 \cdot 10^7$  cells/ml and spotted on agar plates as in the spot test. For wild type cells only  $1 \cdot 10^7$  cells/ml were seeded to avoid overgrowing at high temperatures. The cells were incubated either at 21° C, and 35° C or in an incubator programmed to cycle between 21° C and 35° C every 4 hours for a total of 3 cycles. After the cycles finished the plates were left at 21° C until colony formation. The plates were photographed on the Fujifilm LAS-3000 imaging system.

## 2.7 Liquid culture growth assay

Growth was also assessed in liquid culture by inoculation of  $2 \cdot 10^5$  cell/ml cells and incubation on the wheel at the required temperature. Every day the culture was counted and re-diluted to  $2 \cdot 10^5$  cells/ml. The growth was plotted on a  $\log_{10}$  scale with Sigma Plot.

## 2.8 Scoring of G2/M arrested cells

To monitor cell cycle arrest, pellets from  $1-2 \cdot 10^7$  cells were collected in 70% (v/v) Ethanol. Prior to analysis the pellets were washed with distilled water, stained with  $0.2 \mu\text{g/ml}$  4',6'-diamidino-2 phenylindole (DAPI) from Sigma and sonicated for 3-4 sec. at amplitude 5 microns. The stained cells were scored under a fluorescent microscope as four cell types representing four stages from the cell cycle. Single cells with one nucleus were regarded as cells in G1; budded cells with a bud 50% smaller than the mother cell and with one nucleus represented cells committed to or in S phase; budded cells with a bud greater than 50% of the mother cell with the nucleus in one cell or a nucleus positioned between the mother cell and the bud were considered cells in G2/M phase; cell with two nuclei separated between the mother and daughter cells were scored as late M phase. From every sample, 300 cells were counted and the percentage of cells in different cell phases was calculated.

## 2.9 QAOS assay

Quantitative Amplification Of Single-stranded DNA (QAOS) was performed to quantify the amount of ssDNA generated at a particular locus close to the telomere (Booth et al, 2001). After sample collection DNA was extracted with a modified Qiagen protocol (see below for description). Then the DNA was quantified and equalised by qPCR, relative to standards with known amounts of DNA (40ng, 20ng, 10ng, 2ng and 0.2ng DNA). The standards were prepared from wild type cells.

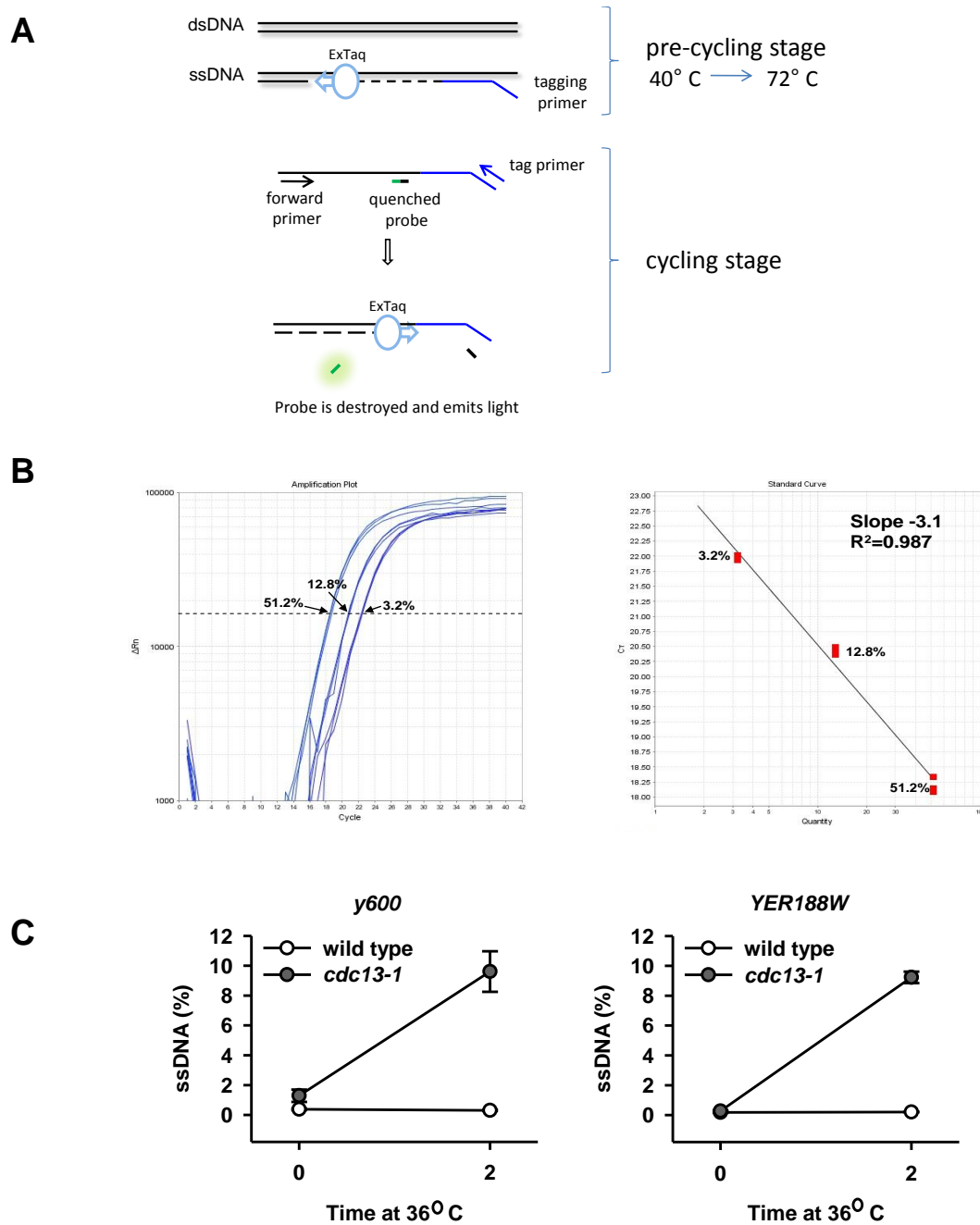
Afterwards ssDNA was detected in specific qPCR conditions with TaqMan probes (Figure 5). In the first part of the PCR the temperature increased gradually from 40° C to 72° C. During this stage the tagging primer could only anneal to ssDNA but not to dsDNA. When the temperature gradually rose the polymerase extended this primer, creating a tagged PCR fragment. In the second stage of the PCR this extended PCR fragment was bound by the probe and the forward and tag primers. Hence only the sequence to which the probe was bound would be amplified.

The probe was labelled with a fluorescent molecule on the 5' (either VIC or FAM) and with a TAMRA quencher on the 3' end. Due to the proximity of the two molecules no light was emitted from the probe as a whole. During every round of amplification the polymerase synthesised a new PCR fragment and destroyed the bound probe through its exonuclease activity. This released the fluorescent dye from the quencher and produced light that corresponded to one round of amplification.

Standards with known amounts of ssDNA were used to quantify the signal. These standards were made by boiling 20mg DNA and mixing dsDNA with ssDNA up to 51.2%, 12.8% and 3.2% ssDNA. An example of a typical QAOS measurement of the standards shows that the CT difference is around 2 as expected (Figure 5B).

To confirm that QAOS shows accumulation of ssDNA, wild type and *cdc13-1* cells were grown for 2 hours at the restrictive temperature 36° C. No ssDNA was detected in wild type cells at two loci close to the telomere, while *cdc13-1* cells accumulated around 10% ssDNA (Figure 5C).





**Figure 5** The theory of QAOS

A. Scheme depicting the principle of quantitative amplification of ssDNA (QAOS)

B. Amplification plot and Standard curve of the 51.2%, 12.8% and 3.2% standards in a typical run measuring ssDNA

C. Single stranded DNA formation in *cdc13-1* (LMY206) and wild type (LMY202) at 36° C at *Y'600* and *YER188W*

## 2.9.1 Genomic DNA extraction

Cells were inoculated overnight at 23°C in the water bath. In the morning the culture was diluted to  $2 \cdot 10^7$  cells/ml and left to shake at indicated temperatures. 30ml samples were taken at various time points and promptly mixed with 300µl 10% sodium azide and 3ml 0.5M EDTA, pH 8.5 on ice. The cells were centrifuged for 2 min at 1000g, 4°C and the supernatant was discarded. Afterwards, the pellet was resuspended in 1ml ice cold water, transferred to an Eppendorf tube and spun again at 16000g for 5 sec. Excess water was aspirated and the pellet was frozen at -80°C until extraction.

All extraction steps were performed on ice or at 4°C in an Sorvall Biofuge PrimoR centrifuge (Thermo scientific), unless otherwise stated. The frozen cell pellets were thawed on ice and resuspended in 1ml NIB buffer. The samples were spun for 7 sec at 16000g and the supernatant was aspirated. The pellet was resuspended in 600µl NIB buffer and transferred to 2 ml Sarstedt screw cap tubes. 1.5ml ice cold, acid pre-washed 0.6mm glass beads were poured into the tubes. Approximately 200µl liquid was left to cover the beads.

The samples were shaken on Precellys24 lysis and homogenization ribolyser (Bertin technologies) 6-7 times for 5sec at 5500 power to break the cell wall of the yeast cells. The tubes were put in ice water between rounds of breakage for 2min. Consequently, the bottoms of the tubes were punctured with a needle and placed in a 1.5ml Eppendorf tube with cut off bottom and cap. Both tubes were fixed into a 15ml Falcon tube and centrifuged 3 times at 1000g for 2 min. Between the spins the beads were washed with 1ml NIB buffer. This step allows the crushed cells to accumulate at the bottom of the Falcon tube free of beads.

The Sarstedt and the eppendorf tubes were removed and the 15ml Falcon tube was spun for 20min at 6500g to pellet the cell debris and nuclei. The supernatant was discarded and the pellet was carefully resuspended in 2ml G2 buffer containing 200µg/ml RNase A. The samples were incubated at 37°C for 30min. Consequently, 55µl of 20mg/ml Proteinase K was added and incubation continued for 1h at 37°C.

The samples were spun at 6500g for 10min and in the meantime 20g Qiagen columns were equilibrated with 1ml QBT buffer. The supernatant of the samples was combined with 2ml QTB buffer, applied on the columns and left to flow through. Then, the

columns were washed 3 times with 1ml of QC buffer. After washing 15ml Falcon tubes were placed under the columns to collect the genomic DNA.

The DNA was eluted with 2ml QF buffer, pre-warmed at 50°C. Consequently, the DNA was precipitated with 0.7 volumes Isopropanol at ambient temperature. The samples were spun at 7700g for 20min and the supernatant was carefully discarded. The DNA pellet was washed with 1ml 70% Ethanol at ambient temperature and centrifuged at 7700g for another 20min.

The supernatant was carefully discarded and the tubes were air dried for 5min at room temperature. Eventually, 400µl TE was added to each sample and the tubes were left to roll on a wheel at 25°C for 2 days.

## **2.9.2 Real-Time PCR measurements of ssDNA**

All DNA samples were measured in triplicates and dH<sub>2</sub>O was run as negative control. The quantity of the isolated genomic DNA was measured at the PAC2 locus with a Step One™ Real-Time PCR (Applied Biosystems) prior to ssDNA measurement. The probe had a VIC fluorescent reporter and a TAMRA quencher. As standards 20, 10 and 2 ng/10µl genomic DNA were used. The samples were loaded on 96 well plates and sealed with a transparent film. The Master Mix for 12wells was prepared as follows:

Master Mix:

124µl dH<sub>2</sub>O

32µl 10 x Ex Taq Buffer (TaKaRa)

24µl dNTPs (2.5mM stock) (TaKaRa)

3.2µl primer (300nM final concentration)

3.2µl probe (200nM final concentration)

1.5µl ExTaq Polymerase (250 Units) (TaKaRa)

10µl DNA sample was mixed with 15µl Master Mix and run at the following conditions:

PCR conditions:

Pre-cycling phase: 95°C, 5 min (one cycle)

95°C, 0.15 min    { 40 cycles  
63°C, 1min        {

Afterwards, the samples were diluted to 20ng/μl and run for a second time with the same PCR conditions. A dilution coefficient was established from the second run in order to minimise dilution errors:

$$dilutioncoefficient = \frac{20}{DNA\ quantity}$$

The diluted DNA samples were next measured for ssDNA quantity at the Y' repeats of the telomere, 600bp from the end of the right arm of chromosome V, locus *Y' 600*. Measurements were also performed at the *YER188W* locus, 8500bp or PAC2, 411,500bp from the end of the right arm of chromosome V. The Tagging primer was designed to have homology with the TG strand and a Tag sequence with no homology to the yeast genome

Master Mix:

124μl dH<sub>2</sub>O

32μl 10 x Ex Taq Buffer (TaKaRa)

24μl dNTPs (2.5mM stock) (TaKaRa)

3.2μl tagging primer (30nM final concentration)

3.2μl tag primer/reverse or forward primer (300nM final concentration)

3.2μl probe (200nM final concentration)

1.5µl ExTaq Polymerase (250 Units) (TaKaRa)

10µl DNA sample was mixed with 15µl Master Mix and run at the following conditions:

PCR conditions:

Tagging primer annealing: 40°C, 5min

Tagging primer elongation: 4% RAMP

72°C, 5min

DNA melting: 94°C, 5min

95°C, 0.15min }  
67°C, 1min } 40cycles

Eventually, the measured ssDNA quantity was multiplied with the dilution coefficient and plotted with Sigma Plot. The standard deviation was used for the error bars. At least two independent experiments were performed, however only one example is shown in each figure.

### 2.9.3 Buffers

Genomic DNA extraction buffers G2, QTB, QC and QF were prepared according to Qiagen's booklet. The NIB buffer contained 170ml Glycerol, 10.46g MOPS, 14.72g Potassium Acetate, 2ml 1M MgCl<sub>2</sub>, 0.55ml 0.9M Spermidine (0.9M stock was kept at -80°C) and 52mg powder Spermine. The reagents were dissolved in 700ml 18.2mQ dH<sub>2</sub>O.

The pH was adjusted to 7.2 and the volume to 1 litre. Afterwards, the buffer was filter sterilized.

Primer	Sequence	Target
Forward	AATAACGAATTGAGCTATGACACCAA	<i>PAC2</i>
Reverse	AGCTTACTCATATCGATTTTCATACGACTT	<i>PAC2</i>
Probe	VIC - CTGCCGCGTTGGTCAAGCCTCAT – TAMRA	<i>PAC2</i>
Tag	GATCTCGAGCTCGATATCGGATCCATT	<i>PAC2</i>
Tagging	GATCTCGAGCTCGATATCGGATCCATTTTCATACGAC	<i>PAC2</i>
Forward	CCGAAATGTTTTATTGCAGAACAGCCCTAT	<i>Y'600</i>
Reverse	GAGATCAGCTTGCCTGGGAGTTACC	<i>Y'600</i>
Probe	FAM - AC GGAATGCCGTCCAATGCGGCACTTTAGA –TAMRA	<i>Y'600</i>
Tag	TGCCCTCGCATCGCTCTCACA	<i>Y'600 TG</i>
Tagging	TGCCCTCGCATCGCTCTCACAGCCCTATCAG	<i>Y'600 TG</i>
Tag	ATGCCCGCACCGCCTCATTG	<i>Y'600 AC</i>
Tagging	ATGCCCGCACCGCCTCATTGCGCTGGGA	<i>Y'600 AC</i>
Reverse	AACGTACAGGTTACGATCGCGTCATTTA	<i>YER188W</i>
Probe	FAM-TAGCCGTTATCATCGGGCCCAAAACCGTATTTCATTG-TAMRA	<i>YER188W</i>
Tag	AAGGAGCGCAGCGCCTGTACCA	<i>YER188W</i>
Tagging	AAGGAGCGCAGCGCCTGTACCATAGCGTGAT	<i>YER188W</i>

**Table 2** Primers for qPCR

## 2.10 Western blotting

### 2.10.1 TCA extraction of proteins

Freshly grown yeast cells were diluted to  $2.10^7$  cell/ml in the morning and 10ml culture was collected at each time point for TCA protein extraction. The cells were harvested at 1600g, dissolved in 200µl 10%TCA and combined with 0.5mm acid-washed glass beads from Sigma in 2 ml Sarstedt screw cap tubes. Then the cells were crushed in a Precellys24 lysis and homogenization ribolyser (Bertin technologies) 3 times for 10 sec at 6500 power. This cycle was repeated for a total of 3 times. In between the cycles the samples were incubated on ice for 5min. Consequently, the bottoms of the tubes were punctured with a needle and placed in a collection 1.5ml tube with cut off cap. Both tubes were fixed into a 15ml Falcon tube and centrifuged 2 times at 1000g for 2 min. Between the spins the beads were washed with 100µl 10% TCA. Afterwards, the collection tube was spun at 16000g and the pellet was dissolved in 2xLaemmli buffer from Sigma. In cases where the Laemmli buffer turned yellow due to change of pH, the colour was restored by drops of 1M Tris Base, pH 8.5 . Finally the protein extracts were boiled for 5min at 95° C and stored at -80°C.

### **2.10.2 SDS-PAGE, blotting and detection**

Proteins extracted with 10% TCA were first separated on 6.5% gels run at 95V for 30min and at 120V for approximately 1h 30min. Then the gel was transferred onto a Nitrocellulose membrane (Amersham) at 300mA for 1 h. Blocking was performed with 5% milk - TBST for 1h. Primary antibodies (1:200 dilution) and secondary antibodies (1:2000 dilution) were diluted in 5% milk – TBST. Incubation with primary antibody was performed on a shaker overnight at 4°C, while secondary antibody was applied for 1h at room temperature. Washes with TBST were performed 3 times for 10min after primary and secondary antibody incubation. Detection was performed with ECL Western Blotting detection reagents and the membrane was imaged on the Fugijilm LAS-3000 imaging system.

### **2.10.3 Buffers**

Running buffer stocks and Transfer buffer stocks were ordered from BioRad. 150ml Methanol was supplemented to 1xTransfer buffer prior use. 10xTBST stock contained 12.11g Tris base, 29.22g NaCl and 10ml Tween 20 per litre. The pH was adjusted to 7.5. The stock was diluted 1:10.

## **2.11 Chromatin Immunoprecipitation (ChIP)**

ChIP was performed as previously described (Aparicio et al, 2004). Liquid cultures grown overnight were diluted in the morning to  $2 \cdot 10^7$  cell/ml and 40ml samples were collected for each time point.

The proteins were crosslinked to DNA *in vivo* by adding 1% final concentration formaldehyde to the sample and incubation at ambient temperature with gentle shaking for 15min. Cross-linking was stopped by 6ml 2.5M glycine for 5 min and the cells were harvested by centrifugation at 1600g for 5min. Afterwards the pellet was washed twice with ice cold TBS, pH7.5 and every time the cells were spun at 1600g for 5min. Finally, the cells were resuspended in ice cold FA lysis buffer (50mM HEPES, pH 7.5 with KOH, 150mM NaCl, 1mM EDTA, pH 7.6, 1% Triton-X, 0.1%

sodium deoxycholate) and spun one last time at 1600g for 5min. The pellets were frozen at -80°C.

In the morning the cell pellets were defrosted on ice and resuspended in 1ml FA lysis buffer containing a final concentration of 2mM phenylmethylsulfonyl fluoride (PMSF). Then the cells were transferred to 2 ml Sarstedt screw cap tubes and spun for 7sec. at 14000g. The pellet was dissolved in 600µl FA lysis buffer containing PMSF and 0.5mm acid-washed glass beads were added up to 0.75ml of the tube. Then the cells were crushed in a Precellys24 lysis and homogenization ribolyser (Bertin technologies) 3 times for 10 sec at 6500 power. This cycle was repeated for a total of 3 times. In between the cycles the samples were incubated on ice for 5min. Consequently, the bottoms of the tubes were punctured with a needle and placed in a collection 1.5ml tube with cut off cap. Both tubes were fixed into a 15ml Falcon tube and centrifuged 2 times at 1000g for 2 min. Between the spins the beads were washed with 200µl FA lysis buffer containing PMSF. The collection tubes were spun at 16000g for 15min at 4°C and the supernatant was discarded.

The pellet was resuspended in FA lysis buffer and sonicated on a Sanyo MSE Soniprep 150 sonicator for 15 sec. at 7 amplitude microns for a total of 5 times with 2 min incubation periods on ice-water between cycles. Following that the sonicated sample was spun at 16000g for 30min at 4°C and the supernatant was combined with 3ml FA lysis buffer, yielding 4ml sonicated sample for immunoprecipitation.

For each sample 80µl were aliquated for Input, 800µl were used for the Immunoprecipitate (IP) and 800µl were used as a control for the background. The Input aliquots were frozen at -80°C. The IP and Background sonicated samples were combined with primary antibody (indicated in the figure legends) or non-specific donkey anti-goat secondary antibody respectively. The primary antibody concentration required for each protein was established empirically by a serial dilution starting with 1µg total antibody. For most proteins tested 3µg specific antibody was sufficient for immunoprecipitation. The donkey anti-goat secondary antibody concentration (sc-2020 Santa Cruz) was the same as the established primary antibody concentration, namely 3µg.

The IP and Background samples were rolled in an end over end rotator. In the mean time tubes with 50µl protein G dynabeads (Invitrogen) were washed three times with 250µl FA lysis buffer for 10min on the end to end rotator. After washing the beads were combined with the IP and Background samples and left to roll on the end over



end rotator overnight. On the next day the beads were washed 5 times for 3 min with 700µl FA lysis buffer. Then the FA lysis buffer was aspirated with a pipette, the beads were resuspended in 100µl ChIP elution buffer (50mM Tris-HCl pH7.6, 10mM EDTA, pH7.6 and 1% SDS) and incubated for 10min at 65°C. This step reversed the crosslinking between the proteins and the DNA. Afterwards the samples were treated with Proteinase K to degrade unwanted proteins as follows:

For each Input sample: 100µl TE, 100µl ChIP elution buffer and 20 µl Proteinase K (20mg/ml stock)

For each IP and Background sample: 80 µl TE and 20 µl Proteinase K (20mg/ml stock)

All samples were incubated in a water bath at 37°C for 2h and then transferred to a heat block at 62°C. Incubation at 62°C was performed overnight to allow removal of formaldehyde from the DNA. In the morning the DNA was column purified with a Qiagen PCR purification Kit. Afterwards qPCR was performed to detect enrichment of immunoprecipitated DNA at *Y' 600* subtelomeric locus and *YER188W* locus. Measurements were also performed at *PAC2* or *ERG26* loci close to the centromere where no enrichment is expected. The following formula was utilised to obtain the percentage of DNA enrichment at the locus of interest:

$$DNA\ enrichment = \left[ \left( \frac{IP}{Input} \right) \times 100 \right] - \left[ \left( \frac{Background}{Input} \right) \times 100 \right]$$

Whenever values were subtracted the corresponding standard deviations were added up with the following formula:

$$SD = \sqrt{SD\ Input^2 + SD\ Background^2}$$

Or in the cases where for example the DNA enrichment at *PAC2* was subtracted from *Y' 600*:

$$SD = \sqrt{SD_{Y'600}^2 + SD_{PAC2}^2}$$

The qPCR utilised was the same one for quantifying DNA in the QAOS protocol and the same forward and reverse primers and probes were used for the loci of interest. At least two independent experiments were performed but only one representative graph was shown in each figure.

