Proteins that function at telomeres: genetic and biochemical investigation



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Abstract

The telomere is a nucleoprotein structure found at the ends of all eukaryotic chromosomes that plays a key role in ageing and cellular proliferation. It prevents chromosome ends being recognised and processed as double strand breaks and therefore preserves genomic integrity. This study aimed to further investigate the role of a telomere capping protein complex found in *S. cerevisiae*, which comprises three proteins, Cdc13, Stn1 and Ten1 forming the CST complex. The CST complex associates with the single stranded G-rich overhangs found at telomere ends acting as a nucleating centre for various telomere associated factors, and preventing access to enzymes that might process the telomere as a double strand break, in particular exonucleases. This is evidenced from genetic studies involving temperature-sensitive mutants of CDC13, STN1, TEN1 all of which accumulated single stranded DNA at the telomere end resulting in activation of the G2/M checkpoint. However, the ability of these proteins to protect telomere-like structures in vitro has yet to be demonstrated. Furthermore, similarities have been drawn between the domain architecture of the components of the CST complex and Replication protein A (RPA) a ubiquitous single stranded DNA binding protein extensively involved in DNA metabolism. This has led to the suggestion that CST could be a telomere specific version of RPA.

In this study, an *in vitro* telomere protection assay was developed and optimised to directly test the ability of CST and RPA to inhibit 5' to 3' resection of telomere mimics and non-telomere controls. It was found that while RPA was able to protect telomere and non-telomere control substrates from resection, Cdc13 only protected telomeres. Furthermore, it was found that the DNA binding domain of Cdc13 was able to inhibit resection by the 5' to 3' exonuclease, λ -exonuclease, and was able to outcompete RPA for binding to the 3' G-rich overhang found at the end of the telomere mimic. The two small subunits of the CST complex, Stn1 and Ten1, were not able to inhibit nuclease resection by λ -exonuclease at telomere mimics or non-telomere controls.

Two synthetic genetic arrays and quantitative fitness analyses were carried out using temperature-sensitive alleles of *STN1* and *RPA3* (the second largest subunit of the CST

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complex and the smallest subunit of the RPA heterotrimer respectively). The aim of these screens was to determine the extent to which the genetic interaction profiles of these screens overlapped with that demonstrated previously for *cdc13-1*. It was found that, similarly to *cdc13-1*, the *stn1-13* temperature-sensitive phenotype was suppressed by deletion of *EXO1* or nonsense-mediated mRNA decay genes. However, deletion of a genome integrity checkpoint protein required for cell cycle arrest in G2/M, *RAD9*, in the *stn1-13* background, enhanced the temperature-sensitive phenotype, suggesting that the G2/M checkpoint was important for the vitality of *stn1-13* strains in contrast to *cdc13-1*. It was also found that deletion of *rpa3-313* strains in contrast to *cdc13-1* and *stn1-13* strains where these deletions had a negative effect on growth. In addition deletion of *EXO1* had no effect on the fitness of *rpa3-313* strains in contrast to its suppressive effect on temperature-sensitivity in the *cdc13-1* and *stn1-13* background.

These results demonstrate biochemically that Cdc13 and RPA inhibit 5' to 3' resection at telomere ends. Furthermore they demonstrate the importance of *STN1* in preserving the telomere end, and the involvement of the nonsense-mediated mRNA decay pathway in disrupting CST-mediated telomere capping. Finally they underline the difficulty of disentangling the role of RPA in telomere capping using genetic techniques due to its extensive involvement in DNA metabolism throughout the cell.

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Abbreviations

AMP	ampicillin
АТР	Adenosine Triphosphate
bp	base pair
BSA	Bovine Serum Albumin
CL	control loop mimic
DAmP	decreased abundance by mRNA perturbation
dATP	deoxyadenosine triphosphate
dCTP	deoxycytidine triphosphate
DDR	DNA damage response
dGTP	deoxyguanosine triphosphate
DMA	deletion mutant array
DNA	deoxyribonucleic acid
DNAse I	DNA endonuclease I
dNTP	deoxyribonucleotide triphosphate
ds	double stranded
DSB	double strand break
DTT	dithiothreitol
dTTP	deoxythymidine triphosphate
EDTA	ethylenediaminetetra-acetic acid
EMSA	electrophoretic mobility shift assay
Exo	exonuclease
g	acceleration caused by gravity
HEX	6'-carboxyl-2',4,4',5',7,7'-hexachlorofluoroscein
HI (buffer)	hepes inositol
HPLC	high pressure liquid chromatography

xv

HR	homologous recombination
IPTG	isopropylthiogalactopyranoside
KAN	kanamycin
Kb	kilobase
K _D	dissociation constant
kDa	kilodalton
MSG	monosodium glutamate
NHEJ	non-homologous end joining
Ni-NTA	nickel-nitrilotriacetic acid
NMD	nonsense-mediated mRNA decay
OD	optical density
ORF	open reading frame
PEG	polyethylene glycol
PNK	polynucleotide kinase
QAOS	quantitative amplification of single stranded DNA
QFA	quantitative fitness analysis
RPA	Replication Protein A
rpm	revolutions per minute
SCGL	synthetic complete glycerol lactic acid
SDS	sodium dodecyl sulphate
SGA	synthetic genetic array
SS	single stranded
TAE	tris-acetate/EDTA
ТВЕ	tris-borate-EDTA
TBS	tris-buffered saline
TE	tris/EDTA
TEMED	N,N,N',N'-Tetramethylethylenediamine

TL	telomere loop mimic
T _M	temperature of melting
UV	ultraviolet light
YEPD	yeast extract peptone dextrose
YEPD	yeast extract/peptone/dextrose
YFG	your favourite gene
YPGL	yeast extract peptone glycerol lactic acid
YPGLA	yeast extract peptone glycerol lactic acid adenine

Yeast genetic nomenclature

Wild type: CDC13

Mutant : cdc13-1

Null: $cdc13\Delta$

Protein: Cdc13

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1. Introduction

1.1 Telomeres

Telomeres are repetitive tandem arrays that form the ends of eukaryotic chromosomes. They serve to buffer the end of the chromosome from attrition as a result of exonuclease activity, limitations of DNA replication and DNA damaging events. The special characteristics of the end of the chromosome were first noticed in the late 1930s and early 1940s by Herman Muller and Barbara McClintock ^{1; 2}. They noticed that while broken chromosomes were susceptible to rearrangements and chromosomal fusions, the same was not true at the chromosome ends, which were preserved, and Herman Muller coined the term "telomere" to describe them.

1.1.2 Discovery

During the 1970s it was discovered that the ends of chromosomes consisted of repetitive elements of DNA ³. In the following decade Elizabeth Blackburn, Jack Szostak and Carol Greider further elucidated the exact nature and function of these terminal end sequences. They found that these repeat sequences protected the ends of chromosomes from degradation and recombination events ⁴. Furthermore, they discovered that a reverse transcriptase called telomerase generates telomere DNA and thereby replenishes the telomere end ^{5; 6; 7}.

1.1.3 Roles in ageing and cancer

In an earlier discovery, it was found that if human fibroblasts were serially cultured they would only divide a fixed number of times before stopping and becoming senescent ⁸. It was proposed that the message to stop dividing came from some internal factor in the cell. While many internal factors in the cell contribute to cellular senescence, telomeres are proposed to be a major factor ⁹ since they shorten with every cell division ¹⁰.

In humans, somatic cells do not generally possess telomerase activity. However embryonic stem cells are pluripotent, meaning they can form multiple cell types, and have high telomerase activity ¹¹. This ensures that they can divide repeatedly without becoming exhausted, enabling for the generation of multiple cell types. Furthermore as these stem cells differentiate into adult stem cells and progenitors, there is a trend towards reduced telomerase activity, until the appearance of somatic cells, where telomerase activity is extremely low or disappears altogether ¹².

The fact that somatic cells have no telomerase activity could be a way of ensuring that these cells do not begin to divide uncontrollably. Such uncontrollable cell division in somatic cells is one of the hallmarks of cancer. Therefore loss of telomerase activity could act as a cancer-defence mechanism ¹³. Interestingly telomerase is re-activated in >85% of human cancers ¹⁴, underscoring its importance in cellular immortalisation. The other 15% do not maintain their telomeres using telomerase and often maintain their telomeres through ALT (alternative lengthening of telomeres) mechanisms which involve recombination ¹⁵. Since >85% of human cancers have active telomerase, this is a useful target for chemotherapeutic drugs. One example is the drug BIBR1532, which was shown to inhibit telomerase processivity as well as interfere with telomere capping in cancer cells, which leads to cytotoxic effects and reduced proliferation ¹⁶.

Telomeres are not only important in cancer cell immortalisation and stem cell differentiation; they are also relevant to age-related diseases. Shortened telomeres are associated with multi-cause mortality in the under-85s ¹⁷, and they are associated with various age-related diseases such as vascular dementia ¹⁸.

Furthermore investigation of naturally occurring genetic diseases in which the telomere maintenance machinery is compromised provide more direct evidence that telomere dysfunction can lead to age-related pathology. Two particularly relevant genetic diseases in respect to this are Dyskeratosis Congenita (DC) and Coats Plus (CP). DC is caused by mutations in the telomerase RNA component (TERC) ¹⁹ or Dyskeratosis Congenita 1 (DKC1) which contributes to telomerase stability ²⁰. CP on the other hand is caused by mutations in the human homolog of CDC13, CTC1 ²¹. However, interestingly both diseases show overlapping pathologies which are characteristic of human ageing including: anemia, dystrophic nails, hypogonadism and loss of bone

mineral density. Therefore while shortening telomeres appear to be a cancer protection mechanism, in later life this process contributes to replicative senescence and age-related diseases.

1.2 The origins of telomeres

One of the intriguing aspects of the eukaryotic domain of life is that generally DNA is organised into linear structures called chromosomes, which are enclosed within a membrane-bound nucleus. This is in contrast to what is generally seen in the other two domains of life: archaea and prokaryotes, where DNA is circular and not separated from the rest of cell. Therefore the question arises: Why do eukaryotes have linear DNA?

It is probable that there was strong selective pressure to develop linear DNA, as there are several costs associated with holding genetic information in this way. Firstly replicating linear DNA is energetically expensive and replication speed is greatly reduced ²². Furthermore since the DNA is organised in a linear fashion, it possesses "ends" which are highly susceptible to being inappropriately "repaired" by the cell ²³. Therefore, eukaryotes had to invest in chromosomal end protection mechanisms, which involved the development of proteins which efficiently hide the telomere end and prevent its degradation. However there are notably exceptions including bacteria which harbour linear chromosomes such as *Streptomyces*. Furthermore *Streptomyces* chromosomes possess a 250-300bp 3' overhang ²⁴. This gap is partially repaired by terminal protein (TP) primed DNA synthesis, and the remaining gap is protected by "capping" proteins, similarly to eukaryotes ²⁵.

So, given the expense of maintaining linear DNA, why does it persist? The evolution of linear chromosomes is probably tightly tied with the evolution of sexual reproduction ²⁶. This is since cells with linear chromosomes are able to undergo two types of cell divison: mitosis and meiosis. The ability of linear DNA to undergo meiosis i.e. the pairing of chromosomes from different parents, and the exchange of genetic material through recombination, gave eukaryotes several evolutionary advantages compared to prokaryotes and archaea.

Firstly, eukaryotes were able to undergo less error prone recombination based repair mechanisms by using a homologous chromosome as a template to repair dsDNA damage ²⁷. However, although the advantages of homologous recombination could explain the evolution of sex, it does not explain its maintenance ²⁸. One argument for why sexual reproduction was maintained is that it enables organisms to dump all of their deleterious alleles into one progeny, rather than gradually accumulating them ²⁹. Finally, and most importantly, meiosis enabled cells to produce progeny with enormous "useful" genetic diversity, compared to their prokaryotic and archael relatives, since they could randomly shuffle alleles of genes ^{26; 30}. How linear chromosomes arose is still an interesting question and possibilities include accidental linearization of a circular chromosome or linearity enforced by the invasion of selfish elements such as retrotransposons or plasmids ³¹.

So, given the fact that linear DNA enabled organisms to have sex and create genetic diversity, there was a clear selective advantage. Therefore when the DNA of some eukaryotic ancestor was linearized in some fortuitous event, the arrangement was preserved. However, the issue of unprotected DNA ends still needed to be resolved, since the consequence is severe genomic instability.

1.3 Budding yeast as a model

Chromosome end protection occurs in all eukaryotes through a largely conserved nucleoprotein structure called the telomere. Efficient telomere end protection is essential to prevent the development of dysfunctional telomeres which play important roles in human diseases such as cancer. The technical challenges of working with human cells can limit research outcomes. Therefore use of simple model eukaryotes such as *S. cerevisiae* can allow more ambitious research goals to be achieved. This, in turn, can then direct human research.

S. cerevisiae is a useful model organism to understand telomere biology because it shares a common evolutionary origin with humans as part of the opisthokont phylum of the eukaryotes ³² (figure 1a). Furthermore telomere capping in this species is relatively well understood, and has conserved mechanisms of telomere end protection with humans ³³ (figure 1b). Budding yeast are also technically simple to work with as

they have short generation times, have a diploid and haploid phase and are easy to manipulate genetically using homologous recombination based techniques.

Budding yeast whose telomerase function has been inactivated also behave similarly to human somatic cells, and divide a fixed number of times before entering senescence ³⁴. Interestingly, similarly to around 10% of human cancers, some of these strains are able to form post-senescence survivors which use recombination-based mechanisms to maintain their telomeres ³⁵.

Temperature-sensitive mutants of genes coding for essential aspects of the telomere maintenance machinery are also available in this species, which can help us to better understand what happens when telomere capping is compromised. *S. cerevisiae* also have a large number of non-essential genes which can be simply deleted by disruption of the open reading frame with a selectable marker. By deleting these genes in the context of temperature-sensitive mutants of essential genes involved in telomere maintenance, it is possible to determine what genes are involved in protecting or processing the end of the telomere. As *S. cerevisiae* are easy to grow, genetically manipulate and has short generation times it is possible to carry out these experiments in a high throughput manner using robotics ^{36; 37}, which allows rapid identification of new pathways of telomere maintenance and processing.

Therefore due to their common evolutionary origin, their simplicity to work with, and a deep understanding of how they maintain and process their telomeres, it is possible to carry out genetic experiments and use this data to inform more clinically relevant research in human cells.



Figure 1. The evolutionary relationship between budding yeast and humans. A. Diagrammatic tree showing the organisation of the eukaryotic domain of life into six major groups, based on phylogenetic data. *Saccharomyces cerevisiae* share a common ancestor with humans as part of the Opisthokont kingdom ³². B. There are conserved mechanisms of telomere end protection in budding yeast, fission yeast and mammals ³⁸.

1.4 Chromosome ends

Linear DNA is characterised as having ends and these structures pose a molecular hazard to cells. Dysfunctional telomeres cause huge genome instability leading to various catastrophic cellular events such as chromosomal end to end fusions, recombination, checkpoint activation and cell death. Exposed chromosomal ends are largely so toxic to the cell because they resemble one half of a form of DNA damage called a DNA double strand break. Double strand breaks can be formed as a result of exposure to ionizing radiation, or the failure of replication at unrepaired DNA damage sites ³⁹. They are also induced to form during meiosis to encourage chromosomal crossover and recombination ⁴⁰. Double strand breaks are highly toxic the cell and need to be repaired, and this can take place via one of three mechanisms: homologous recombination (HR), non-homologous end joining (NHEJ) or microhomology mediated end joining (MMEJ).

1.4.1 Homologous recombination (HR)

HR uses recombination with a homologous chromosome to repair a double strand break. This form of repair is largely error-free (figure 2). The DNA double strand break is initially bound by a suite of proteins whose functions are to generate an intermediate which can be recognised by the cell as DNA damage. In *S. cerevisiae* a heterotrimeric complex called MRX (consisting of Mre11, Rad50 and Xrs2) and Sae2^{41;} ⁴² resect the 5' recessed strands at the double strand break leading to an expanding region of 3' ssDNA. More extensive resection is then carried out by Exo1 and Dna2⁴³. This ssDNA is then bound by another heterotrimeric protein called Replication Protein A (RPA) which recruits the genome integrity checkpoint proteins Mec1 and Ddc2 triggering a checkpoint kinase cascade leading to freezing of the cell cycle⁴⁴. This checkpoint is important since it prevents the cells going through mitosis, leading to the segregation of damaged DNA, which could go on to cause further genome instability. The RPA coated ssDNA then recruits the DNA repair protein Rad51 which forms the nucleoprotein filament to facilitate the invasion of a homologous chromosome and recombination-based repair⁴⁵.