## **2.12 BrdU-IP**

Immunoprecipitation of BrdU labelled DNA was performed in strains containing the URA3::GPD-TK (7x) cassette. The cells were grown in the dark in the presence of 200µg/ml 5-bromo-2'-deoxyuridine (BrdU) from Sigma and 30ml samples were collected. The samples were treated with 300µl 10% sodium azide and 3ml 0.5M EDTA, pH8.5 and spun down for 3min at 1500g. The pellets were stored at -80°C until the BrdU labelled DNA was extracted with phenol-chloroform. The BrdU-IP protocol is based on the ChIP protocol and only the DNA extraction step and the reverse crosslinking step were modified.

### **2.12.1 Phenol-chloroform DNA extraction.**

Phenol-Chloroform extraction was performed as in (Dewar & Lydall, 2010). Cell pellets were resuspended in 1ml lysis buffer (2% Triton-X, 1% SDS, 100mM NaCl, 10mM Tris-HCL pH8, 50mM EDTA pH8) and transferred to Starsted tubes. Then the samples were spun down for 7sec in a bench top centrifuge and the supernatant was aspirated. The washed pellets were resuspended in 400µl lysis buffer and 600mg acid washed glass beads (Sigma) were added to each tube. In a fume hood 400µl phenol:chloroform:isoamyl alcohol (25:24:1, saturated with 10 mM Tris pH 8.0, 1 mM EDTA, Sigma) was added to the tubes. After that the cells were crushed in a Precellys24 lysis and homogenization ribolyser (Bertin technologies) 3 times for 30 sec at 5500 power. This cycle was repeated for a total of 3 times. In between the

cycles the samples were incubated on ice for 2min. 400µl of TE was added to the homogenates and spun for 5min at 14000g in a pre-chilled bench top centrifuge. The aqueous was transferred to 2ml phase lock light gel tubes (5 Prime) and 700µl phenol:chloroform:isoamyl alcohol was added. The samples were spun at 14000g for 5min again and the aqueous phase was poured into a 2ml tube.

The isolated DNA was precipitated for 5min with 1ml 100% ethanol and pelleted for 3min at 14000g. The pellets were air-dried for 5min and RNA was degraded with TE/RNase (0.0075mg/ml RNase in TE) for 30min at 37° C. 26µl 3M sodium acetate, pH 5.2 was added and after briefly vortexing the samples, the DNA was again precipitated with 1ml 100% ethanol for 15min. The DNA was pelleted for 1min at 14000g and air-dried for 30min in a fume hood. Finally the DNA was resuspended in 700µl TE by incubation at 37° C for 30min.

### **2.12.2 Immunoprecipitation of BrdU labelled DNA**

The DNA was sheared in a Sanyo MSE Soniprep 150 sonicator for 10sec at amplitude 7microns for a total of 4 times. In between the samples were kept in ice-water. This produced 500bp fragments similarly to the chromatin fragments in the ChIP protocol. Afterwards 700µ DNA was resuspended in 3ml FA lysis buffer and 80µl was used for Input. For the IP sample 800µl DNA was probed with 3ng anti-BrdU antibody (DB Biosciences) and for background 800µl were incubated with 3ng anti-goat antibody (SantaCruz). The G dynabeads (Invitrogen) were washed 3 times with 250µ FA lysis buffer and combined with IP or background. The tubes were rolled in an end over end rotator at 4° C overnight.

In the morning the beads were washed 6 times with 700µl FA lysis buffer and the bound DNA fragments were eluted in 100µl elution buffer at 65° C for 10min. No RNase treatment and no reverse crosslinking at 62° C were required. After that all samples, including the Input samples were purified with the Qiagen PCR purification kit and measured by qPCR in the same way as for ChIP. At least two independent experiments were performed but only one representative graph was shown in each figure.

## **2.13 Southern Blotting**

Samples collected for Southern blotting were extracted with phenol-chloroform as in the BrdU-IP assay. The samples were measured with the nanodrop, diluted to 27ng/ $\mu$ l and restricted for 3h at 37° C with XbaI (New England Biolabs) in 1x Buffer 2 supplemented with 1xBSA. Afterwards 5 $\mu$  restricted DNA was loaded on 0.5xTBE gel and ran overnight at 25V. The DIG molecular weight marker VII (Roche). In the morning the gel was washed for twice for 10min with 0.25M HCl to depurinate the DNA, followed by denaturation in 1.5M NaCl, 0.5M NaOH twice for 20min and finally neutralised with 1M ammonium acetate twice for 20min. The DNA was gravity transferred for 2 days on a Hydrobond N+ (Amersham) in a sandwich as follows:

500g weight

gel

membrane

2 sheets watmann 3mm paper

15cm white paper towels

Afterwards the membrane was washed briefly in 2xSSC buffer (300mM NaCl, 30mM trisodium citrate, pH7) and cross-linked with 1200J/m<sup>2</sup> UV on a Stratalinker. Then the membrane was pre treated with 15ml DIG easy hybridisation fluid (Roche) at 37° C for 40min. After incubation a digoxigenin (DIG) labelled probe specific for TG repeats was applied in 1:1000 dilution and hybridised with the cross-linked DNA overnight. The probe was synthesised by PCR from a template TG sequence on a plasmid with the PCR DIG probe synthesis kit (Roche) as follows:

PCR mix:

28 $\mu$ l dH<sub>2</sub>O

5 $\mu$ l PCR buffer

5 $\mu$ l PCR DIG mix

10µl primer mix containing

10µM forward (TGCAGGAATTTGGATCACACACTACAC) and

10µM reverse (GCCGGGTAAGGAGTGACAGCG) primers

0.75µl enzyme mix

1.25µl plasmid pDL912 1:200 dilution (from David Lydall)

PCR conditions:

95° C, 3min (1 cycle)

95° C, 30sec

60° C, 30sec

72° C, 1min

} 33 cycles

The probe was boiled at 95° C for 5min and put on ice-water to denature prior application.

After overnight hybridisation with the probe the membrane was washed 2x5min with 50ml 65°C pre-warmed HOT buffer (0.5xSSC, 0.1% SDS) followed by 2x100ml washes for 15min. Then the membrane was rinsed with 1xWashing buffer (Roche) and blocked in 1xDIG blocking solution (Roche) for 30min. Subsequently the labeled probe was detected for 30min with an anti-DIG-alkaline phosphatase conjugated antibody (Roche) that recognises digoxigenin, 1:10000 dilution. Then the membrane was washed twice with 1xWashing buffer, incubated with 1xDetection solution (Roche) and incubated for 5min with CDP-star reagent (New England Biolabs). Finally the signal was detected with the Fugifilm LAS-3000 imaging system.

### **3 Damaged telomeres can “heal” themselves when capping is restored**

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If *cdc13-1* cells really utilise an intrinsic ssDNA re-synthesis mechanism to survive after capping is restored several consequences can be expected. First, polymerases would be recruited to the damage sites, second ssDNA would gradually decrease and third BrdU would be incorporated at the repaired loci. Furthermore polymerase subunits might be essential for this repair This hypothesis will be discussed in the following chapter.

#### **3.1 ssDNA damage is re-synthesised after capping is re-stored**

To understand whether damaged telomeres can be re-synthesised after telomere capping is restored ssDNA production in *cdc13-1* cells was quantified by the QAOS assay. The cells were grown at the non permissive temperature 36° C for 150min to induce uncapping, returned to 23° C and samples were collected every 30min (Figure 6D). To ensure that fluctuations in ssDNA were not caused by cells re-entering the cell cycle, the spindle poison nocodazole was applied to the culture after the temperature was decreased to maintain G2/M phase arrest (Figure 6D). Arrest was monitored by DAPI staining of the nucleus, which allows differentiation between phases of the cell cycle (Figure 6C).

QAOS was performed at two loci close to the telomere (*Y' 600* and *YER188W*) and one centromeric locus (*PAC2*) on the right arm of chromosome V (Figure 6A): *Y'600* is situated in the Y' subtelomere, while the open reading frame *YER188W* is 8500bp away from the chromosome end. After 150min at restrictive temperature *cdc13-1* accumulated ssDNA close to the telomere (*Y'600* and *YER188W* loci, 0min time point) but not at centromere (*PAC2* locus, 0min time point) as expected (Figure 6B). The resection progressed in 5' to 3' direction, because no ssDNA accumulated at the AC rich strand (Figure 6B, *Y'600* grey circles). Interestingly when capping was restored by growing the cells at permissive temperatures the ssDNA gradually

decreased within one hour and then remained constant. The centromeric ssDNA remained below 1% throughout the experiment (Figure 6B, *PAC2*). Loss of ssDNA happened even though 75% of the cells were arrested in G2/M phase with a nucleus in between mother and daughter cells (Figure 6C).

To demonstrate directly that damaged telomeres in *cdc13-1* mutants undergo ssDNA re-synthesis, I conducted a 5-bromo-2'-deoxyuridine (BrdU) incorporation assay. BrdU is a thymidine homologue that can be incorporated into the newly synthesised DNA during DNA synthesis and has been used to study S phase in yeast and human (Lengronne et al, 2001; Maya-Mendoza et al, 2010).

Yeast lacks the enzyme thymidine kinase required for converting deoxythymidine to deoxythymidine monophosphate (dTMP) and instead synthesises dTMPs *de novo* from dUMP. Therefore yeast can not utilise thymidine homologues from the media. However, when the *Herpes simplex* thymidine kinase (*7TK+*) is knocked into the yeast genome, BrdU can be converted to BrdUMP and incorporated into DNA. A yeast strain carrying seven copies of the thymidine kinase under the *GPD* promoter at the *URA3* locus on chromosome V was created by Schwob's group. This strain could incorporate BrdU into DNA without loss of viability and delay in the cell cycle (Lengronne et al, 2001). I crossed this strain with *cdc13-1* and tested how BrdU was incorporated to validate the system.

*Cdc13-1 7TK+* cells were arrested in G1 with alpha factor and incubated with 200µg/ml BrdU for one hour (Figure 7A). Additionally G1 cells were released from the alpha factor arrest and passed through S phase in the presence of BrdU. BrdU incorporation was detected by BrdU-IP with an anti-BrdU antibody at one subtelomeric locus *Y' 600* and one centromeric locus *PAC2* on the right arm of chromosome V. No BrdU incorporation was observed in G1 phase either at *Y' 600* or *PAC2* (Figure 7A). In contrast the cells that went through S phase had incorporated more than 20% BrdU. This data confirms that the strain was behaving as expected, namely DNA synthesis was observed only during S phase but not in G1.

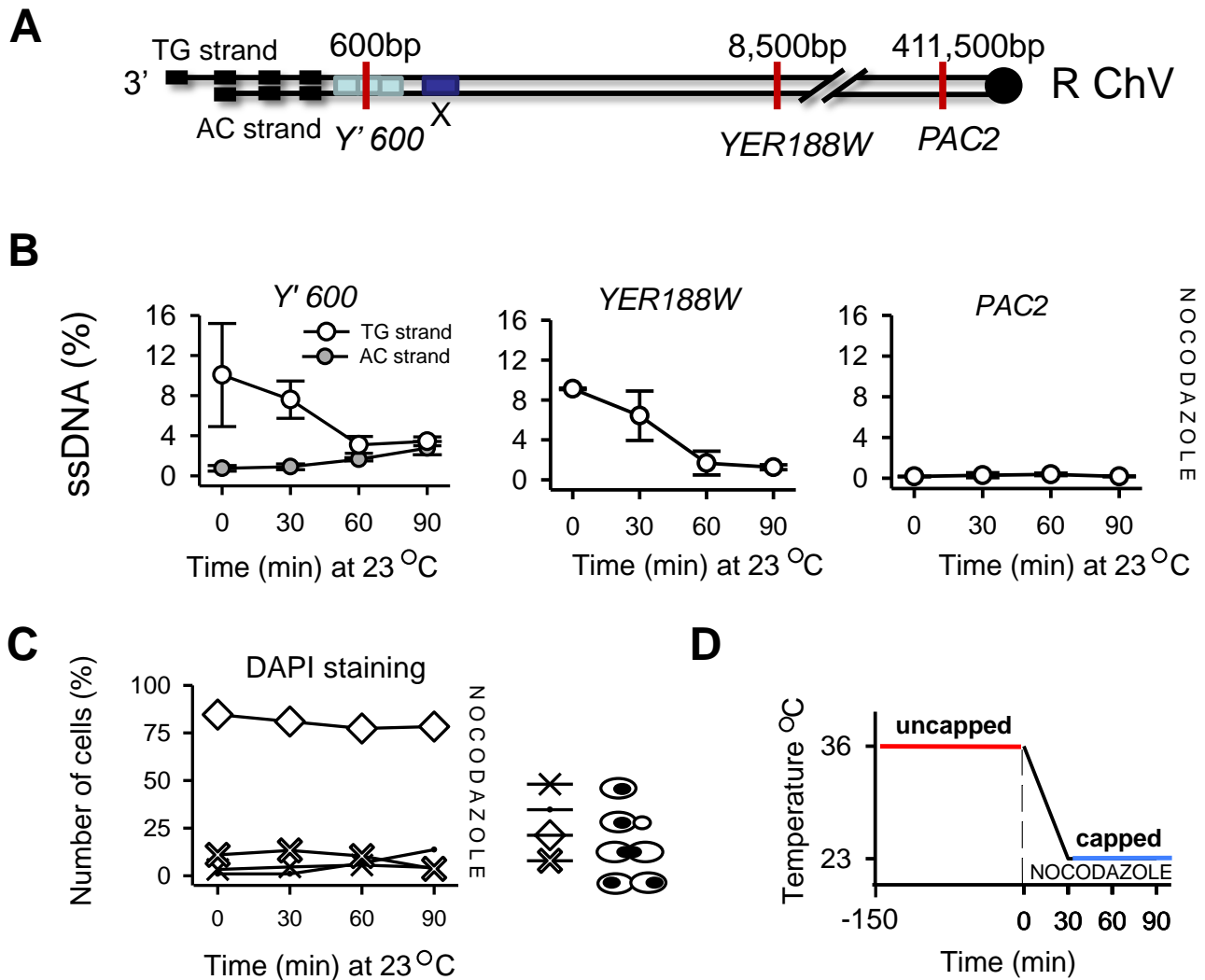
I also tested if BrdU would be incorporated in *cdc13-1* cells arrested at G2/M phase at restrictive temperatures. The cells were grown at 27° C for 3 hours and BrdU was applied for one more hour before sample collection (Figure 7A). Interestingly, *cdc13-1* showed around 5% BrdU incorporation at *Y' 600* and *PAC2* loci even though 91% of the cells were arrested in G2/M (Figure 7A). This suggests that *cdc13-1* cells may have global DNA damage that is actively repaired during G2/M arrest. It is likely that

the observed incorporation is due to post replication repair, which is responsible to fill up any left over ssDNA from replication. A recent publication shows that post replication repair can happen in G2/M and requires traslesion synthesis (Daigaku et al, 2010).

After I have validated the BrdU system in *cdc13-1* cells, I wanted to investigate whether cells with uncapped telomeres synthesise DNA after capping is restored. *Cdc13-1 7TK+* yeast cells were grown at the non permissive temperature 36° C for 150min to induce uncapping, then returned to 23° C and treated with BrdU (Figure 7B). Nocodazole was applied to the culture to keep the cells in G2/M phase.

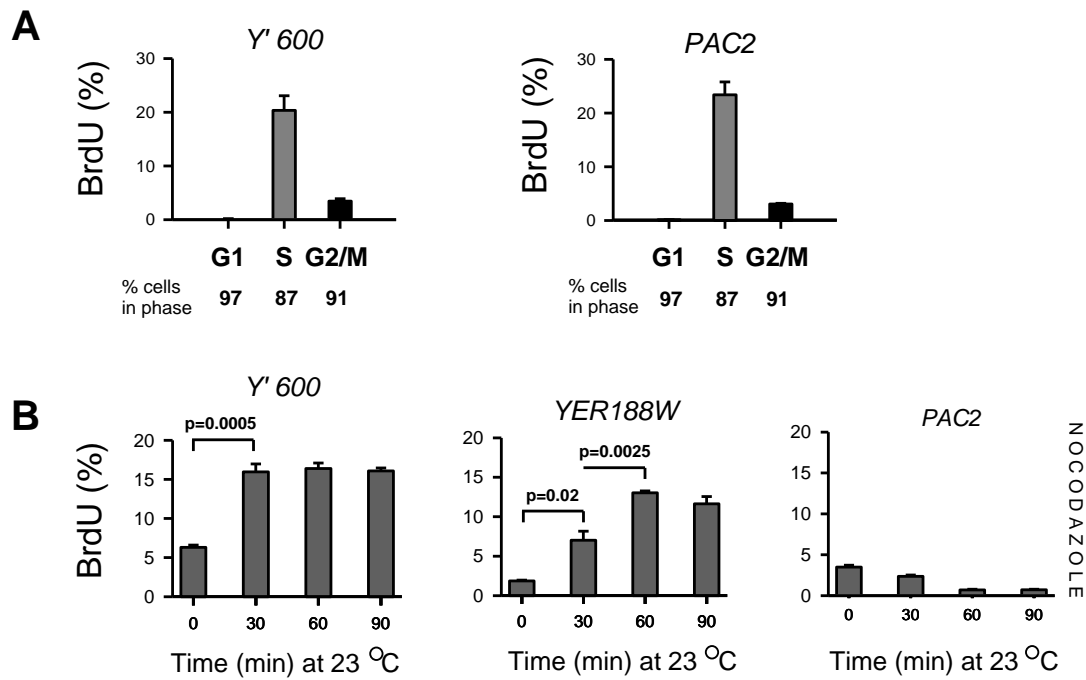
There was almost three fold increase in BrdU incorporation at *Y'600* 30min after the cells were returned to permissive temperatures and no further increase was observed at later time points (Figure 7B, *Y'600*). At *YER188W* BrdU incorporation was slower, reaching maximum values after 60min (3.5h time point) (Figure 7B, *YER188W*). BrdU was specifically incorporated at *Y'600* and *YER188W* because the signal at *PAC2* was lower and did not increase with time (Figure 7B). Thus *cdc13-1* cells re-synthesise ssDNA regions close to the chromosome end when they are returned to permissive temperatures and this can happen even when the cells are arrested in G2/M phase.





**Figure 6** Single stranded DNA production in *cdc13-1* during telomere recapping

- Schematic representation showing the right arm of chromosome V with indicated loci of interest and their distance from the chromosome end.
- Cdc13-1* (LMY206) cells were cultured for 150min at 36° C (restrictive temperature), then returned to 23° C (permissive temperature) and treated with 1.5µg/ml nocodazole to keep the cells in G2/M phase. White circles show QAOS measurements of ssDNA at *Y' 600*, *YER188W* and *PAC2* loci after *cdc13-1* cells were returned to permissive temperatures. Error bars indicate the standard deviation. The experiment was repeated three times. T-test between 0min and 60min time points indicates  $p=0.0953$  for *Y' 600* and  $p=0.0002$  for *YER188W*.
- Percentage of *cdc13-1* cells in the different cell as scored by DAPI staining of the nucleus
- Scheme showing the experimental design



**Figure 7** BrdU incorporation in *cdc13-1* during telomere recapping

- A. *Cdc13-1* 7TK+ (LMY784) cells were arrested in G1 with 33nM alpha factor and treated with 200µg/ml BrdU (indicated as G1) or the cells were left to go through S phase in the presence of BrdU (grey bar). Another culture was grown at 27° C for 3 hours and then treated with BrdU (black bar). Samples were collected 1 hour after BrdU was supplemented. The BrdU labelled DNA was extracted with phenol-chloroform, sheared by sonication and combined with anti BrdU antibody. The bound fragments were pulled down with dynabeads and measured for BrdU enrichment at *Y'600* and *PAC2* relative to the total DNA by qPCR. Error bars represent the standard deviation. This experiment was repeated three times.
- B. *Cdc13-1* 7TK+ cells were grown at 36° C 150min, treated with 200µg/ml BrdU and 1.5µl/ml nocodazole and returned to 23° C. Samples were collected every 30min and BrdU enrichment detected with anti BrdU antibody was measured at *Y'600*, *YER188W* and *PAC2* by qPCR as in A. Error bars represent the standard deviation between three qPCR measurements of the same sample. The experiment was repeated two times. The unpaired t-test was performed with GraphPad.

### 3.2 Polymerase $\delta$ and $\epsilon$ associate with ssDNA during re-synthesis

After I have shown that ssDNA is re-synthesised following telomere recapping I wanted to understand which polymerases perform this re-synthesis. In an UP-DOWN assay serially diluted *cdc13-1* cells were inoculated on agar plates and cycled between 4h incubation at restrictive temperatures (35°C), followed by 4h incubation at permissive temperature (23°C). After 3 cycles the cells are kept at 23°C until colony formation (Figure 8A). Since the cells re-synthesise their ssDNA when returned to permissive temperatures this assay could identify genes required for ssDNA repair at uncapped telomeres.

Several ways for re-synthesis of ssDNA in G2/M can be envisioned. First, HR is likely to operate in *cdc13-1* because the cells arrest in G2/M, the predominant cell cycle phase in which HR is active. Furthermore immortalised *cdc13-1* yeast forms Rad52 dependent survivors (Zubko & Lydall, 2006). *RAD52* and non essential subunits of polymerase  $\epsilon$  (*DPB3* and *DPB4*) and polymerase  $\delta$  (*POL32*) all of which are involved in HR were knocked out in the *cdc13-1* background. Second, PRR is also active in G2/M, thus *RAD18*, responsible for translesion synthesis control was also tested for contribution to ssDNA repair at uncapped telomeres (Daigaku et al, 2010). Deletion of the checkpoint gene *RAD9*, known to be essential for *cdc13-1* survival through the UP-DOWN assay was used as a control (Blankley & Lydall, 2004). *Rad9 $\Delta$  cdc13-1* cells fail to form colonies after the UP-DOWN assay because the cells have a defective checkpoint arrest, allowing them to proliferate during ssDNA accumulation. This leads to rapid cell death due to loss of essential genes.