1.4.2 Non homologous end joining (NHEJ)

NHEJ is an alternative method of repairing double strand breaks which essentially involves ligating the two ends back together (figure 2). Following the formation of a double strand break the DNA ends are bound by the Ku heterodimer. MRX is then recruited and there can be some resection at the DNA ends with the purpose of removing mismatches or damaged bases generating a small overhang. Ku and MRX function together to tether the two DNA ends together. Yku80 and Xrs2 are then proposed to recruit DNA ligase IV to DNA ends, and the overhanging strands are aligned ⁴⁶. Following base pairing, fill-in and processing by polymerases and FLAP-endonucleases the DNA ends are ligated back together ⁴⁷. However, confusingly while MRX is extensively involved in NHEJ it has also been proposed to be integral in determining whether cells repair double strand breaks via HR or NHEJ ³⁹. It has been proposed that Mre11 can use its endonuclease activity to knock the Ku heterodimer off double strand break ends, and allow processive resection by other nucleases such as Exo1 ⁴⁸. Notably in the absence of Ku and ligase IV, end joining is greatly reduced, and occurs through highly mutagenic microhomology-mediated end joining (MMEJ) ⁴⁹.

However, unlike a double strand break, the ends of chromosomes are not blunt, they form a 3' overhanging structure. This overhang is formed on the newly replicated lagging C strand as a consequence of the inability of DNA polymerase to replicate to the end of the 5' recessed strand. This problem was noted simultaneously by both Watson and Olovnikov and it was termed the "end-replication problem" ^{50; 51}. However an overhang is generated on the newly replicated leading strand by the action of nucleases ⁵². The presence of the 3' overhang at the telomere end is significant because it bears a striking similarity to the intermediate structure formed during homologous recombination by resection on the 5' recessed strand by MRX. This means that if left unprotected, a telomere would be treated analogously to a "partially-processed" double strand break, which would be disastrous for the cell. The chromosome ends are organised into nucleoprotein structures called telomeres to prevent the ends of chromosomes being recognised as double strand breaks.



Figure 2. Model showing the two major pathways of double stand break repair: nonhomologous end joining (NHEJ) and homologous recombination (HR). A. In the NHEJ pathway, DNA ends are bound by MRX and Ku which act to tether the DNA ends together. They also recruit various other factors involved in processing this damage, and ultimately DNA ligase IV which ligates the two broken ends back together. B. In the HR pathway, ends are bound by MRX and Sae2 which create short 3' overhanging ssDNA overhangs. Exo1 and Sgs1-Dna2 then create longer overhangs which attract the ssDNA binding protein RPA and trigger checkpoint activation, recruitment of Rad51 and strand invasion ⁴³.

1.5 Telomeres: a unique type of DNA damage

In many ways, telomeres are essentially a double strand break at the end of the chromosome. However, it is essential that they are not recognised as such. So, telomeres have evolved some unique characteristics that enable them to be treated differently to a generic double strand break.

First and foremost, telomeres consist of repetitive TG/CA rich dsDNA ending in a 3' TGrich overhang. The sequences of telomeres vary between species but they are invariably TG rich. *S. cerevisiae* telomeres are unusual in that they are highly heterogeneous consisting of a 3' repetitive TG rich leading strand of the form TG₁₋₃ and a 5' CA rich lagging strand of the form C₁₋₃A ⁵³. The telomere 3' overhang is extended by a reverse transcriptase called telomerase which adds telomere repeats to the 3' overhang through the use of an RNA template. *S. cerevisiae* telomerase consists of four subunits: Tlc1 (which is the RNA component), Est1 (which recruits telomerase to telomere ends through Cdc13), Est2 (the catalytic core) and Est3 (which plays a regulatory role) ^{54; 55; 56} (figure 3).

The heterogeneity displayed by *S. cerevisiae* telomeres is a consequence of some interesting biochemical characteristics of *S. cerevisiae* telomerase: firstly, it can align its RNA template with 3' overhang of the telomere in different registers in each extension cycle and secondly sometimes only part of the RNA template is used for reverse transcription ⁵⁷. This is in stark contrast to humans where telomere repeats are highly uniform and of the form TTAGGG on the leading strand and CCCTAA on the lagging strand.

Secondly, all telomeres have a 3' "G-tail" or "G-overhang" which consists of TG-rich DNA. TG DNA has a tendency to form secondary structures including G-quadruplexes which are formed by stacking of tetrads of guanines ^{58; 59}. These structures are proposed to play a key role in telomere homeostasis, and are proposed to form a rudimentary telomere cap and modulate the activity of telomerase ⁶⁰. Furthermore in humans, where the G-overhang is 150-300bp for most of the cell cycle ^{61; 62}, the 3' overhang invades subtelomeric DNA and results in the formation of looped structures called T and D-loops ^{63; 64}. These looped structures are proposed to help "hide" the

telomere end from the double strand break machinery. In *S. cerevisiae* these looped structures have yet to be demonstrated. Furthermore there are some other relevant inter-species differences since the overhang in *S. cerevisiae* is quite short for the majority of the cell cycle with an average length of 12-15 nucleotides ⁶⁵. However there is a short period between S phase and the second stage of growth (G2) where telomere overhangs increase in length to 30-100 nucleotides ⁶⁶. This increase in length is proposed to be due to a combination of telomerase activity and cell cycle regulated exonuclease activity on the 5' recessed strand ^{67; 68} by several exonucleases ⁶⁹.

Furthermore, not all regions of the telomere are the same, and in *S. cerevisiae* they can broadly be divided into two sections. The most proximal region of the telomere consists of telomere DNA wrapped around histones to form nucleosomes ⁷⁰. The most distal region of the telomere is also referred to as the "simple repeats" and is not associated with histones ⁵³. It is in this region that all of the telomere repeat binding proteins can be found together. This region can be further subdivided into a dsDNA region and an ssDNA region each of which is bound by a unique set of proteins and together these form the telomere proteinaceous "cap" ⁵³. The cap affords telomeres the ability to be treated differently to double strand breaks ²³. This is achieved in a number of ways: through recruitment of telomerase to telomere ends, efficient replication through the telomere, and preventing nucleolytic resection of the 5' recessed strand.

1.5.1 Recruitment of telomerase

It is essential that telomeres retain their length, as when one telomere becomes critically short, telomere capping fails and results in activation of the DNA damage checkpoint ⁷¹. Therefore in *S. cerevisiae* the activity of telomerase is closely regulated, to maintain telomere length homeostasis, and counteract telomere attrition caused by exonuclease degradation and errors in lagging strand synthesis.

The major players in telomerase recruitment are Cdc13 and the Ku heterodimer ⁷² (figure 3), although many other proteins are involved in modulating the activity of telomerase at telomere ends in *S. cerevisiae*. It is thought that the Ku heterodimer interacts with the RNA component of telomerase, TLC1 ⁷³ and encourages telomerase

nuclear import ^{74; 75}. Once inside the nucleus the Est1 subunit of telomerase is then proposed to interact with the telomere ssDNA binding protein Cdc13 which recruits it to telomere ends ⁷⁶. The activity of telomerase is modulated by various other proteins including the Rap1-Rif1-Rif2 polymer which coats telomere dsDNA ⁷⁷ and acts as a measuring mechanism, inhibiting telomerase activity when telomeres become too long ⁷⁸. Another telomere ssDNA associated protein, Stn1, is proposed to be involved in negatively regulating telomerase ⁷⁹ by competing with Est1 for binding to the recruitment domain of Cdc13 ⁸⁰. However telomerase recruitment is still not fully understood, and many factors probably play a role in the recruitment and modulation of telomerase activity.



Figure 3. Recruitment of telomerase to telomere ends in *S. cerevisiae.* It is thought that the Ku heterodimer interacts with the RNA component of telomerase, Tlc1 to import telomerase into the nucleus. The telomerase enzyme is made up of four subunits Est1, Est2, Est3 and Tlc1. Est1 is involved in recruiting telomerase to the telomere end through an interaction with Cdc13. Est2 is the catalytic subunit, while the function of Est3 is still not fully understood. However, it has been proposed that Est3 might interact with ssDNA, in an Est2-dependent manner and form another mechanism of telomerase recruitment ⁸¹.

1.5.2 Preventing C-strand degradation by exonucleases

One of the key functions of telomere capping proteins is to prevent resection of the 5' recessed strand of telomeres, which leads to the generation of ssDNA and activation of the Rad9-dependent G2/M checkpoint ^{82; 83}. The importance of Cdc13 in this protection has been demonstrated genetically using a temperature-sensitive allele called *cdc13-1*. This contains a mutation (P371S) in the second OB fold of Cdc13, which is proposed to be involved in homodimerization. This homodimerization is proposed to be involved in herefore this mutation is proposed to disrupt the Cdc13-Stn1 interaction ⁸⁴. The concequence of this mutation is that it severely disrupts telomere capping, leading to ssDNA generation, G2/M checkpoint activation and poor fitness of *cdc13-1* strains (figures 4a, b).