Interestingly the *POL32* subunit of polymerase  $\delta$  seemed to be required for *cdc13-1* survival because *cdc13-1 pol32 $\Delta$*  cells formed less colonies in the UP-DOWN assay (Figure 8A). This has also been confirmed in a recent paper looking at genes required from *cdc13-1* survival (Addinall et al, 2011). It is known that *POL32* plays a role in recombination-dependent survivor formation presumably because it is required to establish a full replication fork in the absence of an origin of replication (Lydeard et al, 2007). However *POL32* seems to act independently of HR in *cdc13-1* cells because *rad52 $\Delta$  cdc13-1* double mutants survived in the UP-DOWN assay as well as

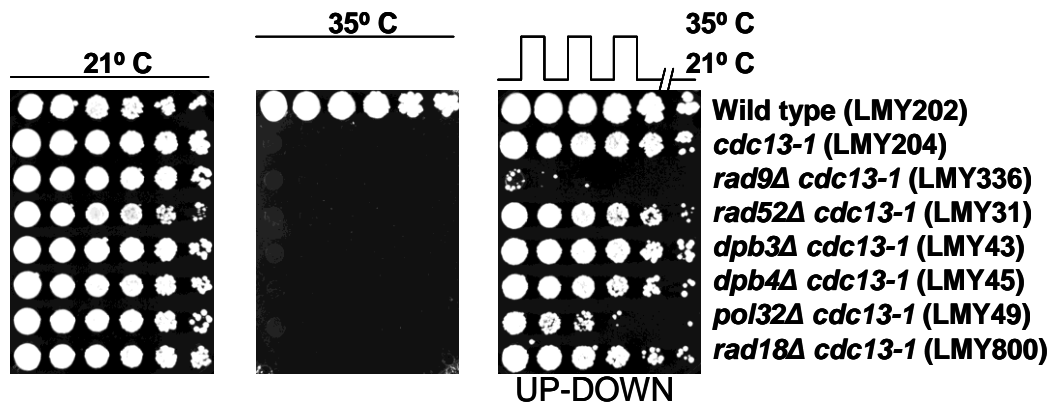
*cdc13-1* only (Figure 8A). The *DPB3* and *DPB4* subunits of polymerase  $\epsilon$  and PRR did not seem to play a role in *cdc13-1* survival.

Since *POL32* was required in *cdc13-1* with uncapped telomeres, I wanted to understand if polymerase  $\delta$  was recruited to the ssDNA damage after capping is restored and thus mediate repair. Polymerase  $\epsilon$  was used as a control because *dpb3 $\Delta$  cdc13-1* and *dpb4 $\Delta$  cdc13-1* survived the UP-DOWN assay. Furthermore I also tested Rap1 as a control for a protein usually associated with telomeres. The cells were first grown in liquid culture at the non permissive temperature 36°C for 2.5h. Then the culture was returned to 23°C and nocodazole was applied after the temperature was decreased to ensure that recruitment happens during G2/M and not because the cells re-entered another cell cycle (as in Figure 6D). Chromatin was isolated from *cdc13-1* cells and the catalytic subunit of polymerase  $\delta$ , Pol3; the catalytic subunit of polymerase  $\epsilon$ , Pol2 and the telomere binding protein Rap1 were immunoprecipitated. *ERG26*, situated close to the centromere, where no DNA damage is present in *cdc13-1* cells, was used as a control locus where no specific polymerase binding is expected (Figure 9).

Interestingly, Pol3 and Pol2, were recruited even at restrictive temperature to both loci close to the telomere but not near the centromere at *ERG26* (Figure 9A and B). Pol3 binding at *Y' 600* remained constant with a modest increase at 3.5h, while at *YER188W* it increased 30min (3h time point) after return to permissive temperature and then continued to decline (Figure 9A). Pol2 binding at *Y' 600* decreased gradually over the time course reaching more than 2 fold difference (Figure 9B *Y' 600*). At *YER188W* Pol2 binding reached a more than 2 fold decrease in the first 30min (3h time point) and remained low for the rest of the time course (Figure 9B *YER188W*).

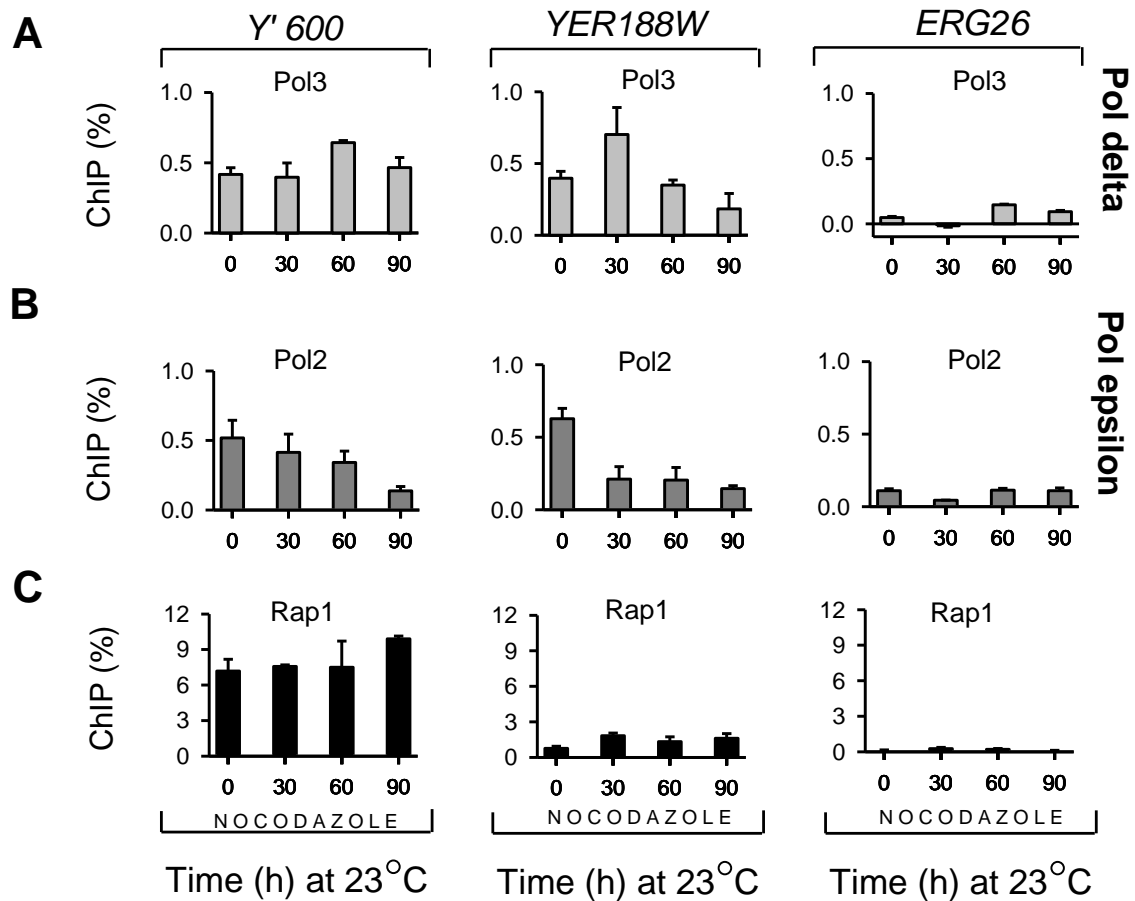
The binding of Rap1 at *Y'600* remained constant throughout the experiment, except for a small increase at 4h that could indicate recapping of the telomere (Figure 9C, *Y'600*). Very little association of Rap1 was found at *YER188W* and *ERG26*, confirming that Rap1 is only binding at repetitive loci close to the telomere even when telomeres are uncapped (Figure 9C)

This data shows that Pol3 and Pol2 are recruited specifically to ssDNA damage even at restrictive temperatures when telomeres were uncapped (Figure 9A and B 2.5h time point). After recapping the binding of these proteins decreased gradually, correlating with ssDNA decrease and BrdU incorporation but Pol2 remained associated for longer (Figure 6B and 9).



**Figure 8** UP-DOWN assay with indicated yeast strains

Yeast strains indicated on the right were serially diluted, spotted on agar plates and incubated at indicated temperatures for 4-5 days. The UP-DOWN plate was incubated for 4h at 21° C followed by 4h at 35° C. This was repeated three times and then the plates were returned at 21° C until day 5. Every strain was tested multiple times and behaved similarly compared to wild type.



**Figure 9** Pol3, Pol2 and Rap1 recruitment during *cdc13-1* telomere recapping

A. Recruitment of Pol3 at *Y' 600*, *YER188W* and *ERG26* (1kb away from the centromere of chromosome 7) as measured by ChIP. *Cdc13-1 POL3::HA* (LMY772) cells were cultured for 2.5h at 36° C (restrictive temperature), then returned to 23° C (permissive temperature) and treated with 1.5µg/ml nocodazole to keep the cells in G2/M phase. Pol3 was immunoprecipitated with 3ng monoclonal anti-HA antibody (11867423001, Roche). Error bars represent the sum of the standard deviations of the IP and background samples between three qPCR measurements. The experiment was repeated two times.

B. Recruitment of Pol2 in LMY772 at *Y' 600*, *YER188W* and *ERG26* as in B. Pol2 was immunoprecipitated with 3ng polyclonal anti-Pol2 antibody (sc-6753, Santa Cruz). The experiment was repeated three times.

C. Recruitment of Rap1 in LMY772 at *Y' 600*, *YER188W* and *ERG26* as in B. Rap1 was immunoprecipitated with 3ng polyclonal anti-Rap1 antibody (sc-6663, Santa Cruz). The experiment was repeated once, but the same result was obtained from Yuan Xue and Michael Rushton in independent experiments.

### 3.3 DNA polymerases are recruited to ssDNA damage at telomeres

In my previous experiments I have seen that polymerase subunits were recruited to telomeres even when the telomeres were uncapped (Figure 9, time point 2.5h). Therefore I wanted to investigate polymerase recruitment during accumulation of telomere damage. *Cdc13-1* mutants were cultured overnight at 23 °C and shifted to the non permissive temperature 27 °C for 480min. Samples for QAOS, DAPI staining and ChIP were collected every 160min. As expected *cdc13-1* cells gradually accumulated up to 10% ssDNA at both loci close to the chromosome end but not near the centromere at *PAC2*, where ssDNA was around 1% (Figure 10A). No ssDNA was observed on the AC strand (Figure 10A, *Y'600* grey circles) DAPI staining revealed that at 0min, when the telomeres were still functional, the cells were in all stages of the cell cycle, confirming that proliferation was not compromised at the permissive temperature (Figure 10B). After 160min at the non-permissive temperature, over 69% of the cells were already arrested in G2/M phase (nucleus between mother and daughter cells), reaching over 85% by 8 hours (Figure 10B).

The ChIP measurements at *PAC2* locus near the centromere were subtracted from *Y'600* and *YER188W* to allow better interpretation of the binding close to chromosome ends. This way the specific binding of proteins to *Y'600* and *YER188W* above background could be visualised (Figure 10 and 11). At 0min, the catalytic subunit of Polymerase  $\alpha$ , Pol1 associated near chromosome ends and this association was 3 times higher at *Y'600* than at *YER188W* (Figure 10C). Pol1 interacts with Cdc13 as well as with *cdc13-1* and this interaction might be required for the synthesis of the AC strand after telomerase elongation (Qi & Zakian, 2000). Therefore the higher association of Pol1 with *Y'600* at 0min could be mediated through its interaction with *cdc13-1*. When the cells were grown at restrictive temperature, binding of Pol1 increased more than three fold at both loci close to the telomere, while it stayed below 0.5% at *PAC2* (Figure 10C).

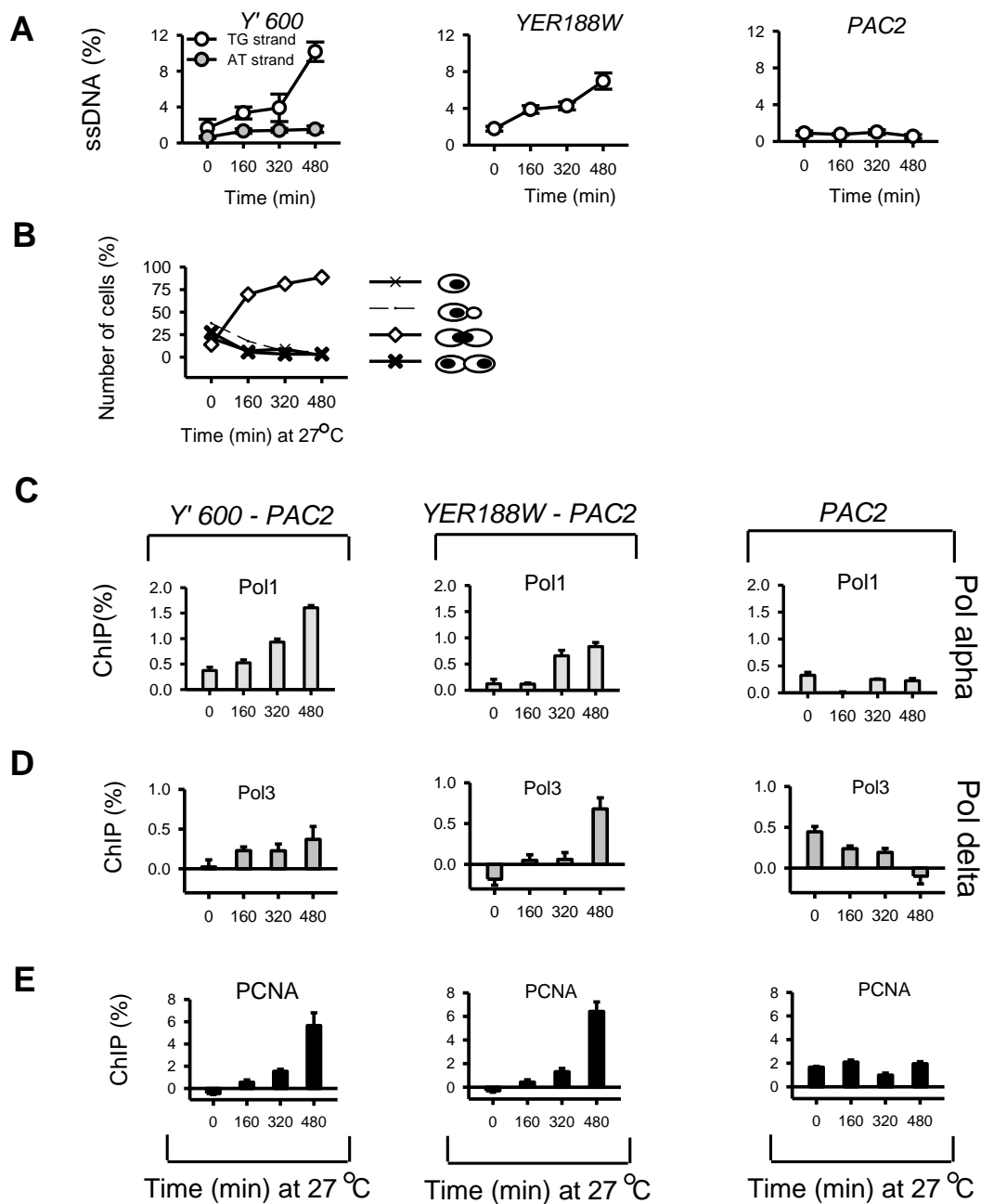
The catalytic subunit of polymerase  $\delta$  and its clamp PCNA behaved similarly close to the telomere. No telomere specific association was observed at 0min, but after uncapping binding increased with time (Figure 10D and E). The association of Pol3 but not PCNA decreased after onset of G2/M arrest at *PAC2*. This decrease was not

due to damage, because no accumulation of ssDNA was observed at that locus. Since polymerase  $\delta$  is extensively involved in DNA replication it is likely that the binding close to the centromere is due to cells in S phase (Budd & Campbell, 1993). When the cells arrested in G2/M the binding decreased at *PAC2* but increased close to the telomere, where ssDNA accumulated (Figure 10D).

Binding of three different subunits from polymerase  $\epsilon$  Pol2, Dpb11 and Dpb2 increased upon telomere uncapping close to the telomere, but remained constant near the centromere (Figure 11A, B and C). Furthermore the telomere binding protein Rap1 decreased binding at *Y'600*, while no increased association was observed at *YER188W* or *PAC2* (Figure 11D). This finding is in agreement with Rap1 binding only to telomeric repeats and shows that upon uncapping Rap1 dissociates from telomeres probably due to loss of the double stranded DNA binding sequence required from Rap1 recruitment to telomeres.

Taken together my data suggests that different polymerase subunits associate specifically to loci that accumulate ssDNA. Furthermore this effect was observed in cells arrested at G2/M phase and can not be explained by association during DNA replication, because only around 6% of the cells were budding at 320 and 480min (Figure 10B).





**Figure 10** Accumulation of ssDNA and recruitment of Pol1, Pol3 and PCNA after telomere uncapping (text continues on next page)

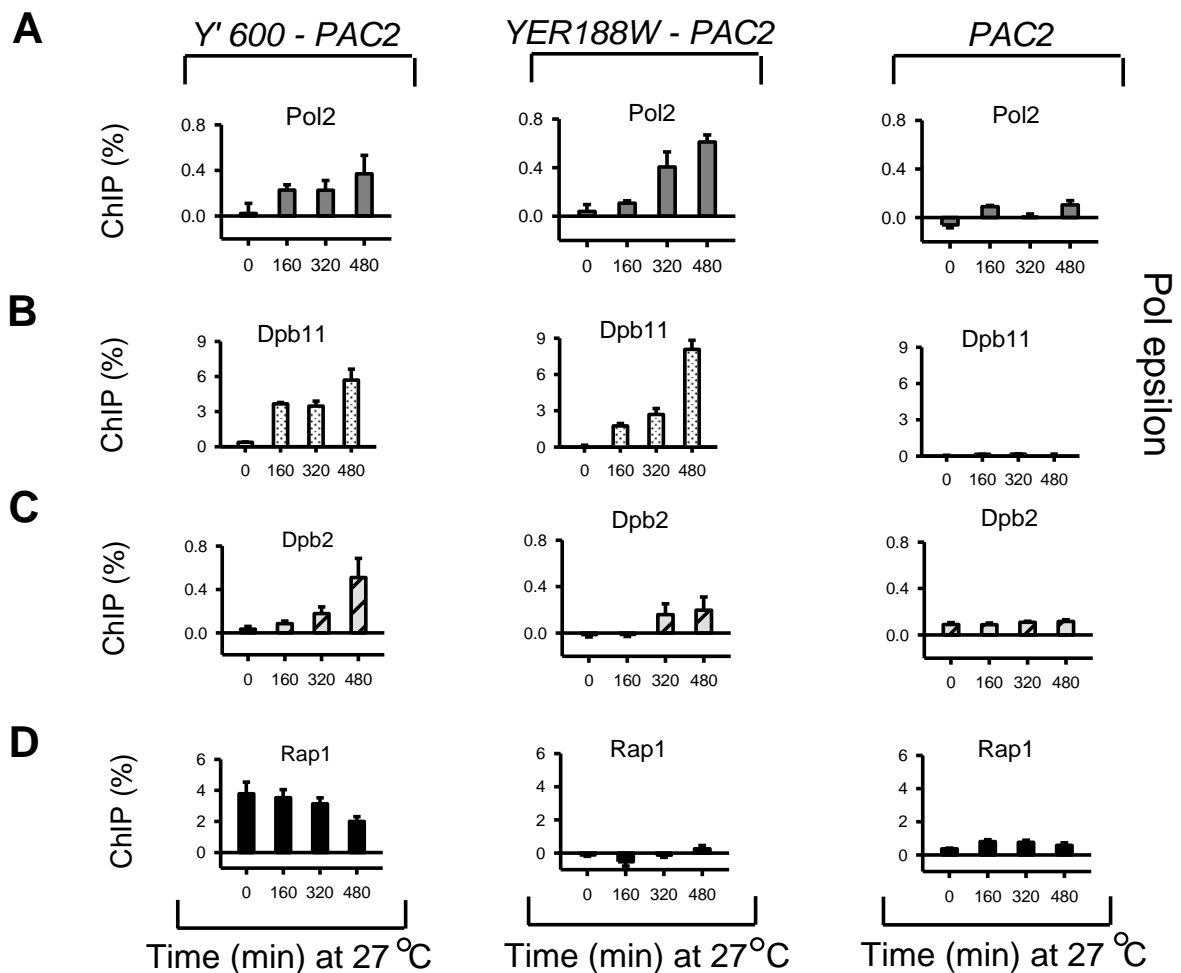
A. QAOS measurement of ssDNA at *Y' 600*, *YER188W* and *PAC2* loci. Error bars show the standard deviation between three replicates from the same sample. LMY 206 was grown overnight at 23° C and diluted to  $2 \cdot 10^7$  cells/ml in the morning. Afterwards the temperature was shifted to 27° C and samples were collected every 160min The experiment was performed four times.

B. Percentile distribution of cells in different stages of the cell cycle as scored by DAPI staining

C. Chromatin immunoprecipitation of Pol1 in *cdc13-1 POL1::HA* cells (LMY746). Pol1 associated DNA was pulled down with 3ng monoclonal anti-HA (11867423001, Roche). The ChIP data at *PAC2* was subtracted from *Y' 600* and *YER188W* signal to show the specific association of the Pol1 to these loci, relative to the *PAC2* background. Error bars represent the sum of the standard deviations of the IP and background samples between three qPCR measurements. Small error bars may not be visible on some bars. The experiment was repeated twice.

D. ChIP of Pol3 in *cdc13-1 POL3::HA* cells (LMY772). Pol3 associated DNA was pulled down with 3ng monoclonal anti-HA (11867423001, Roche). The experiment was repeated twice.

E. ChIP of PCNA in *cdc13-1* cells (LMY205). PCNA associated DNA was pulled down with 6ng monoclonal anti-PCNA antibody (NB500-106, Novus Biological). The experiment was repeated three times.



**Figure 11** Recruitment of Pol2, Dpb11, Dpb2 and PCNA after telomere uncapping

A. Chromatin immunoprecipitation of Pol2 in *cdc13-1 Dpb2::MYC* cells (LMY493). Pol2 associated DNA was pulled down with anti-Pol2 antibody (sc-6753, Santa Cruz). The ChIP data at *PAC2* was subtracted from *Y'600* and *YER188W* signal to show the specific association of the Pol2 to these loci, relative to the *PAC2* background. Error bars represent the sum of the standard deviations of the IP and background samples between three qPCR measurements. Small error bars may not be visible on some bars. The experiment has been repeated three times.

B. Same as in A. but for Dpb11. Dpb11 associated DNA was pulled down with 3ng polyclonal anti-Dpb11 antibody (sc-12007, Santa Cruz). The experiment has been repeated three times.

C. Same as in A. but for Dpb2. Dpb2 associated DNA was pulled down with 3ng monoclonal anti-MYC antibody (Sc-40, Santa Cruz). The experiment has been repeated three times.

D. Same as in A. but LMY772 was used instead and Rap1 associated DNA was pulled down with 3ng polyclonal anti-Rap1 antibody (sc-6663, Santa Cruz). The

experiment was repeated once, but the same result was obtained from Yuan Xue and Michael Rushton in independent experiments.

### 3.4 Discussion

Although telomeres are prone to DNA damage there is currently no data showing telomere repair under normal circumstances in the literature. In yeast and mammals intact telomeres seem to inhibit HR and NHEJ to prevent recombination, telomere fusion and genomic instability (Marcand et al, 2008; Tong et al, 2011). Moreover cells seem to also tolerate telomere damage since healthy mammalian telomeres are hypersensitive to UV irradiation but the pyrimidine dimers are not repaired (Kruk et al, 1995; Rochette & Brash, 2010).

In this chapter I have shown that following telomere uncapping polymerases are also specifically recruited to ssDNA and yeast can use a DNA re-synthesis strategy after telomere recapping (Figure 12). When telomere uncapping was restored the cells gradually lost the ssDNA and re-synthesised the missing 5' strand most probably by using polymerase  $\alpha$ ,  $\epsilon$  and  $\delta$  (Figure12C).

#### **How is ssDNA re-synthesised in *cdc13-1* cells?**

During HR repair of an induced HO cut polymerase  $\alpha$ /primase complex and polymerase  $\delta$  are essential for initiation and elongation of the primer and polymerase  $\epsilon$  is required for further extension of the newly synthesized DNA (Lydeard et al, 2007). However *cdc13-1 rad52 $\Delta$*  cells, defective in HR could form colonies in an UP-DOWN assay, confirming that *RAD52* is not required for *cdc13-1* survival in the presence of DNA damage (Figure 8). Instead polymerase  $\alpha$ ,  $\delta$  and  $\epsilon$  were recruited to ssDNA damage and possibly participate in DNA re-synthesis after recapping of the telomere.

In the case of polymerase  $\epsilon$ , although *DPB3* and *DPB4* were not required for *cdc13-1* survival the catalytic subunit Pol2 as well as the essential subunits Dpb2 and Dpb11 were clearly associated with ssDNA and may still participate in repair. Therefore it seems like polymerase  $\epsilon$  might not require Dpb3 and Dpb4 for DNA synthesis *in vivo*. Polymerase  $\epsilon$  is also involved in the S phase checkpoint because *POL2* mutants activate damage specific gene transcription during G1 but not in S phase (Navas et al, 1996). Thus, another reason for Pol2 association could be due to a checkpoint function. The *DPB11* subunit of polymerase  $\epsilon$  can stimulate Mec1 activation *in vitro* in conjugation with the 9-1-1 complex (Navadgi-Patil & Burgers, 2008). Furthermore

Dpb11-Pol2 interaction is crucial for assembly of the replication complex, suggesting that the checkpoint function of polymerase  $\epsilon$  might be mediated through *DPB11* only (Masumoto et al, 2000). It is not clear whether polymerase  $\epsilon$  is involved in checkpoint activation and/or in repair at uncapped telomeres, however it is known that Dpb11 participates in the checkpoint response to UV damage in G2/M arrested cells with nocodazole (Puddu et al, 2011).

Polymerase  $\delta$  appeared to stay associated with the DNA longer, showing that it may have a different role than polymerase  $\epsilon$  (Figure 9). The decrease in ssDNA measured by QAOS does not necessarily mean that repair has been completed. The assay can only show whether a certain locus is single stranded or double stranded at each time point. Therefore many ssDNA gaps undetected by QAOS might exist and re-synthesis could still be happening even when ssDNA measurements by QAOS are low. This means that polymerase  $\delta$  may still be extending Okazaki fragments. Furthermore during lagging strand replication polymerase  $\delta$  is also important for the creation of a ligatable nick and thus could be associated for longer to aid ligation (Jin et al, 2003).

During telomere replication telomerase adds TG repeats to the 3' overhang leading to a longer ssDNA stretch. This is then re-filled by the DNA replication machinery. Polymerase  $\alpha$ /primase creates short RNA-DNA primers that are then extended by polymerase  $\delta$  and later ligated by the yeast ligase Cdc9 (Waga & Stillman, 1998). Polymerase  $\epsilon$  is not essential for telomere replication but could be important when the overhangs are longer similarly to DSB repair (Budd & Campbell, 1993; Lydeard et al, 2007). Therefore it seems like after recapping, the resected strand is just filled up by DNA synthesis machinery using the 3' overhang as template. However ssDNA re-filling at uncapped telomeres is more complicated because the overhang is much longer and would require several priming sites as shown by recruitment of Pol1 not only at *Y'600* but also at 8500bp away from the telomere. The primers are then extended by at least polymerase  $\delta$  and/or  $\epsilon$  (Figure 12C).

### **Why is ssDNA re-synthesis in *cdc13-1* at restrictive temperatures not sufficient?**

Perhaps the most striking finding was that polymerases were recruited specifically to damaged loci although this did not seem to lead to successful re-synthesis because the cells continued to accumulate ssDNA (Figure10). Repair was only observed when the cells were returned to permissive temperatures, which restores the *cdc13-1* defect. At least two models to explain this effect can be envisioned: a deliberate DNA re-

synthesis block or a constant competition between resection and re-synthesis at uncapped telomeres (Figure 12A and B).

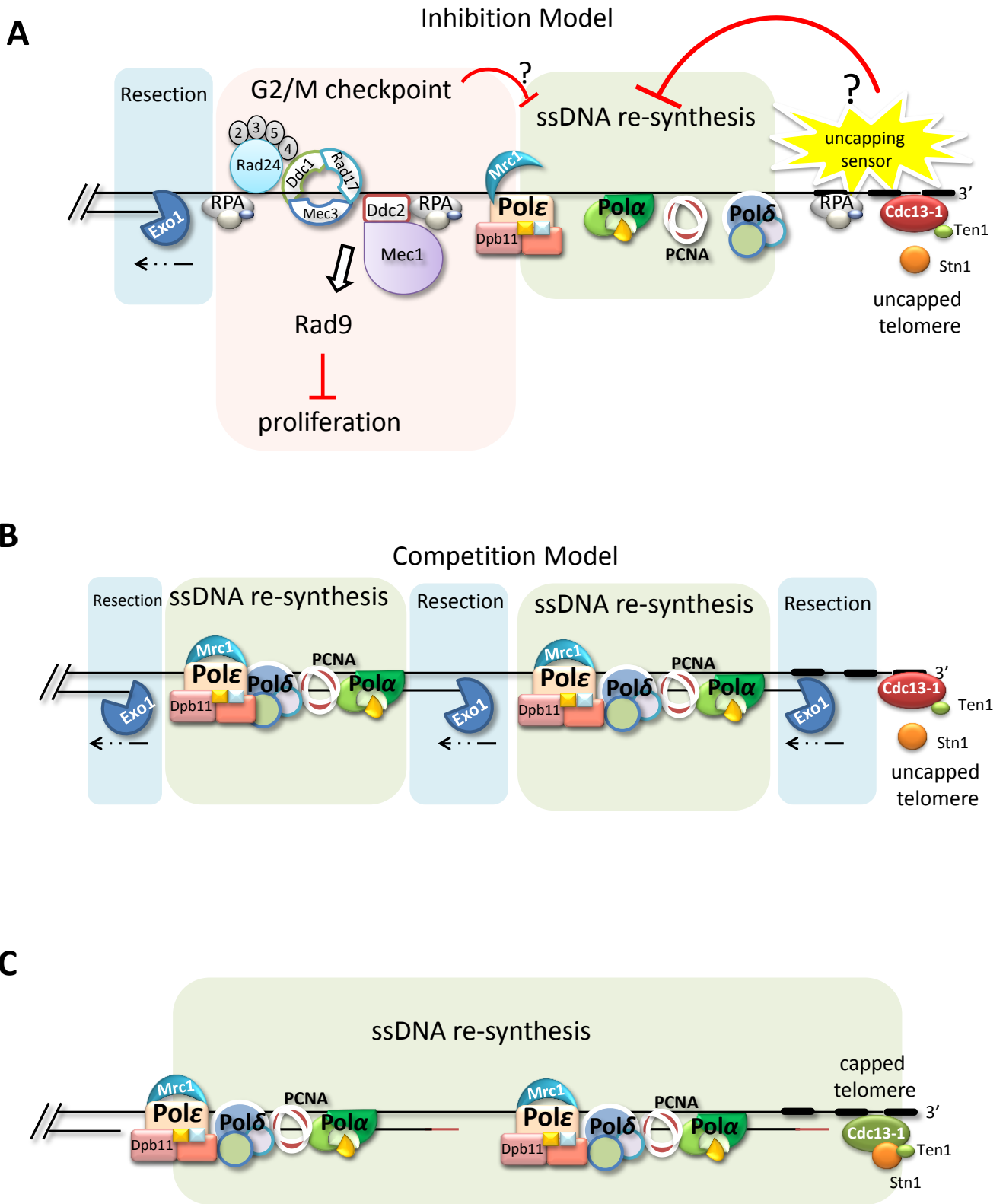
If synthesis is specifically inhibited the cells must pose a telomere uncapping “sensor”, which would be only active during uncapping (Figure 12A). Therefore DNA repair could take place only when the cells were returned to permissive temperatures and proper capping by *cdc13-1* was restored. The literature suggests that *cdc13-1* exhibits reduced interaction with Stn1 at restrictive temperatures (Wang et al, 2000). Therefore Stn1 binding to Cdc13 might function as the “sensor” for uncapped telomeres. Stn1 could influence DNA synthesis by regulating primer formation through its interaction with the Pol12 subunit of DNA polymerase  $\alpha$ /primase. However it is not clear how Stn1 which is supposedly bound to the very end of telomeres would also regulate repair 14kb away at *YER188W* locus. The exonuclease Exo1 can inhibit DSB repair at an induced HO cut, although the mechanism for this inhibition is not clear (Lydeard et al, 2010a). Therefore the checkpoint response itself is also a candidate for ssDNA re-synthesis inhibition at telomeres. Because ChIP can only confirm association with chromatin it is not clear whether recruited polymerase subunits are active or if they are assembled correctly. Therefore it is not clear whether the “sensor” inhibits synthesis or assembly.

In the second model competition between DNA resection and repair occurs (Figure 12B). It is clear that the *cdc13-1* defect leads to accessibility of nucleases to the 5' end. Therefore even if polymerases re-synthesise part of the DNA it can be attacked again by exonucleases and this would continue until the telomere is capped and Cdc13 can protect the 5' end. Once capping is restored the primer most proximal to the telomere end would be elongated. The newly synthesised DNA would not be attacked by nucleases and full repair of the ssDNA would be possible (Figure 12B).

An alternative explanation for the lack of repair at uncapped telomeres is that not all factors required for ssDNA re-synthesis are available at uncapped telomeres. ChIP can only confirm recruitment of a protein in close proximity to the DNA but can not confirm correct loading or activity. A large number of other proteins usually required for DNA replication are also required for DSB repair (Lydeard et al, 2010b). At an HO induced cut, repair could occur in the presence of the DNA replication licensing factor Cdt1 but did not require the pre-replicative complex. All other replication factors were essential for DSB repair, including the helicase Mcm2-7, the DNA synthesis initiation factors Cdc45, Dpb11, Sld3, Mcm10, Ctf4, Cdc7 and the GINS

complex (Lydeard et al, 2010b). Since uncapped telomeres are similar to DSBs, similar requirements for DNA re-synthesis may apply at uncapped telomeres. Thus it is possible that re-synthesis is absent or not efficient due to the lack of some of those factors. ChIP targeting all those proteins might reveal if some are missing and only recruited after recapping. Nevertheless, primers might have been synthesised at uncapped telomeres because PCNA can only be loaded on the primer/template junction (Tsurimoto & Stillman, 1991).





**Figure 12** Models explaining how cells deal with ssDNA at uncapped telomeres and after recapping

## 4 Extracellular salt facilitates repair at uncapped telomeres requires polymerases $\delta$ and $\epsilon$

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In chapter III, I have shown that single-stranded DNA resulting from conditional telomere uncapping could be re-synthesised. This re-synthesis was only detectable after recapping but does not exclude re-synthesis during DNA damage accumulation. Therefore I hypothesise that some factors may stimulate ssDNA damage repair and rescue the lethal phenotype of *cdc13-1* cells with uncapped telomeres. These may include intracellular regulatory pathways that regulate repair. However our laboratory has previously discovered that some extracellular factors may contribute to *cdc13-1* rescue, such as inorganic salts *e.g.* NaCl, KCl, MgCl<sub>2</sub> and CaCl<sub>2</sub> (Maringele unpublished data). This finding provides a potential link between environmental conditions and intracellular responses to DNA damage because *cdc13-1* cells were able to grow at restrictive temperatures in the presence of small amounts of inorganic salts. In this chapter I investigated if and how salt could induce ssDNA re-synthesis. For simplicity only NaCl was used in further experiments. There were several reasons for choosing NaCl instead of any other salt:

- a. To avoid complications arising from cations participating in crucial signalling pathways. For example Ca<sup>2+</sup> is a major intracellular signal mediator and its concentrations in the cells can control different stress response pathways (Araki et al, 2009; Denis & Cyert, 2002).
- b. NaCl is abundant in the environment of uni- and multicellular organisms.
- c. Different types of cells have different environmental requirements. It is not clear what is the natural NaCl concentration surrounding yeast in the wild. However, the concentration of NaCl in yeast media was only 20mM, compared to 150mM in human serum (Table 3). Therefore it is interesting to know how environmental changes could affect cellular responses and in particular DNA repair.

I decided to supplement NaCl to yeast media up to 170mM or 450mM NaCl and study the influence of these NaCl concentrations on telomere uncapping. Yeast media originally contained 20mM NaCl and cells grown in normal media were considered untreated.

Cation content in cell media, human serum and sea water				
Cation	YEPD	D-MEM	Serum	Sea Water*
Na <sup>+</sup>	~20mM	55mM	~150mM	~430mM
K <sup>+</sup>	9mM	2.8mM	5mM	9mM
Mg <sup>2+</sup>	0.3mM	0.17mM	1mM	106mM
Ca <sup>2+</sup>	0.05mM	0.64mM	1.44mM	18.5mM

**Table 3** Cation concentrations in YEDP, D-MEM, human serum and sea water.

The values presented for YEPD and D-MEM were obtained from the manufacturer BD Biosciences and Invitrogen respectively. Human serum is according to (Kramer & Tisdall, 1922) and sea water is according to (Crenshaw, 1972)

\*North Pacific Ocean

#### 4.1 Extracellular NaCl prevents G2/M arrest in *cdc13-1* cells and protects the telomere

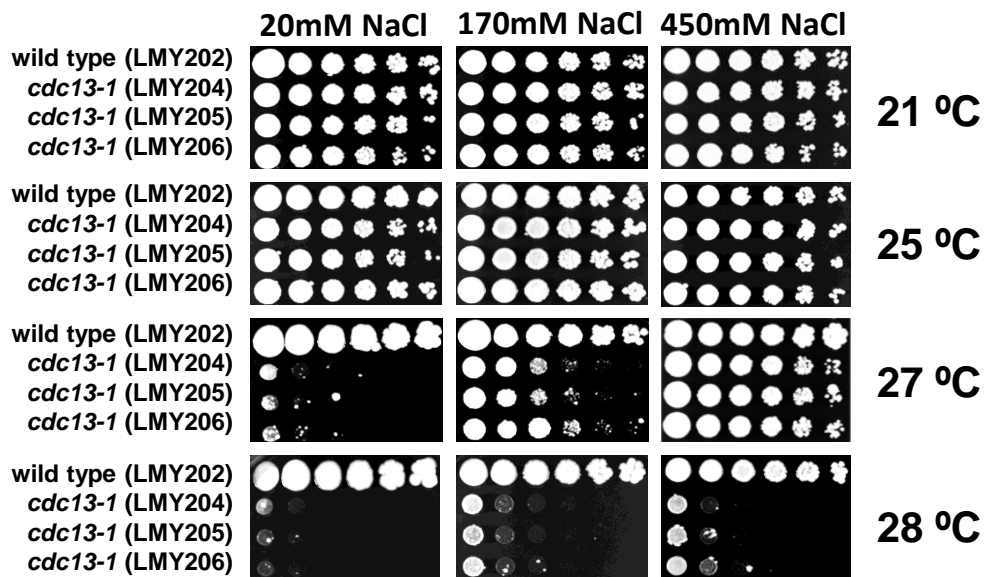
To understand whether extracellular salts can influence cells with damaged telomeres three independent *cdc13-1* strains were spotted on agar plates containing different amounts of NaCl (20mM, 170mM and 450mM NaCl). At the permissive temperatures 21 °C and 25 °C *cdc13-1* cells grew as well as wild type regardless of NaCl concentration in the media (Figure 13). As a result of telomere uncapping at the non-permissive temperature 27 °C, the mutants were not growing in normal conditions while wild type continued to grow well. Intriguingly, when further NaCl was supplemented to the media *cdc13-1* cells were rescued in a dose dependent manner. A total of 170mM NaCl allowed colony formation after 125 fold dilution, while 450mM NaCl produced a fully growing dilution series, similar to wild type. This effect was not observed at 28 °C, where *cdc13-1* failed to grow even with the highest tested concentration of NaCl.

To further understand what could be the biological significance of this mild effect,  $2 \times 10^5$  *cdc13-1* cells/ml were propagated in liquid culture for 5 days at 27° C (Figure 14A). Every day the culture was counted and diluted back to the initial concentration

to establish the growth rate. The advantage of propagation in liquid media over the spot test lies in the ability to distinguish between proliferating cells and cells that do not cycle but increase in size.

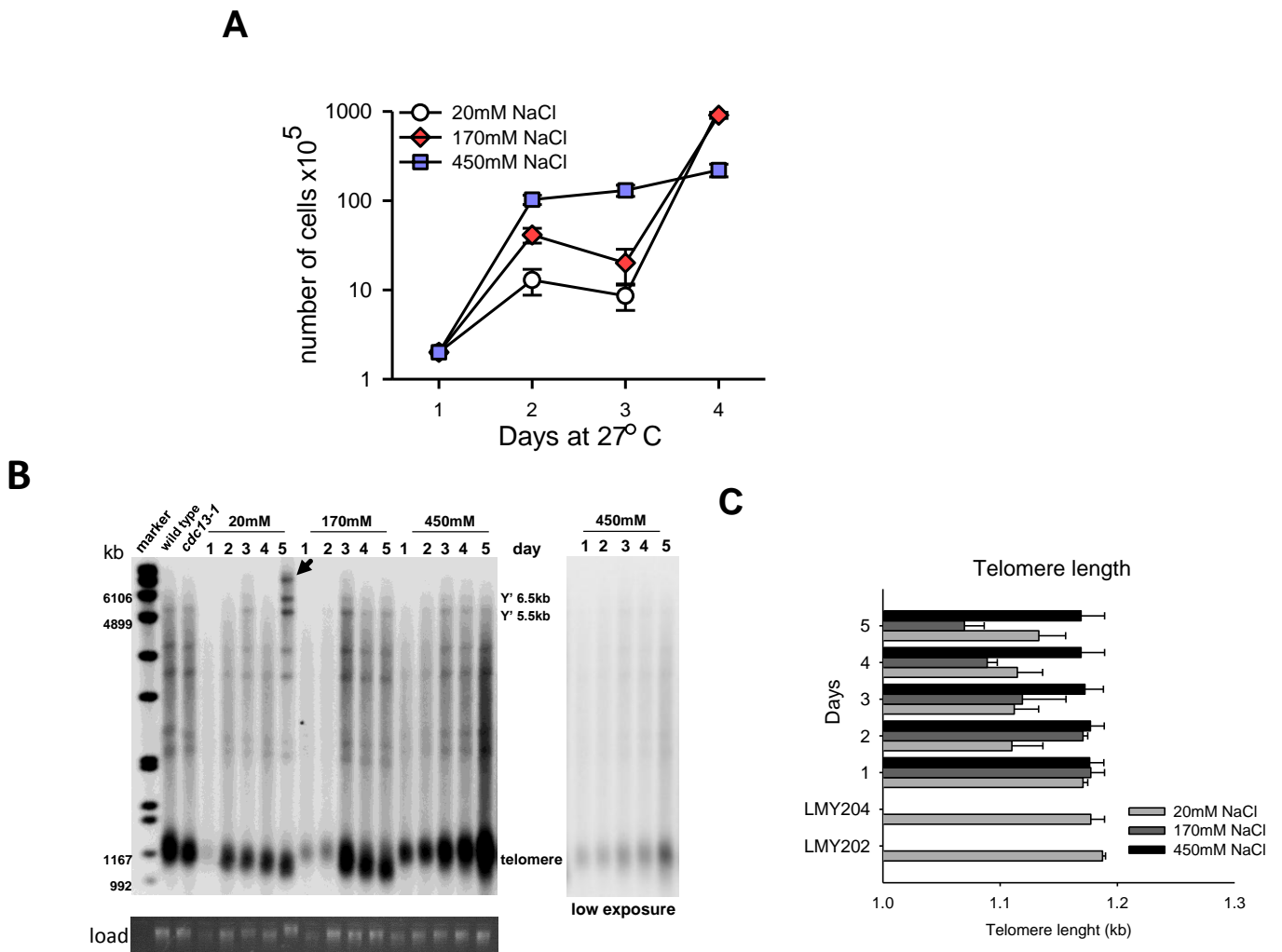
Propagation of *cdc13-1* in liquid culture showed that during the first 3 days, the non-treated cells proliferated very slowly, indicating cell cycle arrest (Figure 14A). After that the cells escaped and started to grow rapidly reaching up to  $1 \times 10^8$  cells/ml at day 4. This rapid growth could be due to formation of survivors. In contrast 170mM NaCl led to a 3 fold increase in the population doublings at day 2. At days 4 these cells also started to grow rapidly, similar to the non treated control. Hence, salt may delay cell cycle arrest because the cells treated with 170mM NaCl only slowed down proliferation at day 3. The cells propagated on 450mM NaCl continued to grow slowly after day 2 but no apparent cell cycle arrest was observed (Figure 14A). This data shows that NaCl allows proliferation of *cdc13-1* cells with uncapped telomeres and although this effect is mild it could significantly influence the survival of cells. Under higher concentration of NaCl in the media the cells coped better in restrictive conditions.

Since propagation of *cdc13-1* showed that the number of cells suddenly increases after day 3 it is possible that survivors may have formed. To test this hypothesis *cdc13-1* was again propagated in liquid culture for 5 days at 27 °C in the presence of different amounts of NaCl. A telomere blot was prepared to elucidate the changes in telomeres (Figure 14B). Interestingly the telomeres of the non-treated cells shortened gradually with every day. 170mM NaCl seemed to delay shortening because there was no change in telomere length at day 1 and 2. Furthermore, no shortening was observed at 430mM NaCl throughout the experiment. These results were confirmed in three independent *cdc13-1* strains, although only one example is shown in Figure 14B. Another interesting finding was that an additional restriction band indicated with a black arrow was observed only in the non treated sample at day 5 suggesting that *cdc13-1* could indeed form survivors at restrictive temperatures. On the contrary NaCl treated samples did not have additional restriction bands indicating that NaCl could delay survivor formation possibly by exhibiting telomere protection. The shortening of the telomere during telomere uncapping in *cdc13-1* cells suggests that the mutant is either defective in telomerase recruitment or the TG strand is degraded.