Interestingly, Cdc13 is proposed to be important for capping in late S-phase and at the G2/M boundary, but not in G1 or early S. This suggests that its function is cell-cycle regulated, and is probably affected by the passage of the replication fork, such that Cdc13 dissociates during replication, and this is evidenced by the fact that C-strand processing increases during telomere replication ⁵³.

However, the role of the CST complex is complicated by the fact that it makes multiple interactions with the lagging strand machinery. This suggests that it may be difficult to disentangle whether the C-strand degradation observed in these mutants is due to resection at the ends or problems with replicating the telomere repeats.

C-strand degradation not only plays a role in checkpoint activation, but it is also important for telomere assembly. This is since the G-tail replicated by the leading strand machinery generates a blunt-end. The 5' end of this blunt ended product needs to be resected by nuclease activity to ensure that the CST complex and other ssDNA-associated proteins can bind, and establish the telomere cap ⁸⁵. This degradation is dependent on the same genes that resect the ends of double strand breaks ⁵³.

MRX is proposed to be involved in resection of the 5' recessed strand at telomere ends, however it is non-essential ⁶⁵. Exo1 and Dna2 are also involved, carrying out processive resection into the telomere to generate ssDNA to signal and respond to

telomere uncapping. This latter type of resection is strongly inhibited by the CST complex and Ku heterodimer ^{79; 83; 86; 87; 88}.





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Figure 4. *cdc13-1* mediated telomere uncapping. A. When *cdc13-1 S. cerevisiae* are grown at the semi-permissive temperature telomeres become "uncapped". Exo1 and other nucleases contribute to the generation of ssDNA which causes Mec1-dependent checkpoint activation ⁸⁹. B. A five-fold dilution series of the indicated genotypes were spotted onto plates and incubated at the indicated temperatures. These spot tests demonstrate that when *cdc13-1* cells are grown at 27.3°C their growth is strongly inhibited. However, when the checkpoint genes *RAD9* or *EXO1* are deleted growth at this temperature is recovered ⁹⁰.

1.5.3 Replicating the telomere

Telomeres are unusual in that they contain a high proportion of G-C base pairs which makes them difficult to replicate by semi-conservative DNA replication because of the high melting temperature and secondary structure formation. Furthermore, the origins of replication at telomeres in *S. cerevisiae* (termed autonomously replicating sequences) fire late near telomeres, due to the telomere position effect ^{91; 92}. This means replication forks move very slowly through telomeric regions and are prone to replication fork stalling ⁹³.

Therefore telomeres are highly sensitive to problems with the conventional replication machinery. For example mutations in DNA polymerase α, Rad27 and Replication Factor C all result in telomere lengthening ^{94; 95; 96} implying that they might negatively regulate telomerase activity. This lengthening is proposed to be mediated through the CST complex as it interacts with DNA polymerase α through Cdc13 and Stn1 ^{97; 98}, and is also involved in the recruitment of telomerase ⁸⁷. It has been proposed that when there are problems with DNA replication, Cdc13 recruits telomerase thereby increasing telomere length ⁵³. Furthermore it is also important that lagging strand synthesis is efficiently completed in S/G2 as this can lead to the accumulation of single stranded DNA. Cdc13 and Stn1 probably play a key role in regulating and tethering lagging strand activity to the telomere end.

The presence of repetitive DNA, secondary DNA structure formation and telomere repeat binding proteins all contribute to the unique way in which telomeres are treated compared to similar DNA substrates in the cell. However the presence of a number of proteins that are involved in DNA double strand break repair at normal wild type telomeres raises the question of how they are contributing to capping at the telomere, but DNA repair elsewhere in the genome.

1.6 The paradox of DDR proteins at the telomere

One of the fascinating things about telomere maintenance is that the nucleoprotein complex which forms at the telomere end incorporates aspects of the DNA repair machinery. This is perhaps not intuitive, as naturally it would expected that the DNA repair machinery would want to be kept away from the telomere end, because of the danger it poses to genome stability through the activation of inappropriate repair activities. One possible reason for this is that the sheer similarity of telomeres and double strand breaks makes it too difficult for these proteins to be excluded ²³. Alternatively it could be more efficient to make use of the local protein resources, rather than evolving new machinery.

Either way, these proteins are present at the telomere end which means that their repair activities are being repressed or redirected to maintaining chromosome ends. This dual functionality is likely mediated by a variety of factors both inherent in the protein structure and modulated by the protein's local environment and substrate.

However, in the initial stages of processing telomeres are treated broadly similarly to double strand breaks. The ends are bound by the Ku heterodimer and the MRX complex and there is some resection of the 5' recessed strand exposing the G-rich telomere 3' overhang. The CST complex is then recruited to the telomere 3' overhang as a result of the high affinity of Cdc13 for telomere G-rich ssDNA ^{87; 99}. This putative complex is proposed to prevent any further resection ¹⁰⁰, and promotes the recruitment of telomerase ^{72; 76} and efficient replication of the telomere ⁸⁰. Therefore establishing the telomere involves cooperation between the DNA repair machinery and telomere-specific factors.

Following establishment of the telomere cap, the Ku heterodimer remains associated with the dsDNA proximal to the CST-bound ssDNA overhang. Not only does this heterodimer act as a another level of protection against exonuclease activity on the C-strand ⁸⁶, it is required for other telomere specific functions including nuclear import of telomerase ⁷⁵, telomeric silencing and the localisation of telomeres at the nuclear periphery ^{101; 102}. Furthermore, the bi-functional nature of the Ku heterodimer is
reflected in its structure with its two "faces", Yku80 and Yku70, performing distinct telomere maintenance and DNA repair roles respectively ¹⁰³.

Once the telomere nucleoprotein structure has been fully formed, Mre11 is proposed to play key roles in recruiting Tel1 specifically to short telomeres, to mediate telomerase recruitment through its kinase activity although the target for phosphorylation is unclear ¹⁰⁴. Furthermore Mre11 was found to be involved in telomere "capping" in the *cdc13-1* background ¹⁰⁵. Mre11 likely contributes to capping through its interaction with Tel1, however this is still unclear. Furthermore it is also clear that Rad9 and checkpoint kinase dependent mechanisms also contribute to telomere capping in the *cdc13-1* background ²³.

Therefore DNA damage response proteins play a key role in telomere maintenance, and their activities seem to be modulated by the local protein-DNA environment at telomeres. Furthermore, there is also evidence that DNA repair proteins have structural alterations that enable them to behave differently at telomere ends versus double strand breaks. However it also evident that CST is essential in managing the behaviour of these DNA damage proteins, to ensure that they do not treat the telomere like a double strand break. Therefore CST appears to act as a nucleating centre for the formation of the telomere cap, and is extremely important for maintaining the integrity of telomere ends.

1.7 The CST complex

Cdc13, Stn1 and Ten1 have been proposed to form heterotrimeric CST complex which is proposed to bind telomere 3' overhangs and play an integral role in telomere homeostasis. Before considering the roles of the individual subunits in capping it is first important to consider why they are proposed to exist as a complex.

Although Cdc13, Stn1 and Ten1 have not been purified as a complex *in vitro* there is biochemical and genetic evidence that they form a complex *in vivo*. The first time Cdc13, Stn1 and Ten1 were referred to as the "CST complex" was in 2001. It was shown that temperature sensitive alleles of the three subunits: *cdc13-1*, *stn1-13*, and *ten1-31* all showed accumulation of single stranded DNA at the telomere and activation of the G2/M checkpoint ^{79; 83; 106}. Interactions were also shown between all three subunits using a combination of co-immunoprecipitation and yeast-2-hybrid analysis ¹⁰⁶. Furthermore structural data from other species showed that Stn1 and Ten1 associated together as a complex in *C. tropicalis* and humans ^{107; 108}. Moreover, in *C. glabrata* the whole CST complex has been successfully purified and was shown to have an unusual stoichiometric arrangement of CST 2:4:2 or 2:6:2 ¹⁰⁹.

These data coupled with the fact that overexpression of Stn1 can rescue *cdc13-1* temperature-sensitivity ⁷⁹ and Ten1 can ablate the long telomere phenotype seen in *stn1* mutants ¹⁰⁶ suggests that CST is behaving like a complex. However, the evidence is still inconclusive and it remains a possibility that CST could form a transient rather than stable complex. Further *in vitro* studies will be necessary to confirm the existence and nature of the CST complex in *S. cerevisiae.* For now though, we will consider the roles of the putative CST complex, which allow it to act as a nucleating centre for the formation of the telomere cap.

Cdc13 forms the "core" of the CST complex and binds to telomere G-overhangs with high affinity through its third OB fold, the DNA binding domain ¹¹⁰. This domain forms an unusual structure when binding telomere ssDNA which includes a long β 2- β 3 loop and a C-terminal helical region. The long β 2- β 3 loop is proposed to mediate the high affinity binding of Cdc13 ¹¹¹, and the DNA binding domain is proposed to bind telomere G-overhangs with 3pM affinity ¹¹² (figure 5a, b). Furthermore, interestingly the binding

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mode shown by Cdc13 is significantly different to that seen in *S. pombe* Pot1 ¹¹³, leaving the idea that they may be homologs in doubt. The hypothesis that Cdc13 acts as the core member of the CST complex and a nucleating centre for various other telomere associated proteins comes from some biochemical work carried out by the Lundblad laboratory. They found that if Stn1 was fused to the DNA binding domain, it could rescue the lethality displayed by cdc13 Δ mutants. Furthermore although telomere replication was defective in these strains, it could also be rescued by fusing telomerase to the DNA binding domain ¹¹⁴. These experiments established Cdc13 as the primary recruiter of Stn1 and telomerase to telomere ends.



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Figure 5. The Cdc13-DBD ssDNA complex. A. Ribbon diagram showing the secondary structure of the DNA binding domain. The β 2- β 3 loop is highlighted in magenta. B. An overview of the structure of DBD bound to telomere ssDNA. The ssDNA binds across the DBD in the cleft formed between the β 1- β 2, β 2- β 3, and β 4- β 5 loops ¹¹¹.

Cdc13 not only acts as a nucleating centre for other proteins with roles at the telomere, it also recruits telomerase through its RNA associated component Est1⁷⁶. Stn1 is proposed to play a key role in regulating the recruitment of telomerase by Cdc13, such that disruption of Stn1 function leads to over-extension of the telomere end⁷⁹. This negative regulation of telomerase by Stn1 is proposed to occur because Stn1 and Est1 have overlapping binding sites on Cdc13^{80; 87}. The dimerization of Cdc13 is proposed to be integral to the proper formation of the Stn1 protein interaction

interpace on Cdc13. Therefore it is hypothesised that the P371S mutation found in *cdc13-1* mutants disrupts this dimerization thereby disrupting the Cdc13-Stn1 interaction and explaining the temperature-sensitive phenotype ⁸⁴ (figure 6a-c).





Figure 6. The location of the P371S mutation of *cdc13-1* **in OB-fold 2 of Cdc13.** A. The domain architecture of Cdc13. OB1, 2 and 4 all play important roles in the homodimerization of Cdc13. B. The tertiary structure of OB2 with the amino acid backbone and side chains of proline 371 highlighted. C. A magnified version of OB2 showing the position of P371S. Structures were created using PyMol⁸⁴ using data taken from Mason et al 2004.

Cdc13 and Stn1 also seem to play key roles in the replication of the telomere end in *S. cerevisiae*. Specifically they seem to interact with the lagging strand machinery thereby tethering C-strand synthesis and telomerase recruitment, ensuring that when the 3' overhang is extended, the 5' recessed strand is filled in, as excessive ssDNA would lead to a DNA damage signal, and freezing of the cell cycle at G2/M. Furthermore, while Cdc13 has been shown to interact with the catalytic subunit of DNA polymerase α ⁹⁷, Stn1 interacts with a regulatory subunit called Pol12 ⁹⁸. It is thought that Stn1 is able to promote pol α activity through this interaction, thereby promoting C-strand synthesis.

Furthermore Stn1 appears to be recruited to non-telomere sites when overexpressed, through this interaction with Pol12¹¹⁵.

Since overexpression of Stn1 rescues the temperature-sensitive phenotype of *cdc13-1* mutants and it plays largely overlapping roles with Cdc13; this has led to questions over the relative importance of the three subunits. Recent work has begun to cast doubts on a Cdc13-centric CST complex, and increasingly there are suggestions that Stn1 and Ten1 could play a more important role than Cdc13 in telomere maintenance. This suggestion came following experiments where it was shown that Stn1 and Ten1 could bind telomeres in the absence of Cdc13. Furthermore it was found that if aspects of the nonsense mediated mRNA decay (NMD) pathway were deleted, *cdc13* Δ mutants which are normally dead could grow. However similar genetic interventions with *stn1* Δ and *ten1* Δ were unsuccessful further fuelling the idea that Stn1 and Ten1 are more important than Cdc13 in telomere maintenance ¹¹⁶. Although it is highly unlikely that Stn1 and Ten1 independently bind telomeres because of their low binding affinity ¹¹⁷, it is possible that they are recruited by other telomere associated proteins including Pol12.

The putative CST complex, therefore, plays a key role in telomere homeostasis and is integral to the formation of the telomere cap in *S. cerevisiae*. The relative importance and roles of the individual subunits are still being elucidated, but recent structural characterization of the three subunits has led to some interesting structural comparisons with other ssDNA binding proteins: in particular Replication Protein A (RPA), a obligate heterotrimer consisting of Rpa1, Rpa2 and Rpa3.

1.7.1 An RPA-like CST complex

RPA is a ubiquitous eukaryotic single stranded DNA binding protein with key roles in DNA metabolism in the cell ¹¹⁸. RPA is proposed to prevent the generation of secondary structure and re-annealing of single stranded DNA during replication, to facilitate progress of the replication machinery. Furthermore RPA plays roles in homologous recombination following DNA damage and during meiosis where it is involved in the recruitment of Rad51 to facilitate strand invasion ^{119; 120}. RPA also plays a role in activating the checkpoint in response to DNA damage by binding to ssDNA

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and recruiting ATM/ATR ⁴⁴. Since both RPA and CST are essential heterotrimeric proteins which utilise OB folds to tightly bind ssDNA, this led to investigation of potential similarities between the two complexes.

Interestingly it was found that significant similarities existed between the sequence domain architecture of Stn1 and the corresponding subunit in RPA, Rpa2 (figure 7). They also found that both proteins utilised wing-turn-helix domains at the C-terminus which created a compact, globular structure to mediate multiple protein-protein interactions ¹⁰⁸ (figure 8). Furthermore it was found that substitution of the essential OB fold of Rpa2 with the corresponding OB fold of Stn1 was sufficient to allow growth in the absence of Rpa2 ¹¹⁷. However, intriguingly the inverse experiment failed perhaps suggesting that the Stn1 OB fold has telomere-specific functions that cannot be replaced by the corresponding domain in Rpa2.

Similar comparisons were made with Ten1, and biochemical characterization revealed that both Ten1 and its proposed RPA-homolog, Rpa3, bind ssDNA very weakly and have slight specificity for telomere DNA. Furthermore both consist of a single OB fold (figure 8), and both form Stn1-Ten1 and Rpa2-Rpa3 complexes. However, intriguingly no sequence similarity was found between Ten1 and Rpa3¹⁰⁸.



Figure 7. The domain architecture of the CST complex and RPA heterotrimer. A. RPA1 consists of 4 OB-folds. DBD-A and B are essential for ssDNA binding. DBD-F, at the N-terminus is required for binding to partially duplex DNA and checkpoint activation. DBD-C is involved in trimer formation and is necessary for the recognition of certain types of DNA damage. RPA2 consists of a single OB fold which mediates interactions with DNA and other proteins. It also possesses a wing-turn-helix domain. RPA3 consists of a single OB-fold and is required for the formation of the RPA heterotrimer ¹²¹. B. Cdc13 consists of 5 OB folds: OB1, 2 and 4 are involved in homodimerization. OB3 is involved in high affinity DNA binding and the recruitment domain mediates interactions with telomerase and Stn1 ¹⁰⁸. Stn1 and Ten1 each consist of a single OB fold which mediates protein-protein and DNA-protein interactions.



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Figure 8. Comparison of the structures of Stn1-Ten1 and Rpa2-Rpa3. Superposition of Stn1N-Ten1 crystal structure on top of Rpa2N-Rpa3 crystal structure, showing significant structural similarity. However Ten1 and Rpa3 do not align well and Ten1 is rotated 15° relative to Rpa3 ¹⁰⁸.

Although there are clear similarities between Stn1/Ten1 and Rpa2/Rpa3 few similarities exist between the large subunits Rpa1 and Cdc13. Various biochemical and structural differences throw doubt on the possibility that Cdc13 and Rpa1 are related. Firstly, although both Cdc13 and Rpa1 are OB-fold containing proteins the domain architecture and sequence of these domains in *C. Albicans are* quite distinct. It has even been proposed that Cdc13 and Rpa1 are an example of convergent evolution, and Stn1-Ten1 could have evolved independently of Cdc13¹²².