**Figure 13** *Cdc13-1* proliferation on salt at different temperatures

Three independent *cdc13-1* strains were diluted 6 times in 5 fold increments and spotted on agar plates containing 20, 170 or 450mM NaCl. The plates were grown for 3 days at the indicated temperatures and photographed. 20mM NaCl was the concentration of NaCl in commercial yeast media.



**Figure 14** Growth in liquid culture and telomere length in *cdc13-1* at restrictive temperatures

A. Three independent *cdc13-1* strains (LMY206, 204 and 205) were inoculated in liquid culture to  $2 \times 10^5$  cells/ml and propagated for 5 days at 27 °C. Every day the cells were counted and diluted to the initial concentration. The number of cells was plotted on a logarithmic scale. Error bars represent the standard deviation between the number of cells in three independent *cdc13-1* strains.

B. Samples for southern blotting were collected daily as in A, the DNA was extracted and equalised with the Nanodrop. Afterwards the DNA was cut with XhoI and the membrane was probed with a TG sequence specific probe. Marker size is indicated on the left. The black arrow highlights a new restriction band at 20mM NaCl, day 5.

C. Quantification of telomere length in three independent *cdc13-1* strains (LMY204, LMY206 and LMY205). The same experiment as in B was performed. Error bars show the standard deviation.

## 4.2 Extracellular NaCl impairs ssDNA accumulation at uncapped telomeres

Since *cdc13-1* cells arrest due to accumulated ssDNA at non-permissive temperatures following telomere uncapping it is plausible that NaCl could influence ssDNA generation. To test this hypothesis ssDNA was measured by QAOS in *cdc13-1* cells at 27 °C in liquid media (Figure 15). Fresh overnight culture was diluted in the morning and the temperature was shifted from 23° C to 27°C. The culture was split in three and either left untreated (20mM NaCl) or supplied with a final concentration of 170mM or 450mM NaCl. Samples were collected every 80min for QAOS and for DAPI staining to allow monitoring of the cell cycle.

The untreated cells accumulated over 10% ssDNA at *Y'600* and around 7% at *YER188W* on the TG rich strand during the time course (Figure 15B). Only around 1% ssDNA was measured on the AC rich strand at *Y'600* locus, confirming that degradation in *cdc13-1* progresses in 5' to 3' direction (Booth et al, 2001; Zubko et al, 2004). When NaCl was supplemented, less ssDNA accumulated at *Y'600* – 5% and only around 3% was found at *YER188W* (Figure 15B). No apparent difference in the ssDNA could be observed between samples treated with 170mM or 450mM NaCl. However, 160min after telomere uncapping over 70% of the non treated cells were arrested in G2/M phase compared to around 40% on 170mM NaCl and only 23% on 450mM NaCl (Figure 15C). This trend remained throughout the time course and indicates that even very small differences in ssDNA may influence commitment to cell cycle arrest.

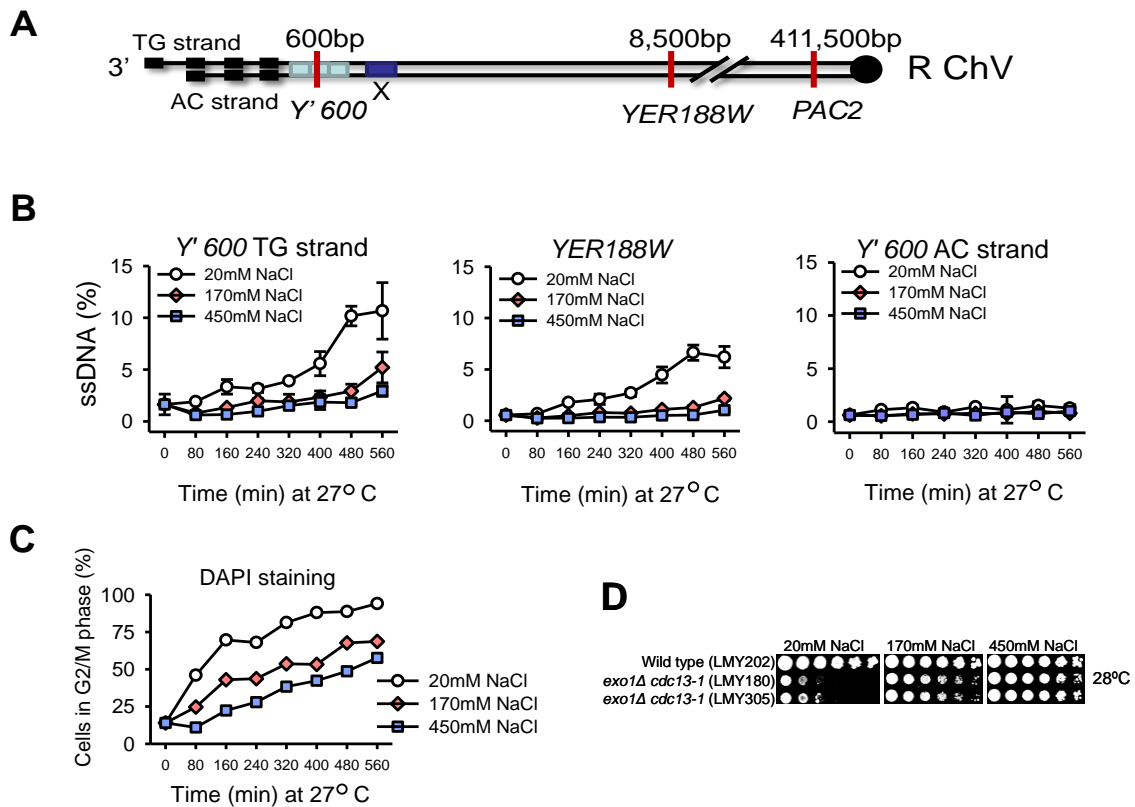
NaCl could abrogate the production of ssDNA by inhibiting the exonuclease Exo1, responsible for resection, by protecting the telomere from degradation or by inducing ssDNA re-synthesis. Deletion of *EXO1* did not seem to abolish the ability of NaCl to rescue the growth of *cdc13-1*, indicating that salt was acting independent of resection (Figure 15D).

Next I used BrdU incorporation to understand if the ssDNA could be repaired on salt. Telomere uncapping was induced at 27° C for 6 hours in *cdc13-1 7TK+* strains and BrdU was added to allow metabolization of the thymidine homologue prior NaCl addition. 30min later (6.5h time point) 170mM NaCl final concentration was supplied

to part of the culture (Figure 16A). The *PAC2* signal was subtracted from *Y'600* and *YER188W* to show specific incorporation at loci with ssDNA.

The cells remained completely arrested in G2/M phase during the time course (Figure 16B). BrdU incorporation was clearly observed at *Y'600* locus, after treatment with NaCl (Figure 16B). Incorporation was also observed at *YER188W* but it was only marginally greater than the background. I have suggested in chapter III that BrdU incorporation during G2/M phase could be due to post replication repair. Deletion of *Rad18* could reduce any PRR background and allow for a better observation of BrdU incorporation. Nevertheless, these experiments confirm that NaCl induces re-synthesis at uncapped telomeres.





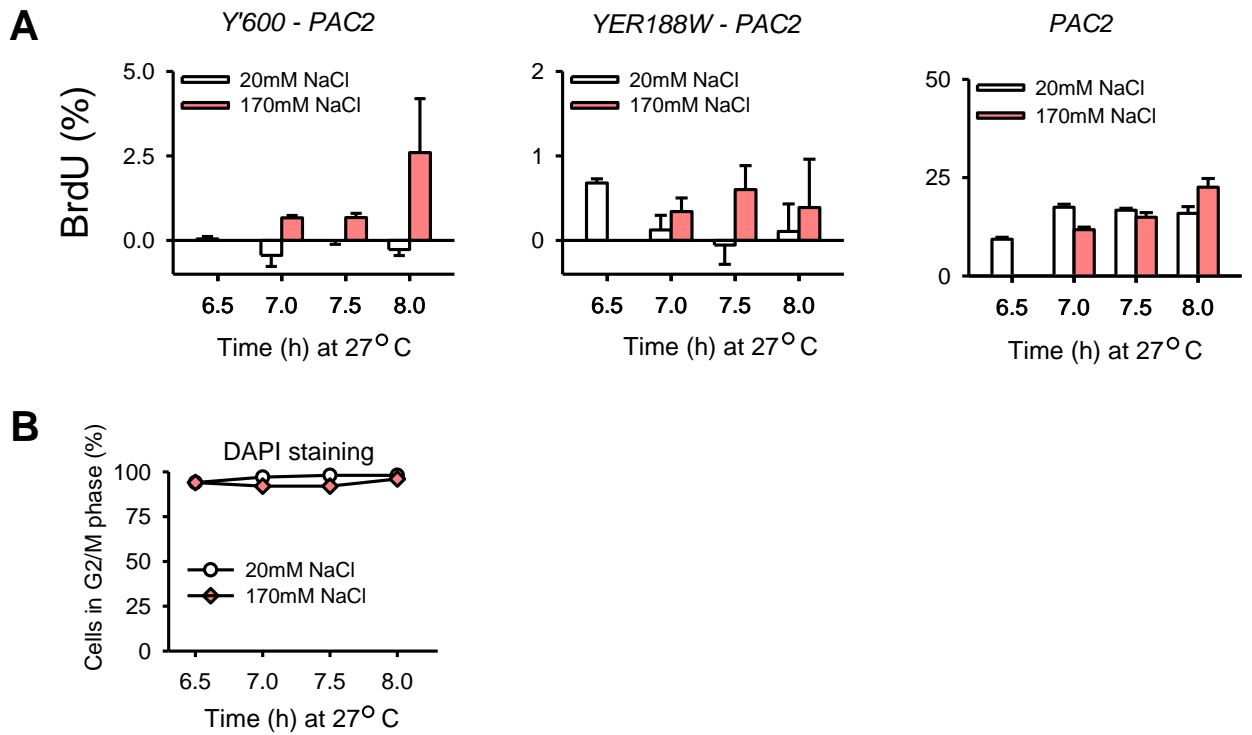
**Figure 15** Single stranded DNA accumulation in *cdc13-1* on NaCl and *exo1Δ cdc13-1* growth

A. Schematic representation of the right arm of chromosome V with indicated loci of interest.

B. QAOS measurements showing accumulation of ssDNA in *cdc13-1* cells (LMY206) at *Y'600* TG rich strand or AC rich strand and *YER188W*. The cells were grown overnight at 23 °C and diluted to  $2 \times 10^7$  cells/ml in the morning. Salt was supplemented to 2 parts of the culture (170mM or 450mM NaCl) and the temperature was shifted to 27 °C for 560min. The cells were counted every 160min and diluted where necessary. Samples for QAOS were collected every 80min. The error bars indicate the standard deviation between three measurements from the same sample. Where not visible, the error bars are behind the symbol. The experiment was performed two times.

C. DAPI staining showing the fraction of cells in G2/M phase. The samples were treated the same way as in B.

D. Five fold dilution series of *exo1Δ cdc13-1* cells propagated at 28° C on solid plates supplemented with indicated amounts of NaCl.



**Figure 16** BrdU incorporation in *cdc13-1* cells supplemented with NaCl

A. BrdU incorporation in *cdc13-1* (LMY784) at *Y'600*, *YER188W* and *PAC2*. The cells were grown at 27° C for 6h and 200µg/ml BrdU was added to the culture to allow sufficient uptake. After 30min (6.5h time point), the culture was split in two and 170mM NaCl final concentration was supplemented to half of the culture. The experiment was repeated four times.

B. DAPI staining showing the fraction of cells in G2/M phase. The samples were treated the same way as in D.

### **4.3 NaCl facilitated ssDNA re-synthesis requires non essential polymerase $\epsilon$ and polymerase $\delta$ subunits but is HR independent**

I have previously shown that subunits of polymerase  $\epsilon$  and polymerase  $\delta$  are recruited to ssDNA damage during telomere uncapping and that after capping is restored the binding decreased, correlating with a decrease in ssDNA and increase in BrdU incorporation. Therefore it is likely that ssDNA re-synthesis at uncapped telomeres is performed by those polymerases. Deletion of *DPB3*, *DPB4* and *POL32* leads to at least 50% decrease in the activity of the respective polymerase *in vitro* (Araki et al, 1991; Gerik et al, 1998; Ohya et al, 2000). If full polymerase activity is required for *cdc13-1* proliferation at 27° C on NaCl, deletion of those subunits should abolish the ability of NaCl to rescue *cdc13-1* growth at non-permissive temperatures. All double mutants were inoculated on agar plates supplemented with different amounts of NaCl (Figure 17). The spot test revealed that *DPB3*, *DPB4* and *POL32* seemed to be required for the NaCl effect at 27° C since the double mutants were not rescued as well as *cdc13-1* on 170mM and 450mM NaCl (Figure 17). Because *dpb3 $\Delta$  cdc13-1*, *dpb4 $\Delta$  cdc13-1* and *pol32 $\Delta$  cdc13-1* grew as well as wild type at 25° C, the lack of growth at 27° C can not be explained with increased temperature sensitivity in the double mutants. This data infers that NaCl facilitated polymerase  $\epsilon$  and  $\delta$  dependent ssDNA re-synthesis in cells with damaged telomeres.

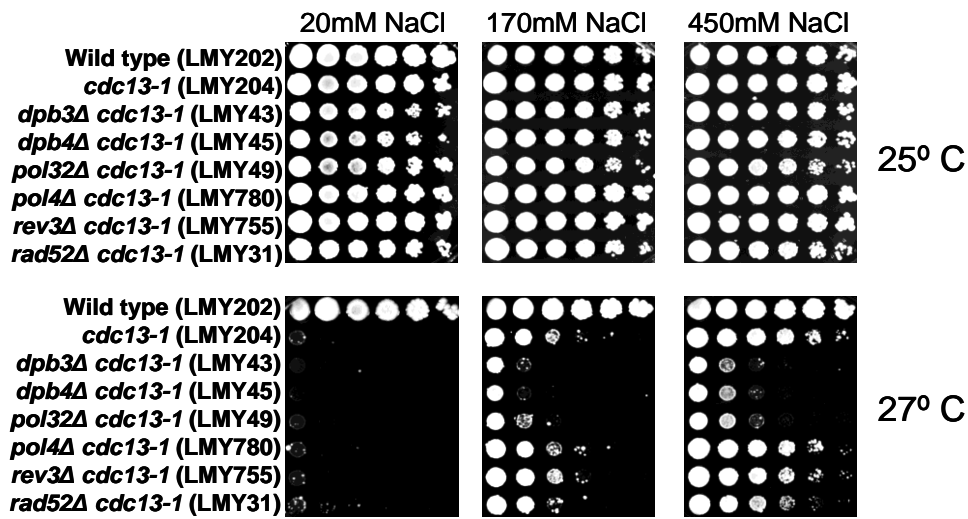
Pol32 participates also in translesion synthesis where it aids polymerase  $\zeta$  (Auerbach & Demple, 2010). To avoid any confusion between the conventional polymerases and the translesion synthesis pathway, polymerase  $\zeta$  catalytic subunit *REV3* was also deleted in *cdc13-1*. Furthermore polymerase IV and the homologous recombination gene *RAD52* were also tested as other possible mechanisms for NaCl facilitated ssDNA repair at uncapped telomere. Polymerase IV is encoded by the *POL4* gene and is usually involved in NHEJ (Tseng & Tomkinson, 2004). In contrast to the subunits of replication polymerase, polymerase  $\zeta$  and IV and HR were not involved because the double mutants were rescued by salt as well as *cdc13-1* (Figure 17).

To provide more evidence for polymerase  $\epsilon$  involvement in ssDNA re-synthesis on NaCl, ssDNA was measured in *cdc13-1* and *dpb3 cdc13-1* mutants (Figure 18). Fresh

overnight cultures were diluted in the morning and one part was treated with 170mM NaCl final concentration (Figure 18 red rhombi), while the untreated control contained only 20mM NaCl found normally in commercial yeast media (Figure 18 white circles). Then the temperature was shifted to 27° C to induce telomere uncapping and ssDNA production. 170mM final concentration NaCl was also added to part of the untreated cells, 400min after telomere uncapping. This allowed visualising how salt influences already accumulated ssDNA (Figure 18, orange triangles).

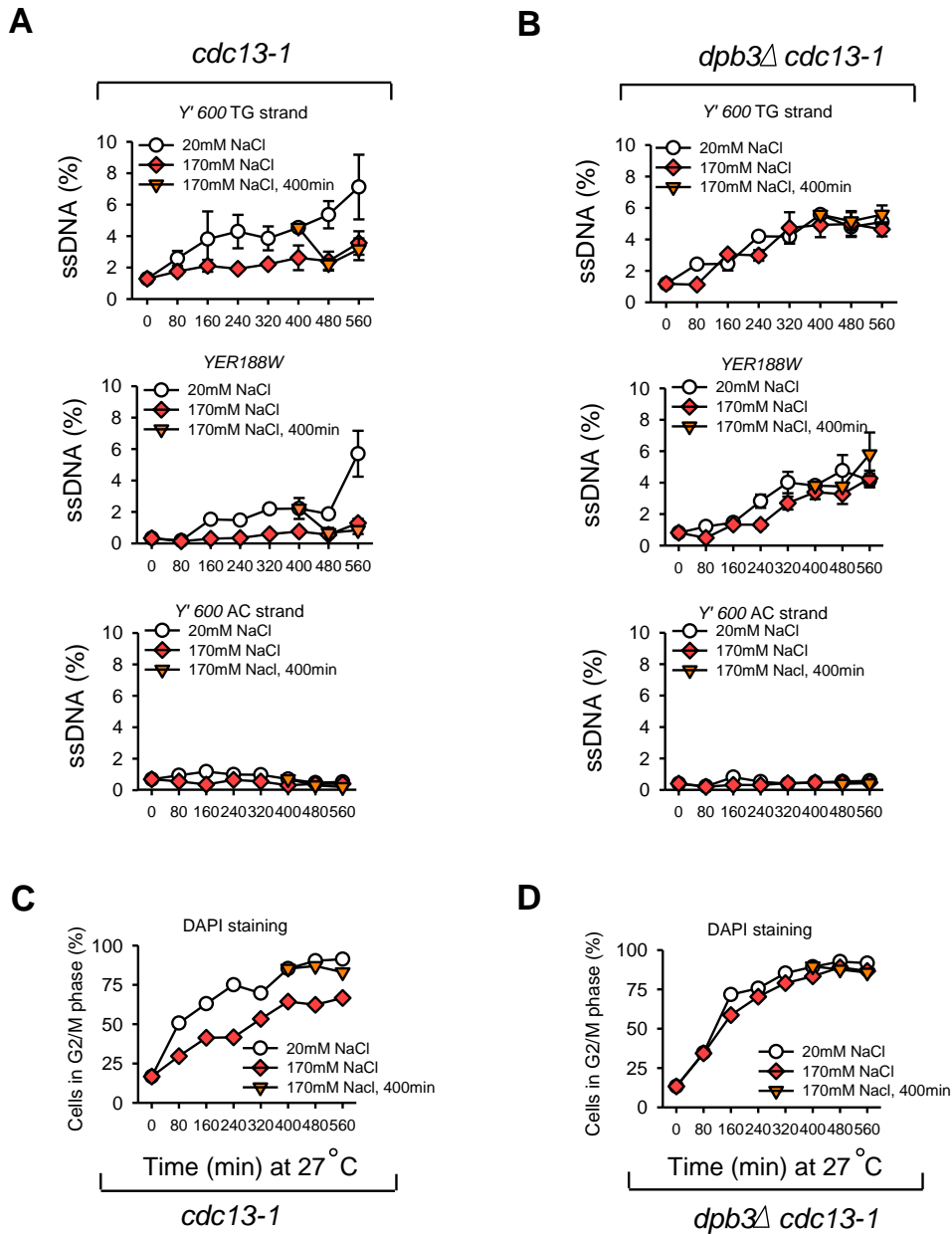
On 20mM NaCl *cdc13-1* cells accumulated up to 7 % ssDNA at *Y' 600* and around 6% at *YER188W*, while the cells with 170mM NaCl accumulated less than 3% ssDNA (Figure 18A, *Y' 600* TG strand and *YER188W*). When NaCl was supplied to cells with uncapped telomeres (400min time point), ssDNA dropped within 80min and stayed at the same level as the culture treated with NaCl from the beginning at both loci (Figure 18A, *Y' 600* TG strand and *YER188W*). No ssDNA was accumulated on the AC strand (Figure 18A, *Y'600* AC strand). Furthermore, the drop of ssDNA can not be explained by cells re-entering the cell cycle because DAPI staining showed that the cells remained in G2/M phase after addition of NaCl at 400min (Figure 18C). Instead DNA re-synthesis seems to occur prior to release from the cell cycle arrest. This data further emphasises that ssDNA re-synthesis at uncapped telomeres occurs in *cdc13-1* cells cultured in the presence of higher salt.

On the contrary, in the *dpb3Δ cdc13-1* double mutant, ssDNA accumulated throughout the experiment up to around 6 % at both loci, independent of treatment with NaCl or addition of NaCl at 400min (Figure 18B). The double mutant also arrested with the same dynamics regardless of treatment with salt (Figure 18D). Thus NaCl facilitated ssDNA re-synthesis during G2/M phase requires the DNA replication polymerases.



**Figure 17** *Cdc13-1* growth on salt in the absence of DNA polymerase subunits and Rad52

*DPB3*, *DPB4*, *POL32*, *POLA*, *REV3* and *RAD52* were knocked out from *cdc13-1* and 5 fold dilution increments were spotted on agar plates supplemented with indicated amounts of NaCl. The plates were grown for 3-4 days at 25° C and 27° C temperatures prior photographing. All strains were tested multiple times and behaved similarly when compared to wild type.



**Figure 18** SsDNA dynamics in *cdc13-1* and *dpb3Δ cdc13-1* cells

A. QAOS assay in *cdc13-1*(LMY206) at Y'600 TG, AC strand and YER188W propagated in media containing 20mM (white circles) or 170mM NaCl (red rombi). 170mM NaCl was also supplemented to cells grown for 400min at 27° C in normal salt conditions (orange triangles). In QAOS, error bars indicate the standard deviation between three measurements from the same sample. Where error bars are not visible, they are behind the symbol. The experiment was repeated twice.