There are clear similarities in structure and biochemical characteristics between the CST and RPA subunits. Furthermore, RPA is extensively involved in DNA metabolism throughout the cell and importantly plays a key role in double strand break repair. Therefore unsurprisingly RPA plays key roles in DNA metabolism at the telomere end alongside CST.

1.8 RPA at telomeres

The overlap in structure and biochemical characteristics between RPA and CST, has led to interest in what RPA might be doing at telomeres. RPA is known to bind to single stranded DNA throughout the genome, including the telomere end and is proposed to bind to telomeres primarily in late S-phase. The over-representation of RPA at the telomere, as measured by ChIP, at this point in the cell cycle could simply be because the replication fork pauses during telomere replication in late S-phase. It could also potentially be over-represented because of cell cycle regulated C-strand degradation which generates single stranded DNA for RPA to bind to ¹²³.

Another reason for the presence of RPA at telomeres in late S-phase is its involvement in telomere extension by telomerase. It is thought that RPA promotes telomerase action at the telomere end through forming a transient complex with Ku, Est1 and potentially Cdc13¹²³.

Furthermore, RPA is also proposed to be involved in C-strand synthesis by DNA polymerase α , since it is proposed to stimulate pol α primase ¹¹⁸. Moreover, RPA has been suggested to interact with Pol12 and Mcm10 which stabilises the catalytic subunit of DNA polymerase α ¹²⁴. The involvement of RPA in lagging strand synthesis has also been reconstituted biochemically. *In vitro* work has shown that RPA reduces replication fork pausing and improves accuracy of lagging strand synthesis of mammalian telomere repeats ¹²⁵. Therefore RPA plays important roles in both telomerase activity and C-strand synthesis, emphasising its key role in telomere maintenance.

Interestingly, recent work ¹²⁶ investigating the large subunit of the RPA heterotrimer Rpa1, has revealed that the role of RPA could extend beyond telomerase extension and efficient replication of the telomere. Specifically it was found that certain temperature-sensitive *rpa1* mutants allowed growth in the presence of *cdc13-1* induced telomere damage similarly to *cdc13-1 exo1* Δ or *cdc13-1 rad9* Δ . However unlike the *cdc13-1* mutants the improved growth was not due to inactivation of aspects of the G2/M checkpoint machinery. In fact, it seems that these rpa1 mutants cells are prevented from dividing via a checkpoint-independent mechanism.

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Furthermore the same study suggested that RPA also protected against inappropriate recombination of the telomere end in the context of a dysfunctional telomere. This is since in the context of the *cdc13-1* mutation, mutants of Rpa1 led to the development of survivors who maintained their telomeres through recombination-based mechanisms. It is hypothesised that this could be due to the accumulation of telomere damage, due to continued division, in the absence of "proper" capping. Therefore it seems possible that similarly to CST, RPA is playing an important role in telomere end protection and maintenance via multiple pathways.

Telomeres evolved in tandem with sexual reproduction, and through the development of specialised DNA and protein structures have become effective at preventing the ends of linear DNA being recognised as DNA damage. The CST and RPA complexes play an integral role in maintaining this structure through their interactions with DNA and through recruitment of a diversity of other proteins. Developing our understanding of how these essential protein complexes maintain and protect the telomere, will help us better understand what happens when telomere capping is compromised in human disease.

1.9 Aims

The putative CST complex has a well-established role in maintaining chromosome integrity. However the relative importance and role of the three subunits is still being unravelled. Recent biochemical and structural comparisons between RPA and CST have underlined some interesting similarities between the two complexes. Furthermore the role of RPA in general DNA metabolism including replication and DNA repair are well-documented, and it has recently been established that RPA also plays a key role in telomere homeostasis, as well as its wider roles elsewhere in the genome.

This study used a combination of high-throughput genetic screening and a novel biochemical assay to further elucidate the role of individual members of the CST complex in telomere capping, and determined to what extent the roles of CST and RPA in the maintenance of genomic stability overlap. This was achieved in two complementary ways:

- Establishing a novel *in vitro* telomere end protection assay to determine if the RPA heterotrimer and members of the CST complex were capable of inhibiting C-strand resection by 5' to 3' exonucleases.
- Searching for epistatic interactions between temperature-sensitive alleles of STN1 and RPA3 and a library of deletions/RNA knockdowns of essential and non-essential genes, using high-throughput genetic screening.

2. Materials and Methods

2.1 Reagents

Reagent	Components
HPLC deoxyoligonucleotide buffer A	5% acetonitrile and 0.6% acetic acid
	adjusted to pH 6.5 using triethylamine
HPLC deoxyoligonucleotide buffer B	65% acetonitrile and 0.6% acetic acid
	adjusted to pH 6.5 using triethylamine
10x DNA loading buffer A	62.5% glycerol, 6.25% SDS, 1%
	bromophenol blue and 1% xylene cyanol
10x Tris-Boric Acid-EDTA (TBE) buffer	1M Tris, 1M Boric acid and 0.02M EDTA
His-column buffer A (HCA)	25mM Tris-HCl pH7.5, 250mM NaCl, 10%
	glycerol, 10mM imidazole, 1mM DTT
His-column buffer B (HCB)	25mM Tris-HCl pH7.5, 250mM NaCl, 10%
	glycerol, 1M imidazole, 1mM DTT
Hepes-inositol (HI) buffer	30 mM HEPES (from a 1 M stock at pH 7.8),
	0.25 mM EDTA, 0.25% (w/v) myo-inositol, 1
	mM dithiothreitol (DTT), and 0.01% (v/v)
	Nonidet-P40
TBS (Tris-buffered saline)	50mM Tris-HCl pH7.4, 150mM NaCl, 1mM
	EDTA
SDS-PAGE running buffer	25mM Tris-HCl pH7.4, 250mM glycine, and
	0.5% SDS
5 x SDS-PAGE loading buffer	0.25M Tris-HCl pH6.8, 15% SDS, 50%
	glycerol, 25% B-mercaptoethanol, 0.01%
	bromophenol blue
SDS-PAGE staining buffer	10% acetic acid, 10% isopropanol and
	0.25% bromophenol blue
SDS-PAGE de-staining buffer	10% acetic acid and 10% isopropanol
SDS-PAGE separating gel	7-15% Design A Gel 37.5:1
	acrylamide:bisacrylamide (National
	Diagnostics), 375mM Tris-HCl (pH8.8), 0.1%
	SDS, 0.05% ammonium persulphate (APS)
	and 0.05% N,N,N',N'-
	tetramethylethylenediamine (TEMED)
SDS-PAGE stacking gel	4% acrylamide:bisacrylamide (37.5:1)
	(National Diagnostics), 125mM Tris-HCl
	(pH6.8), 0.1% SDS, 0.05% ammonium
	persulphate (APS) and 0.05% N,N,N',N'-
	tetramethylethylenediamine (TEMED)
2x stop buffer	80% formamide, 10mM EDTA and 10mM
	NaOH
2x EMSA loading buffer	40% glycerol, Tris pH8, 3mM EDTA
1-step S. cerevisiae transformation	0.2M lithium acetate, 40% PEG, 100mM

buffer	DTT
12% native (non-denaturing) gels	12% acrylamide (National Diagnostics), 1x
	TBE, 10μL N,N,N',N'-
	tetramethylethylenediamine (TEMED),
	200μL 10% ammonium persulphate (APS)
	made up to 40mL with water
17% non-native (denaturing) gels	17% acrylamide (National Diagnostics), 8M
	Urea, 1x TBE, 10μL N,N,N',N'-
	tetramethylethylenediamine (TEMED),
	200μL 10% ammonium persulphate (APS)
	made up to 40mL with water
GST column buffer A	30mM HEPES pH7.4, 10% glycerol, 2mM
	EDTA, 0.02% Nonidet P-40, 2mM DTT,
	300mM NaCl
GST column buffer B	30mM HEPES pH7.4, 10% glycerol, 2mM
	EDTA, 0.02% Nonidet P-40, 2mM DTT,
	300mM NaCl and 20mM glutathione
Heparin column buffer A (HeCA)	25mM Tris-HCl pH7.5, 150mM NaCl, 10%
	glycerol and 1mM DTT
Heparin column buffer B (HeCB)	25mM Tris-HCl pH7.5, 1M NaCl, 10%
	glycerol and 1mM DTT

Fable 1. A list of the components	of the different bu	uffers used in this study.
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2.2 E. coli strains used

Strain	Genotype
	F^{-} ompT hsdS _B (R _B ⁻ m _B ⁻) gal dcm λ (DE3 [lacl
Rosetta (DE3) pLysS	lacUV5-T7 gene 1 ind1 sam7 nin5])
	pLysSRARE (Cam ^R)
	F ⁻ mcrA Δ(mrr-hsdRMS-mcrBC)
Top10 chemically competent <i>E. coli</i>	f80lacZ∆M15 ∆lacX74 recA1 araD139
	Δ(ara-leu) 7697
	galU galK rpsL (StrR) endA1 nupG λ-
	F ⁻ mcrA Δ(mrr-hsdRMS-mcrBC)
DH10 Bac Competent Cells	Φ80 <i>lac</i> ZΔM15 Δ <i>lac</i> X74 <i>rec</i> A1 <i>end</i> A1
	araD139 Δ(ara, leu)7697 galU galK λ ⁻ rpsL
	nupG/pMON14272/pMON7124

Table 2. The genotypes of the different strains of *E. coli* used during this study

2.3 Media used

Media	Components
Lysogeny broth (LB) liquid	10g tryptone, 5g yeast extract and 10g NaCl (in 1L)
Lysogeny broth (LB) solid	10g tryptone, 5g yeast extract, 10g NaCl and 15g agar (in 1L)

Table 3. Media used to grow E. coli.

2.4 Oligodeoxynucleotides used for this study

Oligodeoxynucleotides were produced by Eurofins MWG Operon and stored at 200uM at -20°C in water. All oligodeoxynucleotides displayed in the table below are shown in the 5' to 3' orientation.

Oligodeoxynucleotide	Sequence (5' to 3')	Details
M3501	GTTCTTTGGATCCTACTA	Primers used to amplify
	TTCAACGATCCATGCATC	Rad55-Sed1 intergenic
	TGCCAGATTC	yeast integrative
M3502	GTTCTTTGGATCCGAGAT	plasmid pRS405
	GACATCGAGAAGGGATG	
	GATCTTTAG	
M3519	GTTCTTTCATATGAGCCA	Primers used to amplify
	GTTAGTTCTAGACTTAAA	Ten1 ORF for cloning
	ATG	
M3520	GTTCTTTCATATGTTAGG	-
	AGTTAGAGATGTTCAGAA	
	ACTTCTC	
M3517	GTTCTTTGTCGACATGGA	Primers used to amplify
	TAAGTACGGACATATAGC	Stn1 ORF for cloning
	TCATCAAGAAG	
M3518	GTTCTTTGTCGACTTATGA	1
	ATATGCTGCTGCTGCGTA	

	TTCC	
M3515	GTTCTTTCATATGGATACC	Primers used to amplify
	TTAGAAGAGCCTGAGTG	the Cdc13 ORF for
M3516	GTTCTTTGCGGCCGCTTA	
	CGAGGTGGGAACGGCTC	
	CGGCGTCATCGTC	
M3521	GTTCTTTCTCGAGATGAG	Primers used to amplify
	CCAGTTAGTTCTAGAC	the Ten1 ORF for cloning
M3522	GTTCTTTCTCGAGCACCT	
	GTATACTATTATTTACATT	
	ACATAAC	
M3526	GTTCTTTGCGGCCGCATG	Primers used to amplify
	GATAAGTACGGACATATA	Stn1 ORF for cloning
	GCTCATC	into prio+25 G/L.
M3527	GTTCTTTGCGGCCGCAAT	
	ATATGCATAAAGTTATAGT	
	ATCTTATTTTG	
M3528	GTTCTTTGTCGACATGGA	Primers used to amplify
	TACCTTAGAAGAGCCTGA	Cdc13 ORF for cloning
	GTG	
M3529	GTTCTTTGTCGACAGCAT	
	CAAGAAGGAAGGAAGACC	
	TTTC	
M3504	TGACCCCACATAAATCTC	Primers used for
	CAACGTCAATGC	introduce a stop codon
M3505	GAGATTTATGTGGGGTCA	near the start of the
	ACTCTTGGTGTTTC	ORF (position 34 G \rightarrow T).
M3508	CATAAAACGAATATTCATC	Primers used for
	AGTAACGGGCGGTGGCTT	mutagenesis of Rpa2 to introduce a stop codon
	TG	near the start of the
M3509	TCGCCCCTCTAAAGTTGA	ORF (position 12 T \rightarrow A).
	ACACTGCCCATG	
M3506	CAACTTTAGAGGGGGCGAT	Primers used for
	TTTCATAGCATCTTC	mutagenesis of Rpa1 to

M3507	GCCCCTCTAAAGTTGAAC ACTGCCCATG	introduce a stop codon near the start of the
M2405	GTTCTTTCTCGAGTTGTTG	ORF (position 20 C \rightarrow A).
1013435	CGAAGGCCTCTTTCTTG	Rpa3-Cps1 intergenic
M2406	GTTCTTCTCCACCCCTC	region for insertion into
1013430	CAAAAAGCGTCGTTC	plasmid pRS405
M3524	GTTCTTTCATATGGATAA	Primers used to amplify
	GTACGGACATATAGCTC	Stn1 for cloning into
	ATC	pET28a
M3525	GTTCTTTCATATGTTATG	
	AATATGCTGCTGCGTATT	
	С	
M3513	GTTCTTTCATATGGATACC	Primers used to amplify
	TTAGAAGAGCCTGAGTG	the Cdc13 ORF for
M3514	GTTCTTTGCGGCCGCTCG	cioning into perzzo
	тсаттатсстсстсстттт	
	TCAC	
M3531	Hex:GTGGGTGTGGTGTG	Hexachlorofluorescein
	G	labelled 15bp
		consisting of telomere
		sequence
M3532	Hex:CTACTTCGCGCAACA	Hexachlorofluoroscein labelled 15bp
		deoxyoligonucleotide
		consisting of sequence
		Mata/Mat α mating type
		locus
M3534	Hex:GGTGTGTGGTGTGTGTGTGT	Hexachlorofluorescein labelled 50bp
	GIGGIGIGIGGGIGIGGGGI	deoxyoligonucleotide
	GIGIGGGGIGIGGIGG	consisting of telomere
M3535	Hex:TGGCACGCGGACAAA	Hexachlorofluoroscein
		labelled E0bp
	ATGCAGCACGGAATATGG	
	ATGCAGCACGGAATATGG GACTACTTCGCGCAACA	deoxyoligonucleotide consisting of sequence
	ATGCAGCACGGAATATGG GACTACTTCGCGCAACA	deoxyoligonucleotide consisting of sequence taken from the
	ATGCAGCACGGAATATGG GACTACTTCGCGCAACA	deoxyoligonucleotide consisting of sequence taken from the Mata/Matα mating type

		1
M3536	CACCCACACACCACACCA	Fluoroscein labelled
	CACACCACACCACACCTT	self-complementary 90bp telomere
	TTGGTGTGGTGTGGTGTG	oligonucleotide
	TGGTGTGGTGTGTGGGTG	labelled at the 3' end with fluorescein (X)
	TGTGGGTGTGGTGTGGXC	
M3537	CCCATATTCCGTGCTGCA	Fluoroscein labelled
	TTTTGTCCGCGTGCCATT	self-complementary 90bp telomere
	TTTGGCACGCGGACAAAA	oligonucleotide
	TGCAGCACGGAATATGGG	labelled at the 3' end with fluorescein (X)
	ACTACTTCGCGCAACAXC	
		1

Table 4. Oligodeoxynucleotides used in this study.

2.5 Oligodeoxynucleotide design, synthesis, and purification

2.5.1 Oligodeoxynucleotide design and synthesis

Oligodeoxynucleotides were designed using the Clone Manager Professional Suite version 8.0 (Scientific and Educational Software, 600 Pinner Weald Way Ste 202, Cary NC27513, USA) and synthesised by Eurofins MWG Operon with the exception of the looped oligonucleotides which were synthesised in house on an applied Biosystems 392 DNA/RNA synthesiser using columns and reagents from Proligo (6200 Lookout Rd Boulder, CO 80301, USA). Following synthesis, oligodeoxynucleotides were cleaved from their glass bead supports by incubation at 55°C in 35% ammonia for 4-15 hours. The ammonia was then removed by evaporation using a Savant Speed Vac SC100 and the glass bead supports separated from solution by filtration through a 22uM Millipore Millex syringe driven filter unit.