B. Same as B. but for *dpb3Δ cdc13-1*(LMY43).

C. DAPI staining showing the fraction of *cdc13-1* cells in G2/M phase

D. DAPI staining showing the fraction of *dpb3Δ cdc13-1* cells in G2/M phase

## 4.4 NaCl facilitates ssDNA re-synthesis in *cdc13-1* independently of Hog1 pathway

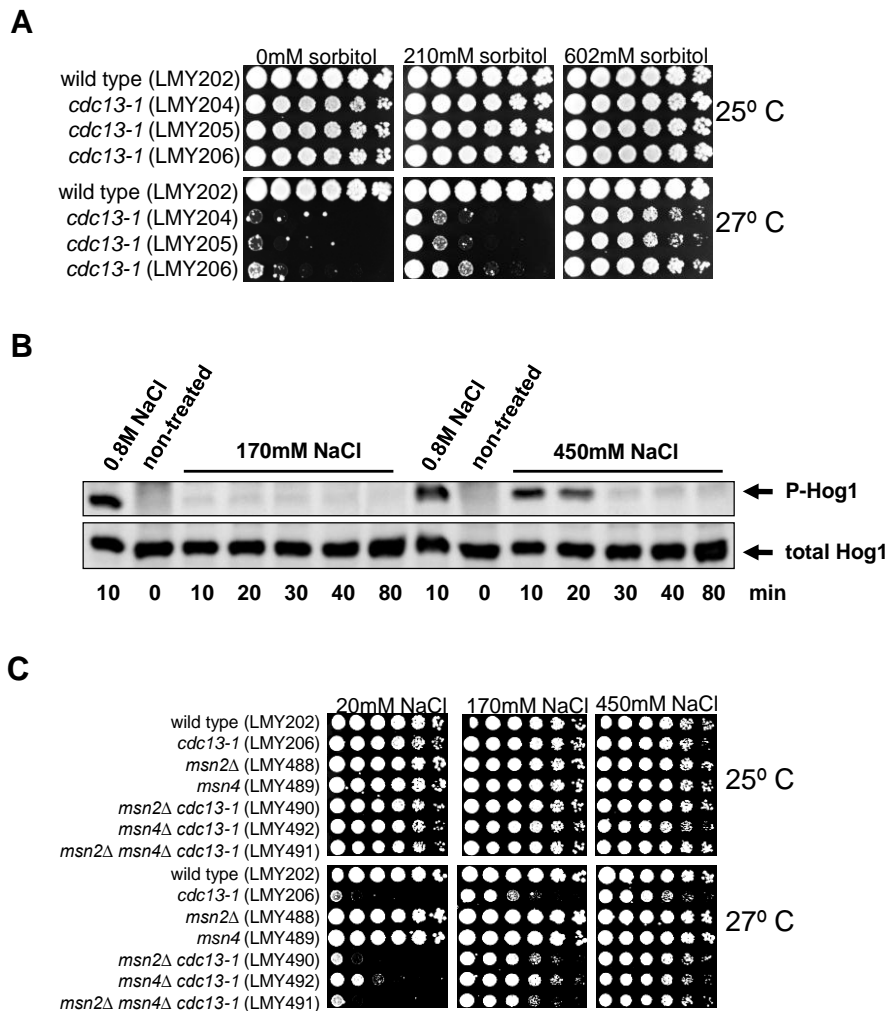
NaCl poses a threat to cells because it leads to loss of turgor and poisons metabolic processes. Cells have developed two strategies to deal with these problems. First,  $\text{Ca}^{2+}$  is increased in the cytosol, inducing expression of the P-type  $\text{Na}^+$ -ATPase that ensures  $\text{Na}^+$  export and prevents ion toxicity. This response lasts for only few minutes (Park et al, 2001). Second, the HOG signalling pathway is activated to produce glycerol and restore turgor by changing the mRNA stability of many yeast genes (Romero-Santacreu et al, 2009). To understand whether ssDNA repair at telomeres is just an effect of osmotic stress, *cdc13-1* cells were treated with the sugar alcohol sorbitol. Sorbitol is used as osmotic stress inducer instead of NaCl because it activates HOG without exhibiting ion toxicity. To create the same pressure on the cells as 170mM and 450mM NaCl, sorbitol was supplemented in a 1:1.4 ratio. This means that for every mol NaCl, 1.4 mols sorbitol were required. Thus 210mM and 602mM final concentration sorbitol were supplied in the media.

The spot test revealed that Sorbitol, similarly to NaCl could rescue the growth of *cdc13-1* in a dose dependent manner up to 27 °C (Figure 19A). To understand if mild osmotic stress can activate Hog1, *cdc13-1* cells were treated with 170mM and 450mM NaCl and phosphorylation of Hog1 was monitored with a previously validated anti phospho-p38 antibody (Figure 19B) (Marques et al, 2006). Cells treated with 0.8M NaCl were used as positive control, while untreated cells were used as negative control. The total Hog1 protein was detected with an anti-Hog1 antibody. No Hog1 phosphorylation was detected in the cells treated with 170mM NaCl 10min after NaCl addition. Therefore, no Hog1 phosphorylation occurs at this concentration, or it occurs earlier than 10min. The cells treated with 450mM NaCl showed a phosphorylation band only at 10 and 20min, after which no signal was detected (Figure 19B). Hence, Hog1 phosphorylation is brief and transient and it is questionable if the prolonged repair observed at the telomere is directly under Hog1 control.

To reinforce this idea two important Hog1 dependent transcription factors *MSN2* and *MSN4*, that act downstream of the kinase were knocked out in *cdc13-1* either independently (*msn2 $\Delta$  cdc13-1*, *msn4 $\Delta$  cdc13-1*) or together (*msn2 $\Delta$  msn4 $\Delta$  cdc13-1*). Deletion of none of those transcription factors had any effect on the ability of NaCl to

rescue *cdc13-1* at non-permissive temperatures (Figure 19C). Although NaCl is acting through osmotic stress, the signal does not appear to be transmitted through the Hog1 pathway.





**Figure 19** The role of osmolarity in *cdc13-1* with uncapped telomeres

A. Three independent *cdc13-1* strains were diluted in 5 fold dilution increments and spotted on agar plates supplemented with indicated amounts of sorbitol. The plates were grown for 3-4 days at different temperatures prior photographing.

B. Western blotting against phosphorylated and total Hog1 protein. The proteins were separated on 10% gel. Phosphorylation of Hog1 was assessed at different salt concentrations. Total Hog1 was detected with Hog1 (yC-20) goat polyclonal IgG (sc-6815), while phosphorylated Hog1 was visualised with a phospho-p38 MAPK (Thr180/Tyr182) antibody (9211S, NEB). P38 is the mammalian homologue of Hog1 and this antibody has been used previously to detect phosphorylated Hog1. The experiment was performed once, but similar data has been published multiple times in the literature.

C. Same as A. but with *MSN2* and *MSN4* single or double mutants

## 4.5 Inositol polyphosphate signalling is important for *cdc13-1* survival

Since I have shown that the HOG signalling pathway is unlikely to control NaCl induced telomere repair I asked what other pathways could be mediating this effect. A search in the literature revealed another pathway that could be activated upon osmotic stress – the inositol polyphosphate (IP) signalling pathway (Figure 20).

Briefly IP signalling starts when the cell membrane bound phosphatidylinositol 4,5-bisphosphate (PIP<sub>2</sub>) is hydrolysed by phospholipase C (Plc1) to membrane bound diacylglycerol (DAG) and soluble inositol 1,4,5-triphosphate (IP<sub>3</sub>) (Figure 20). IP<sub>3</sub> is then free to diffuse to the nucleus where it is converted to inositol 1,3,4,5,6 – pentakisphosphate (IP<sub>5</sub>) by the kinase Ipk2 in two subsequent phosphorylation reactions. IP<sub>5</sub> can have two distinct fates. It is either converted to the inositol pyrophosphate PP-IP<sub>4</sub> by the kinase Kcs1 or to IP<sub>6</sub> through Ipk1-dependent phosphorylation. Vip1 and Kcs1 then convert IP<sub>6</sub> to different IP<sub>7</sub> and IP<sub>8</sub> inositol pyrophosphates (Figure 20). Furthermore dephosphorylation of some inositol polyphosphates is governed by the phosphatase Ddp1. Mammalian cells utilise the same pathway for pyrophosphate production as yeast but the initial phosphorylation steps converting IP<sub>3</sub> to IP<sub>5</sub> may be conducted by an alternative set of reactions (Figure 20). Inositol isoforms that contain phosphates on more than one carbons in the inositol ring are called inositol polyphosphates (PIP<sub>2</sub>, IP<sub>3</sub>, IP<sub>4</sub>, IP<sub>5</sub> and IP<sub>6</sub>), while if another phosphate group is attached to an already phosphorylated carbon, the inositol isoform is called inositol pyrophosphate (PP-IP<sub>4</sub>, IP<sub>7</sub> and IP<sub>8</sub>).

The IP pathway controls various aspects of cell biology including cell death, DNA repair, heat shock and osmotic stress responses in both yeast and mammalian cells (Bennett et al, 2006). In yeast telomere length and vesicular trafficking were also affected in *kcs1Δ* mutants. Deletion of genes involved in IP signalling alters the inositol polyphosphate profile in cells as shown in Table 4.

The IP pathway might provide the connection between NaCl and DNA repair in *cdc13-1* because a low activity polymerase  $\alpha$  isoform can hydrolyse PIP which leads to increase in DNA binding and synthesis (Sylvia et al, 1988). Furthermore IP signalling was present in cells under hyper-osmotic and hypo-osmotic stress and during hyper-osmotic shock, accumulation of inositol polyphosphates was HOG independent (Dove et al, 1997; Perera et al, 2004).

To test whether NaCl could signal through the IP pathway all major genes of the pathway were knocked out in wild type or in combination with *cdc13-1* (Figure 21). The single mutants were also tested for NaCl and sorbitol sensitivity by spot test, because it was reported that *PLC1*, *IPK1* and *KCS1* deletions might be salt sensitive but unaffected by osmotic changes (Abe & Minegishi, 2008; Dubois et al, 2002). Indeed higher concentrations of NaCl impeded the growth of *plc1Δ* and *ipk2Δ* and sorbitol could rescue this growth defect (Figure 21A). *Kcs1Δ* and *ipk2Δ* were less salt sensitive and 170mM NaCl had no effect on their growth (Figure 21A). All other single mutants grew as well as wild type at any salt or sorbitol concentration.

When *PLC1*, *IPK1* and *KCS1* were deleted in *cdc13-1* they failed to grow above 25° C (Figure 21B). Interestingly neither NaCl nor sorbitol could fully rescue their growth. Only 602mM sorbitol allowed partial rescue of the cells. On the other hand *IPK1*, *DDP1* and *VIP1* had the opposite phenotype when combined with *cdc13-1*. Deletion of *IPK1* allowed *cdc13-1* to grow slightly better at 27° C while *ddp1Δ cdc13-1* and *vip1Δ cdc13-1* behaved like *cdc13-1* single mutant (Figure 21B). NaCl and sorbitol could rescue these double mutants as well as *cdc13-1*.

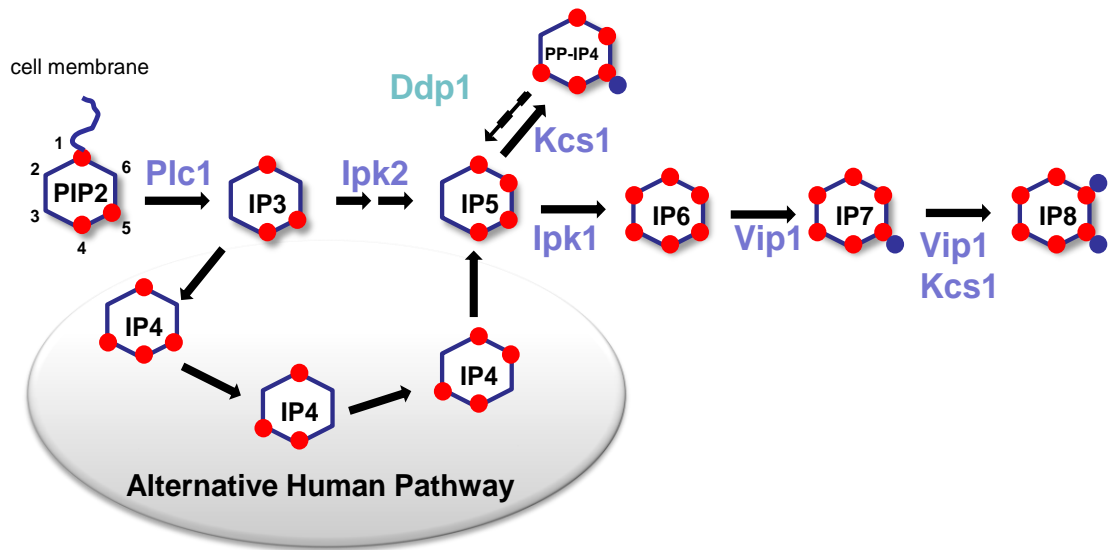
It is difficult to draw a definitive conclusion on the role of *PLC1*, *IPK2* and *KCS1* in NaCl facilitated re-synthesis at the telomere because of the salt sensitivity of the cells and because sorbitol could rescue their growth partially. However, *KCS1* was the least salt sensitive and was not rescued by 170mM or 210mM sorbitol providing limited evidence that it might be involved in DNA repair

Interestingly *plc1Δ*, *ipk2Δ* and *kcs1Δ* can not synthesise inositol pyrophosphates and this seems to correlate with the poor growth and lack of rescue by NaCl in the double mutants indicating that these molecules might be required for *cdc13-1* viability (Figure 21B and Table 4). Deletion of *IPK1* increases PP-IP<sub>4</sub> and IP<sub>8</sub> production, confirming that these compounds are important for cells with damaged telomeres (Figure 21B and Table 4). Furthermore *PLC1*, *PIK2* or *KCS1* deletion leads to slight telomere elongation and *IPK1* deletion to slight telomere shortening (York et al, 2005). However, this did not seem to be the reason why the cells were not rescued by NaCl, because deletion of *RIF1* in *cdc13-1* cells which also leads to telomere elongation and temperature sensitivity at 25° C was rescued by salt (Figure 21C) (Anbalagan et al, 2011).

To better understand whether inositol polyphosphate signalling is required for ssDNA repair at uncapped telomeres, the least NaCl sensitive mutant *kcs1Δ cdc13-1* was

grown at restrictive temperature at different NaCl concentrations and ssDNA was measured by QAOS (Figure 22). NaCl could still prevent ssDNA accumulation at *Y'600* and *YER188W* at the early time points, however later on at 560min similar amounts of ssDNA were found in treated and non-treated samples (Figure 22A). No accumulation of ssDNA was found on the AC strand, confirming that resection was progressing in 5' to 3' direction as expected. Even though NaCl seemed to impair ssDNA accumulation, *kcs1Δ cdc13-1* cells arrested faster than *cdc13-1* (compare Figure 22B and Figure 15C). For example at 320min the difference between ssDNA in NaCl treated and untreated cells was biggest at both loci *Y'600* and *YER188W* (Figure 22A). Yet over 80% of the cells were in G2/M phase, suggesting that *KCS1* may not play a role in NaCl induced ssDNA re-synthesis. Instead lack of *KCS1* may increase the sensitivity to uncapped telomeres (Figure 22B).

It is known that inositol pyrophosphates produced by *KCS* can inhibit Tel1 and/or Mec1, therefore *kcs1Δ cdc13-1* mutants may have a more active checkpoint leading to earlier cell cycle arrest and death (Saiardi et al, 2005). Furthermore, Mec1 has been linked to *cdc13-1* apoptosis after telomere uncapping due to increased ROS and caspase activity (Qi et al, 2003). Therefore *kcs1Δ cdc13-1* cells might grow poorly at 25° C because they are more sensitive to ssDNA and due to increased apoptosis.

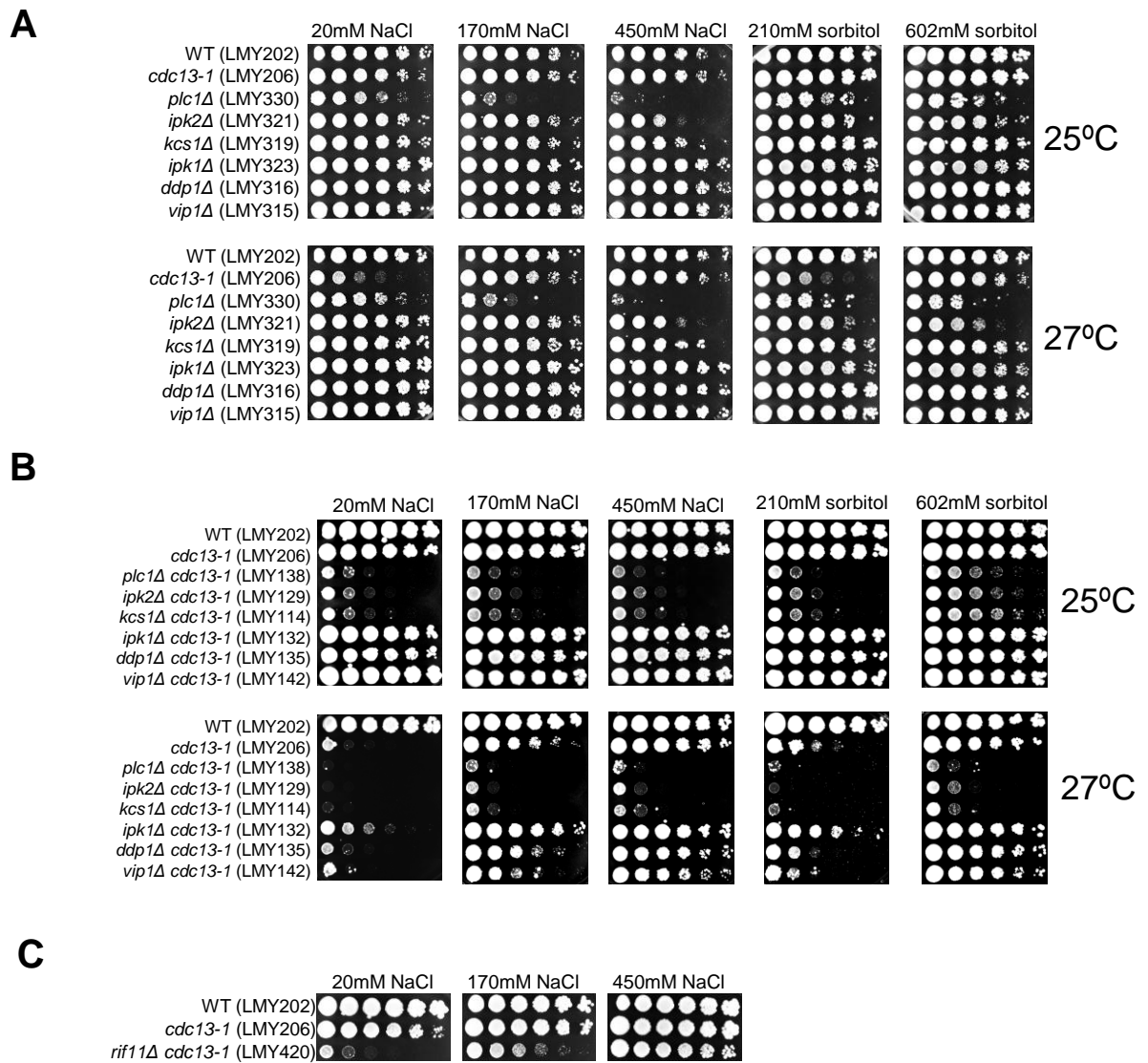


**Figure 20** Scheme showing the current understanding of the inositol polyphosphate (IP) signalling pathway

	IP3	IP4	IP5	IP6	PP-IP4	IP7	IP8	Reference
wild type	-	-	-	+	-	+	+	(Saiardi et al, 2005)
<i>plc1Δ</i>	-	-	-	-	-	-	-	(York et al, 1999)
<i>ipk2Δ</i>	+	-	-	-	-	-	-	(Odom et al, 2000; Perera et al, 2004)
<i>kcs1Δ</i>	-	-	-	+	-	-	-	(Saiardi et al, 2002; York et al, 2005)
<i>ddp1Δ</i>	-	-	-	+	-	+	-	(York et al, 2005)
<i>ipk1Δ</i>	-	+	+	-	+	-	+	(Perera et al, 2004; York et al, 1999)
<i>vip1Δ</i>	-	-	-	+	-	+	+	(Onnebo & Saiardi, 2009)

**Table 4** Inositol polyphosphate profile in different IP signalling mutants.

The plus sign indicates presence and the minus sign represents absence of the respective inositol isoform. IP3, IP4, IP5 and IP6 are inositol polyphosphates, while PP-IP4, IP7 and IP8 are inositol pyrophosphates



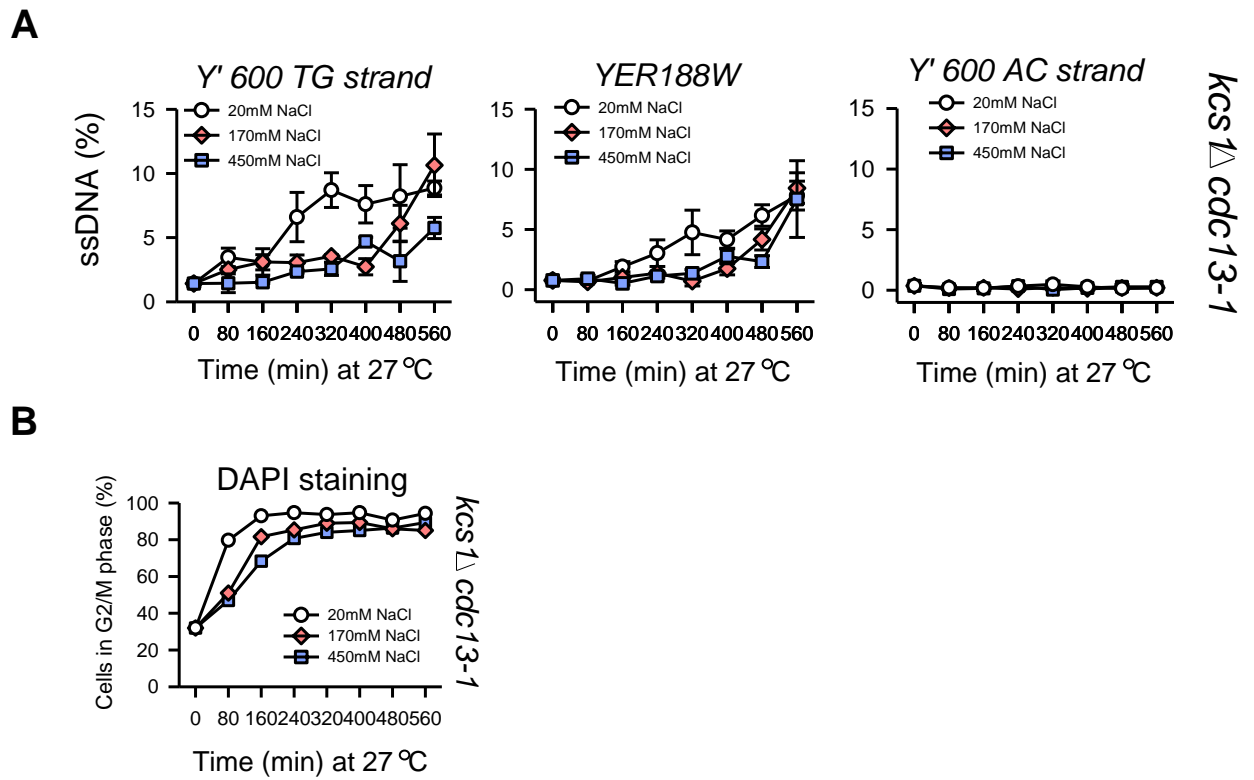
**Figure 21** Growth of inositol polyphosphate mutants and *rif1Δ cdc13-1* on NaCl and sorbitol

A. *PLC1*, *IPK2*, *KCS1*, *IPK1*, *DDP1* and *VIP1* were knocked out in wild type, the strains were diluted in 5 fold increments and spotted on agar plates supplemented with indicated amounts of NaCl or Sorbitol. The plates were grown for 3-4 days at different temperatures prior photographing.

B. Same as A but for double mutants with *cdc13-1*

C. Same as A but for *rif1Δ cdc13-1*

Two biological replicates were tested for each strain and behaved similarly to wild type.



**Figure 22** Single stranded DNA accumulation in *kcs1Δ cdc13-1* on NaCl

A. QAOS measurements of ssDNA in *kcs1Δ cdc13-1* (LMY114) cells at *YER188W*, *Y'600* TG strand and AC strand. The cells were grown at 27° C for 560min at indicated NaCl concentrations. Error bars represent the standard deviation between three qPCR measurements of the same sample. Wherever not visible, error bars are behind the symbol. The experiment was repeated three times.

B. DAPI staining showing the fraction of cells in G2/M phase. The samples were treated the same way as in A.



## 4.6 *KCS1* overexpression suppresses *cdc13-1* temperature sensitivity

To further investigate what is the role of inositol pyrophosphates at uncapped telomeres, *PCL1*, *IPK2* and *KCS1* were put under the *GAL1* promoter to test how their overexpression would influence *cdc13-1* growth (Figure 23). Gene expression from the *GAL1* promoter is suppressed on glucose or raffinose media and induced upon addition of galactose. The *GAL1::PLC1 cdc13-1*, *GAL1::GST::IPK2::cdc13-1* and *GAL1::GST::KCS1 cdc13-1* strains were created by substituting 300bp upstream of the start codon with a *GAL1* or *GAL1::GST* cassette. Correct substitution was tested by PCR and overexpression was confirmed in the GST tagged strains by Western blotting (data for *IPK2* is not shown).

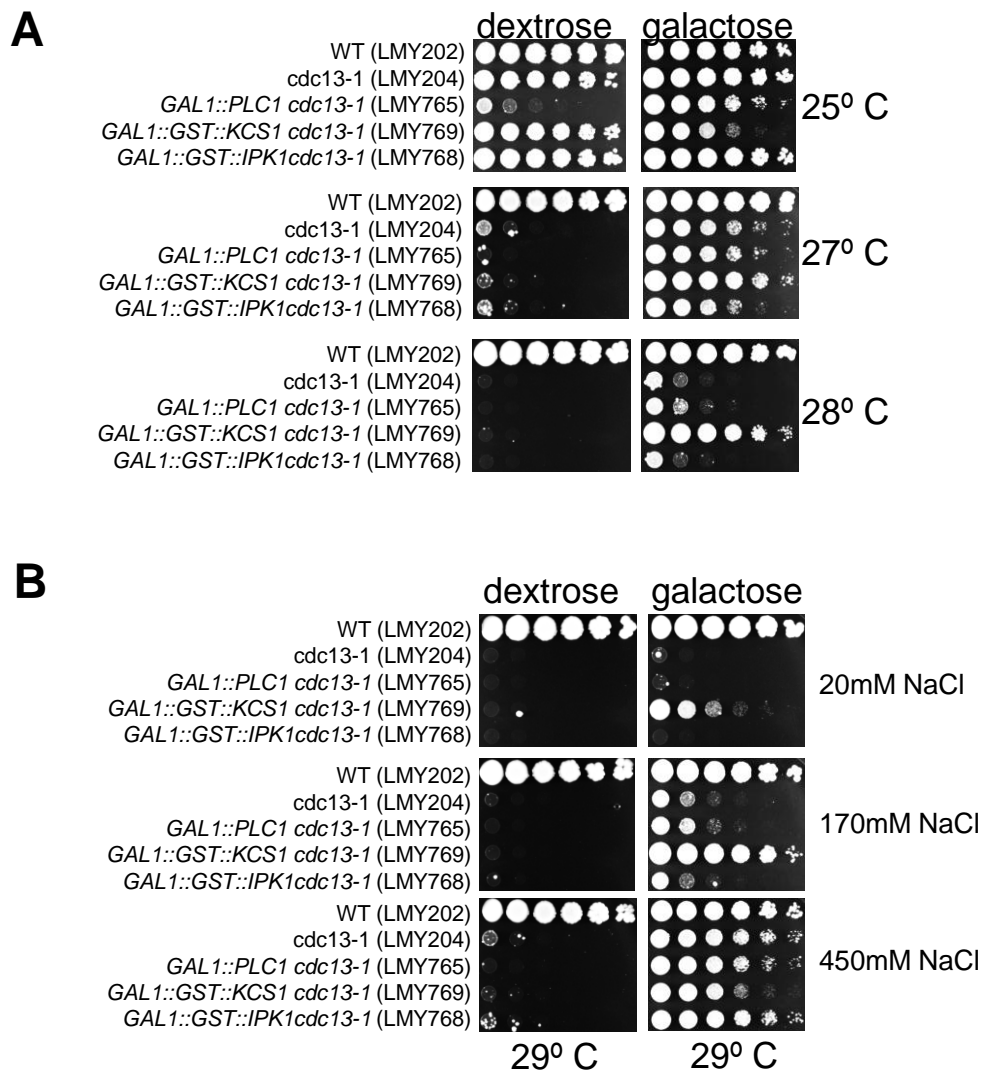
When *GAL1::PLC1 cdc13-1* was propagated on dextrose, no colonies were formed at 25° C, similarly to a *plc1Δ cdc13-1* deletion (Figure 23A). However *GAL1::GST::KCS1 cdc13-1* and *GAL1::GST::IPK1 cdc13-1* formed colonies up to 27° C, suggesting that endogenous *KCS1* and *IPK1* could be expressed despite deleting 300bp upstream of the respective start codon. Previous attempts to overexpress *KCS1* on a plasmid under the *GAL10* promoter also yielded a wild type phenotype and it was proposed that the *GAL10* promoter might be slightly leaky (York et al, 2005). Although no *KCS1* or *IPK1* band was detected by Western blotting on raffinose, it is possible that a very low level of expression under *GAL1* is also responsible for the wild type phenotype on raffinose (Figure 14E and G and data not shown). Hence, cells must be able to synthesise sufficient inositol polyphosphates even in the presence of limited amounts of IP kinases.

Galactose increased the temperature resistance of *cdc13-1* to 27° C and all tested strains formed colonies at that temperature (Figure 23A). Only overexpressed *KCS1* could rescue *cdc13-1* growth up to 29° C, while *PLC1* and *IPK1* overexpression did not seem to influence *cdc13-1* temperature sensitivity (Figure 23A and B). This clearly shows that *KCS1* overexpression reduces the temperature sensitivity of *cdc13-1* independently of IP signalling.

When *PLC1*, *KCS1* and *IPK1* were overexpressed on salt, *cdc13-1* cells were able to form more colonies at 170mM and 430mM NaCl than at 20mM NaCl, confirming that IP signalling improves *cdc13-1* growth in a separate pathway than NaCl (Figure 23B). Nevertheless, *KCS1* overexpression may induce partial ssDNA repair independently of NaCl. To test this BrdU incorporation assay was performed in *GAL1::GST::KCS1 cdc13-1 7TK+* cells (Figure 24A).

Telomeres were uncapped at 29° C in raffinose for 6.5 hours and then *KCS1* overexpression was induced by supplementing 2% galactose (Figure 24A and C). No convincing BrdU incorporation was observed specifically at *Y'600* or *YER188W*, showing that *KCS1* overexpression rescues *cdc13-1* growth independently of ssDNA. Interestingly DAPI staining revealed that although no BrdU incorporation was observed a small fraction of cells (around 10%) escaped G2/M arrest at 8h, correlating with the strongest expression of *KCS1* (Figure 24A,B and C). This can again be explained by the genetic interaction between *KCS1* and *MEC1*. If inositol pyrophosphates produced by Kcs1 are inhibiting Mec1, then *KCS1* overexpression could down regulate checkpoints and allow escape in the presence of ssDNA damage.

Finally, I wanted to understand whether *KCS1* is specifically recruited to damaged telomeres where it may regulate checkpoints directly. ChIP revealed that overexpressed *KCS1* did not appear to associate to *Y'600* or *ERG26* (Figure 24D and E). Thus *KCS1* might not directly regulate checkpoints.

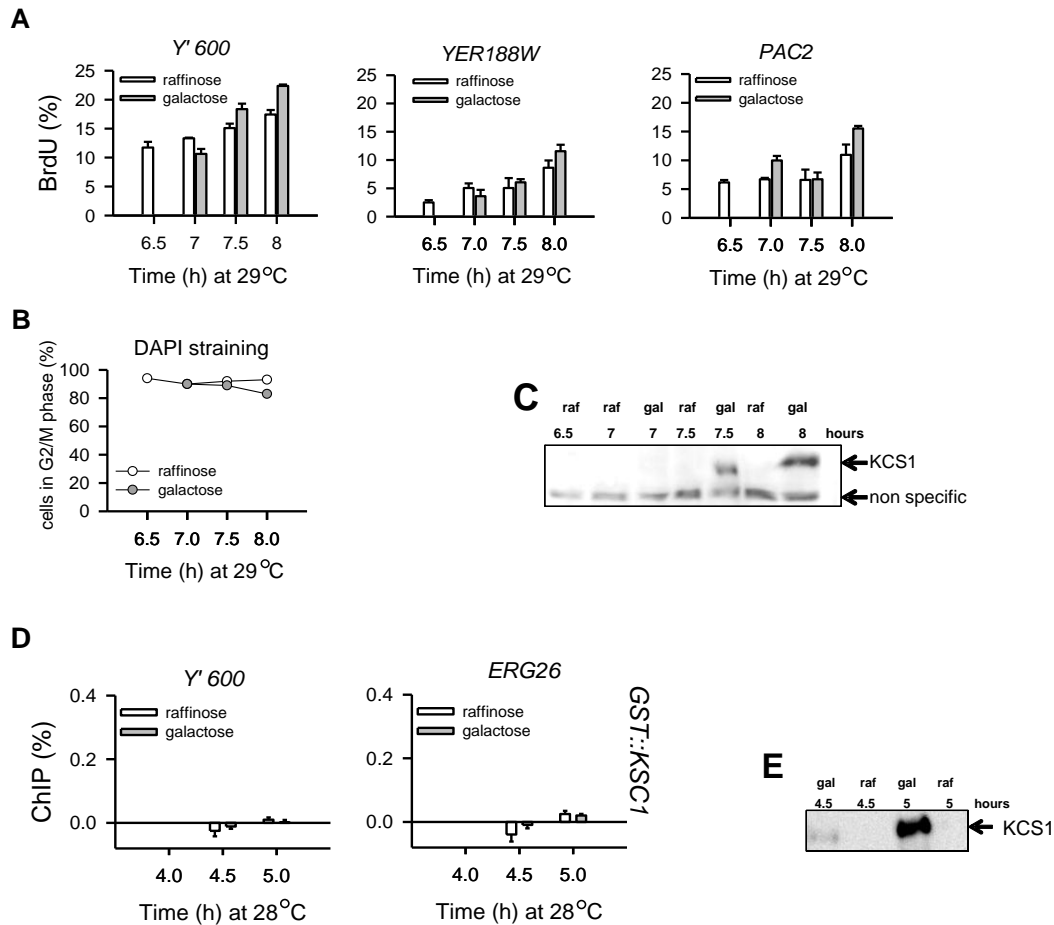


**Figure 23** The effect of *KCS1* overexpression on *cdc13-1* survival

A. *PLC1*, *KCS1* and *IPK1* were overexpressed under the *GAL1* promoter and grown either on dextrose or galactose at indicated temperatures.

B. Same as A. but the cells were grown at 29° C and at different amounts of NaCl as indicated on the right.

Two biological replicates were tested for each strain and behaved similarly to wild type.



**Figure 24** BrdU incorporation and recruitment of overexpressed *KCS1* to damaged telomeres

A. BrdU incorporation in *GAL1::GST::KCS1 cdc13-1 7TK+* (LMY785). The cells were grown in raffinose for 6.5hours and then 2% galactose was supplied to induce expression. BrdU incorporation was measured at *Y'600*, *YER188W* and *PAC2*. The error bars indicate the standard deviation between three measurements from the same sample. Where not visible, the error bars are behind the symbol. The experiment was repeated twice.

B. DAPI staining showing the fraction of cells in G2/M phase. The samples were treated the same way as in A.

C. Western blotting confirming overexpression of *KCS1* on galactose. *KCS1* was detected with an anti-GST antibody on a 6.5% gel. The samples were treated the same way as in A.

D. Recruitment of overexpressed *KCS1* to uncapped telomeres by ChIP. LMY785 was grown in raffinose for 4hours at 28° C and *KCS1* overexpression was induced with 2% galactose. ChIP measurements were performed at *Y'600* and *ERG26*. The experiment was repeated twice.

E. *KCSI* overexpression was monitored by Western blotting. The samples tested are the same as in D.

## 4.7 Discussion

In this chapter I have shown that relatively small amounts of NaCl can enhance the growth of *cdc13-1* at restrictive temperatures. This had a profound effect on long term survival. NaCl facilitated polymerase  $\delta$  and  $\epsilon$  dependent ssDNA re-synthesis at chromosome ends. More importantly, re-synthesis was taking place at restrictive temperatures, while the cells were arrested in G2/M. This ssDNA re-synthesis seemed to be the consequence of osmotic stress because sorbitol could also rescue *cdc13-1*.

### **What are the functions of polymerase $\epsilon$ and $\delta$ during ssDNA re-synthesis at uncapped telomeres on NaCl?**

NaCl facilitated ssDNA re-synthesis at uncapped telomeres required the non-essential subunits *DPB3/DPB4* and *POL32* for polymerase  $\epsilon$  and polymerase  $\delta$ . More importantly ssDNA decrease on NaCl required full polymerase  $\epsilon$  activity because the *dpb3 $\Delta$  cdc13-1* double mutant accumulated ssDNA regardless of NaCl treatment (Figure 18). In contrast neither *DPB3* nor *DPB4* were required for *cdc13-1* survival during the UP-DOWN assay (Figure 8), showing that repair following recapping did not need these subunits.

Two possibilities can explain these differences. First, polymerase  $\epsilon$  might participate only in the checkpoint response during recapping, as suggested in the discussion of chapter III. At uncapped telomeres, however NaCl could stimulate polymerase  $\epsilon$  activity and aid ssDNA re-synthesis. This would explain why *DPB3* and *DPB4* are only important for NaCl facilitated ssDNA re-synthesis at uncapped telomeres but not after recapping.

Second, both polymerase  $\epsilon$  and polymerase  $\delta$  may be participating in re-syntheses after recapping as well as upon increased NaCl at uncapped telomeres. However when telomeres are recapped *DPB3* and *DPB4* might not be essential, the same way as they are not required during normal replication. An uncapped telomere might be more difficult to repair and full activity of polymerase  $\epsilon$  might be required. For example Rap1 bound to telomeres naturally pauses the replication fork (Makovets et al, 2004). If the ssDNA at uncapped telomeres or any proteins associated with it are a barrier for repair, full polymerase activity might be required to overcome this barrier at uncapped telomeres but not after recapping.

Finally competition between 5' to 3' degradation at uncapped telomeres may exist as suggested in chapter III (Figure 12B). NaCl would then favour enhanced activity in polymerase  $\epsilon$  and/or  $\delta$  and lead to successful repair at uncapped telomeres.

### **How is NaCl regulating ssDNA repair?**

I have shown that ssDNA repair can be induced in hyperosmotic conditions at uncapped telomeres. However, this repair did not seem to be mediated through the HOG signalling cascade because deletion of the transcription activators *MSN2* and *MSN4* did not abolish the increased proliferation in *cdc13-1* cells on NaCl.

A recent high throughput study involving latest state of the art mass spectrometry shows that apart from fold changes in protein levels, 400mM NaCl can also induce changes in the phosphorylation of many proteins, including proteins not connected previously to the HOG pathway (Soufi et al, 2009). Hence, NaCl can induce many other pathways, apart from HOG and IP signalling and provided new insights in osmotic stress regulation.

The phosphorylation data in this study provided evidence for two ways in which polymerase  $\alpha$  can be regulated, by Ser-209 phosphorylation in Pol1 and de-phosphorylation of Pol12 at several residues (Soufi et al, 2009). This finding was very interesting because Pol12 de-phosphorylation in yeast has been linked to correct loading of polymerase  $\alpha$ /primase during DNA replication (Foiani et al, 1995). Moreover, Pol12 seemed to be phosphorylated during replication stalling and G2/M arrest following nocodazole treatment. No studies on Pol1 phosphorylation were conducted in yeast but in mammalian cells the conserved Ser-209 is phosphorylated in a CDK-dependent manner during S phase (Schub et al, 2001). The mammalian Pol12 was also phosphorylated but in this case both phosphorylation events on Pol1 and Pol12 were required for the *in vitro* activity of polymerase  $\alpha$ /primase. Furthermore, the Ser-209 phosphorylation in yeast might not be conferred by CDK1 as in human, because NaCl down regulated the activity of the yeast CDK1, through phosphorylation of the inhibitory tyrosine residue 19 (Soufi et al, 2009). This shows some differences between yeast and mammalian polymerase  $\alpha$ / primase regulation.

NaCl could also regulate polymerase  $\delta$  by inducing phosphorylation on two residues in the non-essential subunit Pol32 (Chi et al, 2007; Soufi et al, 2009). However, no functional information about this phosphorylation sites is available in yeast. Mammalian Pol32 is phosphorylated during early S phase and this could contribute to

assembly of the replication machinery and polymerase  $\delta$  activity (Lemmens et al, 2008). No other subunits of replicative polymerases were phosphorylated during osmotic stress (Soufi et al, 2009).

Polymerase  $\epsilon$  might not be regulated directly by NaCl, however the binding partner of Pol2 called Mrc1 is strongly phosphorylated on NaCl (Soufi et al, 2009). Interestingly the interaction between Pol2 and Mrc1 is important for polymerase  $\epsilon$  stability and DNA replication (Lou et al, 2008). Mrc1 and Pol2 interact with each other via their C and N terminal. When Mrc1 is phosphorylated by Mec1 and/or Rad53 following replication stalling the interaction via the N terminal is lost, leading to polymerase  $\epsilon$  destabilisation at the replication fork (Lou et al, 2008). Therefore it is possible that at uncapped telomeres Mrc1 is phosphorylated and polymerase  $\epsilon$  is unstable. Osmotic stress, however, dephosphorylates Mrc1 and thus could contribute to ssDNA repair.

Lastly, the PRR gene Rev1 encoding polymerase  $\eta$  was dephosphorylated strongly on 400mM NaCl (Soufi et al, 2009). The literature suggests that Rev1 phosphorylation by Mec1 might be essential for its function in translesion synthesis during DNA damage (Sabbioneda et al, 2007). Therefore, it seems like NaCl could also influence the DNA re-synthesis pathway choice by down regulating Rev1

It is also not clear whether NaCl would produce the same phosphorylation pattern when the cells are in G2/M phase, since the osmotic stress screen was performed in freely proliferating cells (Soufi et al, 2009). During DNA damage some proteins might already be phosphorylated or de-phosphorylated and it is difficult to predict how NaCl would affect their modification. Nevertheless there are at least three possible ways for NaCl to induce polymerase  $\epsilon$  and  $\delta$  dependent repair at uncapped telomeres. First, through phosphorylation or de-phosphorylation of polymerase  $\alpha$ /primase subunits Pol11 and Pol12. Second, through phosphorylation of the polymerase  $\delta$  subunit Pol32; and third through de-phosphorylation of Mrc1. Further experiments would have to be conducted to confirm whether NaCl regulates any of those modifications at uncapped telomeres.

### **NaCl and telomere length regulation at uncapped telomeres**

I found that *cdc13-1* cells experience telomere shortening when propagated at restrictive temperatures (Figure 14). Furthermore this was also apparent in cells that have escaped cell cycle arrest (Figure 14B, day 3-5). It is not clear whether this was due to lack of telomerase recruitment or degradation of the TG rich strand. It has been



suggested that only *cdc13-2* has a telomerase recruitment defect, while *cdc13-1* conferred only dysfunctional telomere capping and the interaction of Est1 with Cdc13 was not affected in the *cdc13-1* mutant (Nugent et al, 1996). However actual propagation of *cdc13-1* cells at any restrictive temperature has not been published.

It is possible that Cdc13-1 is indeed proficient in telomerase recruitment and the telomere loss is due to TG strand degradation at restrictive temperatures.

At 27° C *cdc13-1* managed to propagate for 3 days, exhibiting a senescence phenotype, before escape and formation of survivors (Figure 14). During this time telomeres clearly shortened, which correlated with slow growth, but continued to shorten even after survivors appeared. Interestingly 170mM NaCl could delay telomere shortening with two days, while the cells grown in 450mM NaCl did not seem to lose their telomere for at least 5 days and no reduction in growth was observed (Figure 14).

The ability of NaCl to prevent telomere shortening might be *RAD52* independent, as *RAD52* deletion did not abolish NaCl induced growth at restrictive temperatures in *cdc13-1* (Figure 17). Further experiments would have to be conducted to understand why telomeres shorten at restrictive temperatures in *cdc13-1* cells. For example deletion of different checkpoints and *EXO1* could show if shortening is due to a certain checkpoint or due to resection.

### **What is the role of inositol pyrophosphates in *cdc13-1* with damaged telomeres?**

In this chapter I have shown that *KCSI* but not any other kinases involved in IP signalling is important for *cdc13-1* survival. This was not due to induction of ssDNA re-synthesis (Figure 24) but instead could be due to Mec1 regulation through inositol pyrophosphates (Saiardi et al, 2005).

Overexpressing *KCSI* allowed *cdc13-1* cells to escape G2/M arrest in the absence of ssDNA repair, meaning that *KCSI* might be involved in checkpoint adaptation to DNA damage. Following DNA damage and checkpoint activation cells remain arrested until repair is conducted. However sometimes yeast and mammalian cells escape the checkpoint arrest and continue to proliferate in the presence of DNA damage (Syljuasen, 2007). This process is possible only after the checkpoint is down regulated, hence the cells have adapted to the checkpoint signal.

Several proteins have been involved in adaptation such as the casein kinase II, responsible for completion of anaphase, Yku70 and the Rad53 interacting phosphatases Ptc2 and Ptc3 (Lee et al, 1998; Leroy et al, 2003; Toczyski et al, 1997). If *KCSI* is indeed a checkpoint adaptation gene, overexpressing *KCSI* might down regulate the checkpoint through inhibiting Mec1.

Kcs1 was not recruited to uncapped telomeres, suggesting that the inositol pyrophosphates produced by Kcs1 are more likely to regulate checkpoints. Recent studies show that IP7 can further pyrophosphorylate proteins already phosphorylated by casein kinase II, a kinase implicated in adaptation (Bhandari et al, 2007). Pyrophosphorylation is a novel post translational protein modification where a pyrophosphoinositol can directly give away its phosphate to an already phosphorylated protein, creating a high energy di-phosphate or pyrophosphate. Proteins modified by IP7 are more resistant to dephosphorylation but their biological significance is not clear. It is not clear whether IP7 synthesised by Kcs1 could target Mec1 for pyrophosphorylation and thus lead to Mec1 inactivation. Nevertheless, inositol pyrophosphates seem to be extensively involved not only in telomere length regulation as shown before but also in telomere uncapping (Saiardi et al, 2005).

## 5 The role of NaCl at other DNA damage types

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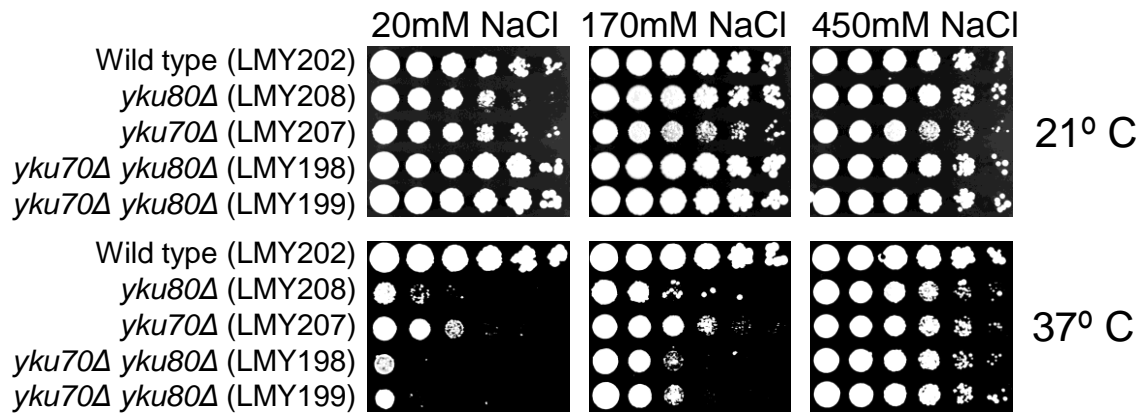
I have shown that uncapped telomeres can be repaired by polymerase  $\alpha$ ,  $\delta$  and  $\epsilon$  in the presence of NaCl (Chapter IV). Another important question is whether this effect is telomere specific. To test this hypothesis I used the *yku70* $\Delta$  and/or *yku80* $\Delta$  telomere uncapping system where ssDNA is also created at telomeres to confirm that the results in the previous chapters are not *cdc13-1* specific.

I also wanted to understand if repair can happen at any ssDNA damage, not necessarily associated with telomeres. Single stranded DNA is produced in cells after different types of DNA damage. For example 3' overhangs are found at DSBs or after processing of pyrimidine dimers and DNA alkylation; rising the possibility that NaCl facilitated repair could contribute to other types of DNA damage (Daley & Wilson, 2005; Giannattasio et al, 2010; Pascucci et al, 2005). The involvement of the NaCl induced repair in global DNA damage response can be tested by utilising the DNA damaging agents hydroxyurea (HU), methyl methanesulfonate (MMS) and ultra violet (UV) light.

### 5.1 The effect of NaCl is *cdc13-1* independent

Telomere uncapping can also happen in yeast lacking the telomere binding yKu heterodimer. Loss of either *YKU70* or *YKU80* leads to short telomeres, temperature sensitivity at 37° C and Exo1 dependent ssDNA accumulation. However *yku70* $\Delta$  cells accumulate less ssDNA than *cdc13-1* and require only Mec1, Rad9 and Chk1 for G2/M arrest (Maringele & Lydall, 2002). Nevertheless, this system allows a more comprehensive interpretation of the data because uncapping is due to complete loss of a telomere protecting complex and not a single point mutation.

Serial dilution of *yku70* $\Delta$  and *yku80* $\Delta$  cells revealed that NaCl can rescue the growth defect of *yku70*, *yku80* or *yku70/80* double mutants in a dose dependent manner, similarly to *cdc13-1* (Figure 25). Therefore the effect of NaCl seems to be specific to the ssDNA damage produced after telomere uncapping, rather than dependent on the model system or the downstream checkpoint activity.



**Figure 25** Growth of *yku70Δ* and/or *yku80Δ* mutants on NaCl

A 5 fold dilution series of *yku70Δ*, *yku80Δ* and *yku70Δ yku80Δ* mutants was grown at indicated amounts of NaCl at the permissive temperature 21° C and the restrictive temperature 37° C. The plates were incubated for 3-4 days prior photographing. The strains behaved the same in different experiments when compared to wild type.

## 5.2 NaCl plays no role in UV and hydroxyurea induced DNA damage but rescues MMS treated cells

After I confirmed that the effect of NaCl is not *cdc13-1* specific, I wanted to understand if salt can also influence cells with ssDNA lesions produced during UV damage, double strand breaks and at stalled replication forks (Figure 26 and 27).

UV light can be absorbed by double bonds in pyrimidines (thymidine or cytosine). This opens the bond and a reaction with neighbouring molecules is feasible, leading to the production of pyrimidine dimers (Goodsell, 2001). Such lesions can be repaired through post replication repair and nucleotide excision repair (Broomfield et al, 2001; Giannattasio et al, 2010).

Following UV irradiation nucleotide excision repair (NER) creates short ssDNA gaps that can be further processed by Exo1 to longer gaps (Giannattasio et al, 2010). Furthermore this processing happened when the short ssDNA gaps created by NER could not be repaired, leading to checkpoint activation. UV DNA damage repair requires also HR and PRR, suggesting that pyrimidine dimer can be processed to double strand breaks (Chang et al, 2002).

In contrast hydroxyurea (HU) blocks DNA synthesis by decreasing the availability of dNTPs (Koc et al, 2004). Presumably this leads to replication fork stalling and arrest in S phase. Only HR and PRR genes are sensitive to HU treatment, thus NER is not required for repair after replication fork stalling (Chang et al, 2002).

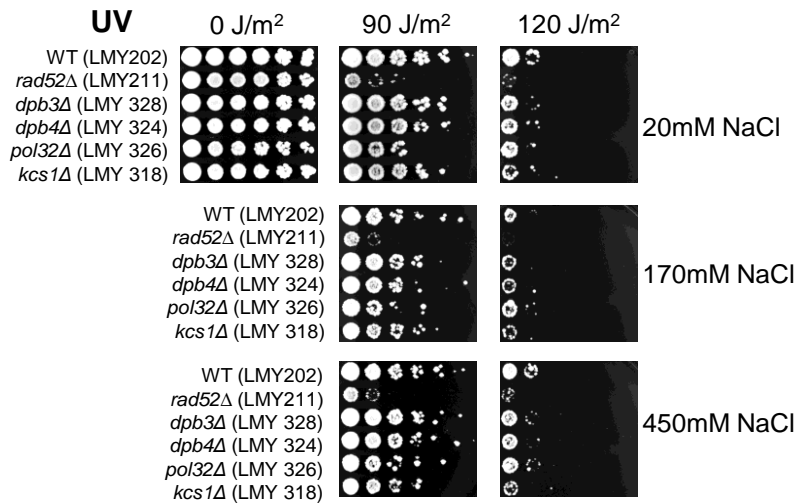
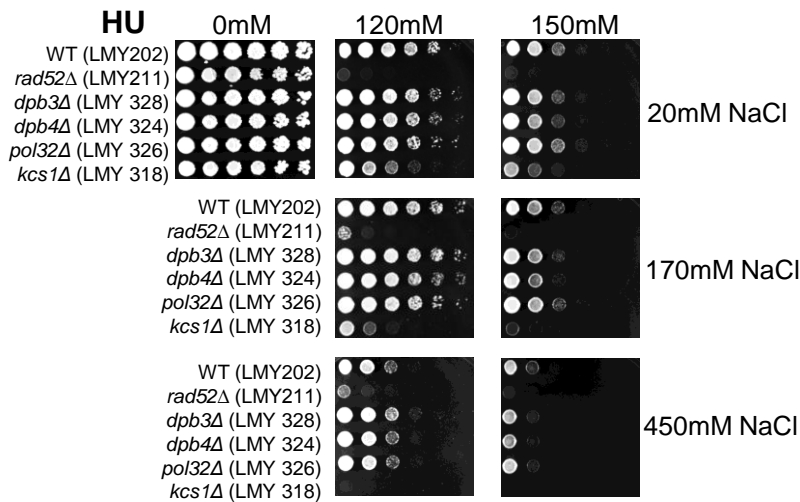
The third DNA damaging agent I tested was the DNA alkylating drug methyl methanesulfonate (MMS). MMS can methylate adenine and guanine, producing N<sup>7</sup>-ethylguanine and N<sup>3</sup>-ethyladenine *in vitro* and *in vivo* (Beranek, 1990). Repair of methylated adenine leads to creation of ssDNA *in vivo* (Wong & Dewey, 1981). However, no DSBs could be detected by pulse field gel electrophoresis in mammalian and yeast cells but it is known that even a single DSB is sufficient to completely arrest the cell cycle (Lundin et al, 2005; Lydeard et al, 2007).

I tested whether NaCl could rescue cells damaged with UV and HU (Figure 26). *RAD52* deletion was used as a control, since this mutant is sensitive to many DNA

damaging agents. I further wanted to understand if *DPB3*, *DPB4* and *POL32* are also required for NaCl on UV and/or HU agents. Finally, since *KCSI* may play a role in adaptation to DNA damage, sensitivity to DNA damage was also assessed in the *kcs1Δ* mutant.

Only *POL32* was sensitive to UV light (Figure 26A) and this has been shown previously (Chang et al, 2002). NaCl could not rescue cells damaged by UV light (Figure 26A). Similarly NaCl could not rescue HU treated cells as well, instead salt reduced further the viability of the damaged cells (Figure 26B).

Interestingly the *kcs1Δ* mutant was more HU sensitive than wild type, indicating that inositol pyrophosphates might play a role in replication fork stalling (Figure 26B). In contrast *kcs1Δ* cells were more resistant to MMS treatment than wild type cells and formed colonies at 0.016% MMS even in the least diluted spot (Figure 27). These mutants remained salt sensitive, as can be seen by the poor growth of *kcs1Δ* at 450mM NaCl (Figure 26 and 27). More importantly, NaCl managed to improve the growth of wild type, *dpb3Δ*, *dpb4Δ* and *pol32Δ* on MMS, showing that salt might indeed play a role in certain types of global DNA repair (Figure 27).

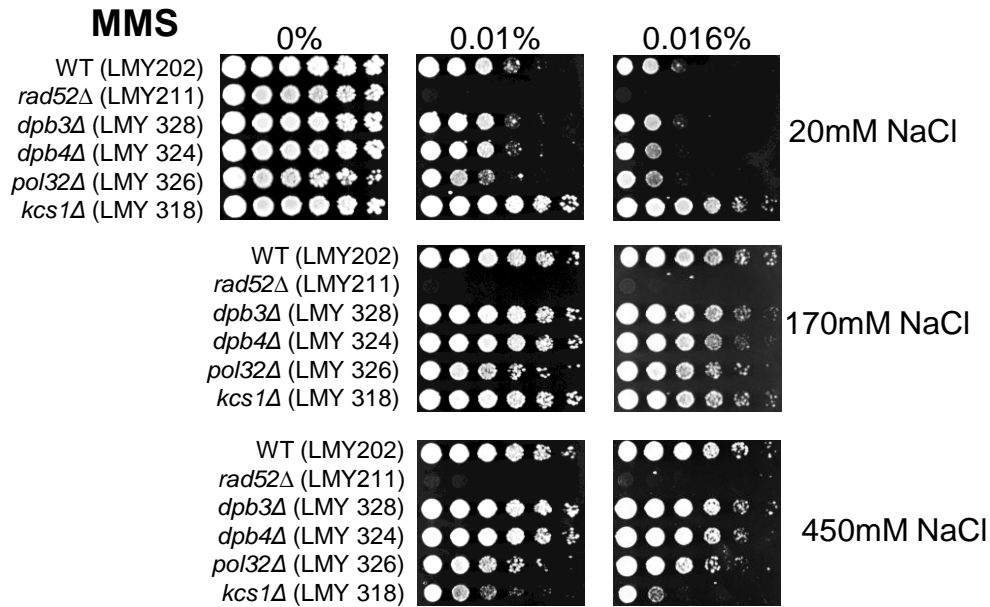
**A****B**

**Figure 26** Growth of wild type, *rad52Δ*, *dpb3Δ*, *dpb4Δ*, *pol32Δ* and *kcs1Δ* cells in the presence of NaCl and the DNA damaging drugs MMS and HU

A. Indicated strains were diluted in 5 fold increments and spotted on agar plates supplemented with 20mM, 170mM or 450mM NaCl. Then the plates were placed under UV light until they received 90J/m<sup>2</sup> or 120J/m<sup>2</sup> UV. The plates were grown for 3 days at 25° C prior photographing

B. Agar plates were supplemented with a combination of NaCl and 120mM or 150mM hydroxyurea (HU). Then the indicated strains were grown on the plates for 3 days at 25° C prior photographing

The strains behaved similarly between independent experiments and across biological replicates.



**Figure 27** Growth of wild type, *rad52Δ*, *dpb3Δ*, *dpb4Δ*, *pol32Δ* and *kcs1Δ* cells in the presence of NaCl and the DNA damaging drug MMS

Agar plates were supplemented with a combination of NaCl and 0.01% or 0.016% methyl methanesulfonate (MMS). Then the indicated strains were diluted in 5 fold increments and grown on the plates for 3 days at 25° C prior photographing.

The strains behaved similarly between independent experiments and across biological replicates.



### 5.3 Discussion

In this chapter I have confirmed that NaCl can rescue cells with other telomere capping defects and cells undergoing certain types of DNA damage. This is probably a consequence of NaCl induced ssDNA repair as seen in *cdc13-1* cells with uncapped telomeres because ssDNA can arise in *yku70Δ yku80Δ* cells and after MMS treatment. However I can not exclude a different mechanism, especially in the case of MMS because ssDNA can be created during UV and HU damage as well. Yet NaCl could not rescue cells threatened with such DNA damaging agents.

It is possible that NaCl can only induce repair at certain types of ssDNA damage and this would indicate that the damage after UV, HU and MMS treatment is fundamentally different. Indeed there are subtle differences between the genes required for survival after damage with those agents. For example MMS requires four genes which are not sensitive to either UV or HU. These are the chromatin assembly protein Cac2, the 3-methyl-adenine DNA glycosylase Mag1 important for initiation of BER, the PRR polymerase  $\zeta$  catalytic subunit Rev3 and the endonuclease Slx4 required for DNA processing during repair (Chang et al, 2002).

Furthermore while MMS and UV damaged cells arrest in G2/M phase, similarly to *cdc13-1* with uncapped telomeres, HU treated cells are blocked in S phase. Therefore it is likely that different DNA damages induce different ssDNA intermediates and NaCl can only resolve some of them

One of the most intriguing findings was that NaCl did not require either *DPB3/DPB4* or *POL32* for rescuing MMS treated cells, while *POL32* was required for repair at uncapped telomeres. Therefore the mechanism through which NaCl allowed proliferation on MMS might be different than the one at uncapped telomeres. It is also possible that these non essential subunits are not required for NaCl induced repair. For example only Pol32 was important for *cdc13-1* survival after recapping but both Dbp3/Dpb4 and Pol32 were required for NaCl induced repair at uncapped telomeres, showing that the same type of damage requires different subunits depending on capping. Hence, NaCl facilitated ssDNA repair at other loci might be still depend on polymerase  $\epsilon$  and  $\delta$  but not on their non-essential subunits.

Another interesting finding in this chapter is that NaCl reduced the viability of cells growing on HU but not on UV. This could be explained in two ways.

First, NaCl can induce further damage to HU treated cells that overwhelms the repair pathways. Second, NaCl could further enhance the activity of HU.

Although the exact mechanism of HU activity is not known, HU treated cells have reduced dNTP pools and this leads to replication fork stalling (Koc et al, 2004). Interestingly NaCl treatment strongly phosphorylates the yeast ribonucleotide reductase (RNR) required for synthesis of dNTPs *in vivo* (Soufi et al, 2009). Such phosphorylation has been previously identified in other high-throughput phosphorylation studies but the function is not clear (Albuquerque et al, 2008). If NaCl induced phosphorylation of RNR further reduces dNTP levels, this could explain why the cells grew less on media with HU and 430mM NaCl.

Finally one of the gene deletions I tested behaved fundamentally different upon different DNA damage. Deletion of *KCSI* had no effect on cell viability under UV damage but was slightly more sensitive to HU (Figure 26). In contrast loss of *KCSI* was beneficial for cells treated with MMS because the *kcs1Δ* mutant grew much better than wild type (Figure 27). Yet again *KCSI* managed to surprise with the variability of responses following loss or overexpression of this gene.

The ability of *KCSI* to rescue cells on MMS was probably not mediated through Mec1, because this checkpoint should be more active in the *kcs1Δ* mutant. Therefore it seems like *KCSI* may regulate cell viability in distinct ways depending on the type of DNA damage and further experiments should be conducted to understand the exact function of this gene. A recent publication shows that *kcs1Δ* is also growing better than wild type in the presence of H<sub>2</sub>O<sub>2</sub> (Onnebo & Saiardi, 2009). *KCSI* was important for mediating sensitivity to H<sub>2</sub>O<sub>2</sub> and this chemical specifically inhibited the activity of *KCSI* (Onnebo & Saiardi, 2009). Hence *KCSI* might also be mediating sensitivity to MMS. Most probably the inositol pyrophosphates synthesised by *KCSI* are the culprits behind the diverse responses observed through *KCSI* deregulation.

Inositol pyrophosphates are an emerging group of very interesting molecules that are involved in various aspects of cellular biology. These molecules can bind proteins directly and pyrophosphorylate phosphoproteins. The specificity of their targets and the biological outcome of those activities remain elusive (Alcazar-Roman & Wentz, 2008; Bhandari et al, 2007).

Interestingly mice lacking the *KCSI* homologue IP6K2 exhibit resistance to ionizing radiation but had increased incidence of cancer after administration of the tumorigenic drug 4-nitro quinolone 1-oxide that causes DNA lesions repaired by NER (Morrison et al, 2009). Thus inositol pyrophosphates exhibit differential functions in different DNA damage in mammals as well as in yeast. Moreover deregulation of IP signalling might either cause or prevent cancer and the molecular mechanisms governing these responses should be elucidated in the future.

## 6 Single stranded DNA re-synthesis and significance for cellular biology

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Until now telomere uncapping that leads to resection has been perceived as a terminal event that inevitably leads to prolonged cell cycle arrest, cell death or formation of HR dependent survivors. Although telomere resection clearly leads to checkpoint activation, no consequent ssDNA re-synthesis had been described before.

In this thesis found evidence for targeted ssDNA re-synthesis following telomere uncapping. When telomeres become uncapped and ssDNA is created by exonucleolytic activity, checkpoints are recruited to halt the cell cycle. However, DNA repair proteins are also recruited; including subunits from polymerases  $\alpha$ ,  $\epsilon$  and  $\delta$  and their clamp PCNA. Under uncapping conditions re-synthesis remained unsuccessful because it was prohibited by an unknown telomere uncapping sensor or because it was competing with resection. Only when capping was restored re-synthesis of the resected AC strand became apparent. More importantly, I found a way to stimulate this intrinsic ssDNA repair by applying mild osmotic stress on the cells. Interestingly osmotic pressure facilitated repair might also occur in cells damaged with MMS. This suggests that NaCl induces the activity of one or several polymerases involved in repair at the telomere or during recovery from DNA alkylation.

### **Why does ssDNA re-synthesis exist?**

SsDNA re-synthesis could simply be tightly coupled to normal checkpoint activation. Usually checkpoints are important to guide the repair machinery to damage sites and may even actively participate in DNA synthesis (Kai & Wang, 2003; Szyjka et al, 2008). Furthermore some polymerase subunits are also checkpoint sensors, showing that checkpoint and repair are closely collaborating in ensuring normal cell survival (Navadgi-Patil & Burgers, 2008; Navas et al, 1996). Hence, it is very likely that DNA polymerases are naturally recruited to DNA damage together with checkpoint proteins.

This may be beneficial for telomeres because if any capping protein becomes transiently damaged or can not associate to telomeres due to mutations, this may

induce telomere resection and arrest the cell cycle. If repair proteins were deliberately excluded from telomeres, as in the case of short telomeres, no repair would occur even if telomere capping is restored at a later point. This would be devastating for cells undergoing protein damage, for example when cells have increased ROS.

On the other hand if ssDNA at telomeres was always repaired following checkpoint activation, this would allow proliferation of cells that may have permanent telomeric protein damage or loss, for example due to mutations in the genes encoding these proteins. This by itself might cause severe chromosomal instability. Perhaps instead cells have developed a sensor that distinguishes between a capped and uncapped telomere that can regulate when ssDNA can be repaired at the telomeres or they ensure arrest through competition between re-synthesis and resection.

The fact that osmotic stress also induced ssDNA re-synthesis is probably telomere independent because NaCl could also rescue MMS treated cells. Hence, salt probably stimulates one or several polymerases *per se*. High osmotic stress induces double stranded breaks on chromosomes, thus activation of DNA polymerases and repair may have evolved as a mechanism to defend cellular integrity (Dmitrieva et al, 2005; Kultz & Chakravarty, 2001).

### **Importance for mammalian telomere biology**

NaCl induced repair might be active in some tissues undergoing osmotic stress. For example human kidney cells can tolerate up to 500mM NaCl without any loss of viability, in the presence of DSBs (Kultz & Chakravarty, 2001). These cells are usually cultured in 300mM NaCl and can proliferate freely in those conditions despite having DSBs, suggesting that such salt concentrations might promote repair in the kidney (Kultz & Chakravarty, 2001; Zhang et al, 2002).

NaCl facilitated rescue of damaged cells could be beneficial for some cells that have not been damaged substantially but may also allow proliferation of cells with accumulated mutations, leading to increased cancer risk. Indeed there are some reports in the literature that link high NaCl diet with stomach cancer (Peleteiro et al, 2011; Tsugane et al, 2004). Furthermore, patients experiencing osmotic pressure like diabetics or patients with high blood pressure have increased risk to develop certain cancers (Chow et al, 2000; Coughlin et al, 2004; La Vecchia et al, 1994). However it is not possible to predict if any of those correlations between hyperosmotic conditions and cancer are due to osmolality-driven proliferation of precancerous cells.

Overall my results answered the questions outlined in my aims and brought some new insights into telomere uncapping, ssDNA re-synthesis and repair. I have shown that resected telomeres can be repaired naturally after recapping by re-synthesizing the lost AC strand. This repair could be stimulated even when telomeres were uncapped by mild osmotic stress. Salt-mediated ssDNA repair was not only limited to telomeres but also important for some other types of DNA damage.

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