2.5.2 Oligodeoxynucleotide purification

Purification of oligodeoxynucleotides was carried out by reverse phase high pressure liquid chromatography (HPLC). Purification was carried out at 60 °C with an Apex C18 octadecylsilyl 0.5 micron column (Jones Chromatography, Llanbradach, Wales). Two buffers were used in the purification of the looped oligonucleotides: Buffer A (5% acetonitrile; 0.6% acetic acid, adjusted to pH 6.5 using triethylamine) and buffer B (65% acetonitrile; 0.6% acetic acid, adjusted to pH 6.5 using triethylamine). Preequilibration of the column was carried out at 1mL/min for 20 minutes, and samples were loaded at 1mL/min, and eluted using a 25mL linear gradient (0-20%) of buffer A to B. The 5' dimethoxytrityl (DMT) group was removed by incubation at room temperature with 80% Analar acetic acid. Acetic acid was then removed by evaporation on a rotary evaporator and DNA resuspended in 20mL of nanopure water. This wash was repeated twice, before finally resuspending the DNA in 1mL of water.

2.5.3 Kinase-treatment of looped oligodeoxynucleotides

As the oligodeoxynucleotides were synthesised, they did not possess a 5' phosphate group which is necessary for λ exo to initiate resection. Therefore 100pMol of looped oligonucleotides were treated with 30 units of polynucleotide kinase (Promega) in 1x reaction buffer for 1 hour at 37 °C. DNA was then separated from the reaction mixture using a PCR purification kit (QIAGEN).

2.5.4 Determining oligodeoxynucleotide concentration

Oligodeoxynucleotide concentrations were calculated using the Beer-Lambert Law:

$$C = A_{260}/\epsilon.1$$

Where:

C = mM concentration of oligodeoxynucleotide

 A_{260} = absorbance of light of 260nM wavelength

 ε = Extinction coefficient (mM⁻¹ cm⁻¹) (calculated by adding the individual extinction coefficients of each nucleotide and fluorescent label together) (see Table 5)

1cm = path length of quartz cuvette

Deoxynucleotide	Extinction coefficient (mM ⁻¹ cm ⁻¹)
dA	14.7
dC	6.1
dG	11.8
dT	8.7
dU	10.0
Hexachlorofluoroscein	31.6
Fluoroscein	38.8
Table 5. Extinction coefficients	of individual base components of

oligodeoxynucleotides at 260nM in a single stranded context.

Since the looped oligonucleotides were partially double stranded, this reduced their A260 value due to base stacking. This hypochromicity was accounted for using the following equations:

$$h = (0.059 \times fGC) + (0.287 \times fAT)$$

 $\varepsilon_{dsDNA} = \varepsilon_{complement} + \varepsilon_{reverse complement} \times (1 - h)$

$$\varepsilon_{loop} = \varepsilon_{dsDNA} + \varepsilon_{ssDNA}$$

Where:

h = hypochromicity

*f*GC = Fraction GC base pairs

*f*AT = Fraction AT base pairs

2.5.5 PCR reactions

20μL scale PCR reactions were performed using the following mix: 1 x reaction buffer [Velocity GC or Hifi (Bioline) Taq Mg-free buffer (New England Biolabs)], 1.5mM MgCl₂ (for Taq polymerase), 200uM dNTPs, 0.2uM forward primer, 0.2uM reverse primer, Genomic DNA (variable) or Plasmid DNA (~1ng), 2U polymerase (Taq or Velocity) and nanopure water.

Thermocycling was performed on a Veriti 96 well thermocycler (Applied Biosystems) using standard cycling conditions (Table 6).

Stage	Temperature	Time	Cycles
Denaturation	95 °C	2 mins	1
Denaturation	95 °C	30s	
Annealing	55-69 °C	30s	15-35
Extension	72 °C	30s/kb	
Final extension	72 °C	2x extension	1

Table 6. Standard PCR conditions used in this study.

2.6 General DNA manipulation

2.6.1 Agarose gel electrophoresis

Vectors and PCR products were analysed by agarose gel electrophoresis in 1x TBE buffer using 1x TBE/1% agarose gels supplemented with 1ug/mL ethidium bromide. Fermentas Generuler 1kb or 100bp DNA ladder were run as size markers in parallel lanes. Samples were supplemented with 6.25% glycerol, 0.625% SDS, 0.1% bromophenol blue and 0.1% xylene cyanol. Gels were run at 100V, 100mA, 10W for as long as was necessary to achieve suitable resolution.

2.6.2 Extraction of DNA bands

Where PCR products or restriction enzyme treated vectors were required for downstream applications, DNA bands were manually excised from the gel using a surgical blade following electrophoresis. The excised band was then weighed and DNA extracted using the QIAquick gel extraction kit (QIAGEN). To further purify the DNA, an extra step was often performed where elutes from the gel extraction were subjected to further purification using the QIAquick PCR purification kit (QIAGEN).

2.6.3 Quantification of purified DNA

To determine the concentration and purity of DNA samples, absorbance using a Nanodrop 100 spectrophotometer (Thermo Scientific). The A₂₆₀ reading was used to determine the concentration of DNA using the Beer Lambert law, however it was assumed that the extinction coefficient for nucleic acids was $50ng/\mu$ L (1cm pathlength) for double stranded DNA and $33ng/\mu$ L (1cm pathlength) for single stranded DNA. The A₂₆₀:A₂₈₀ ratio was used as an indication of purity, with values of 1.6 or above considered satisfactory.

2.7 Molecular cloning

2.71 Plasmids used in this study

Plasmids used in this study (which were not made in-house) are listed in the table below and were stored at -20°C in water.

Plasmid	Details	Source
pDL1633 (pETDuet-1)	Plasmid used for the co-expression of	Novagen
	two target genes under the control of the	
	T7 promoter (Ampicillin resistant).	
pCR4-TOPO	Plasmid used for subcloning (Ampicillin	Life
	and Kanamycin resistant)	technologies

pDL1634 (pET28a)	Plasmid used for expression of single N- terminal his-tagged gene under the control of the T7 promoter (Kanamycin resistant)	Novagen
pDL1635 (pET22b)	Plasmid used for the expression of a single C-terminal his-tagged gene in <i>E. coli</i> under the control of the T7 promoter (Ampicillin resistant)	Novagen
pDL1241 (pRS405)	Yeast integrative plasmid (Ampicllin resistant) (carries LEU2 biosynthesis gene)	D. Lydall
pDL1636 (pRS426-GALGST)	Bi-directional GAL1-10 promoter drives expression of two genes, one of which is GST tagged (Ampicillin resistant) (carries URA3 biosynthesis gene) in <i>S. cerevisiae</i>	P. Burgers
pDL1637 (pRS425-GAL)	Bi-directional GAL1-10 promoter drives expression of two untagged genes (Ampicillin resistant) (carries LEU2 biosynthesis gene) in <i>S. cerevisiae</i>	P. Burgers
pDL1630 (pRSET.A)	An expression vector incorporating an N- terminal hexahistidine tag (Ampicillin resistant)	H. Gao
pDL1624 (pET21a-DBD)	T7 promoter drives the expression of the sequence coding for the DNA binding domain of Cdc13 and incorporates a C-terminal his-tag. (Inserted between the <i>Xhol</i> and <i>Ndel</i> sites) (Ampicillin resistant)	D. Wuttke
pDL1625 (P11d-tRPA)	Synthetic operon containing sequence coding for the three subunits of <i>S</i> .	M. Wold

	<i>cerevisiae</i> RPA under the control of the T7 promoter (Ampicillin resistant)	
pDL1045 (pAG32)	Construct containing the hygromycin B gene. It does not contain direct repeats (Ampicillin resistant)	D. Lydall
pDL1306 (PCN284)	Ycp plasmid containing TEN1 under the control of its native promoter (Ampicillin resistant)	C. Nugent
pDL1259 (pVL1051)	Stn1 under the control of the GAL promoter (Ampicillin resistant).	D. Lydall
pDL1648 (pVL2848)	Stn1 and N-terminal FLAG tag under the control of the T7 promoter (Ampicillin resistant).	H. Gao

Table 7. A list of plasmids used in this study which were not made in-house.

2.7.2 Plasmids produced in this study

Plasmids produced in this study are listed in the table below and were stored at -20 $^{\circ}$ C in water.

Construct	Details	Source
pDL1619	Plasmid used for the co-expression of N- terminal his-tagged Stn1 and untagged	This study
	Ten1 in <i>E. coli</i> . Derived from the pETDuet	
	vector (Novagen).	
pDL1620	Plasmid used for the attempted expression of N-terminal his-tagged Stn1 in <i>E. coli</i> . Derived from the pET28a vector	This study
	(Novagen).	

pDL1600	Yeast integrative plasmid used to integrate LEU2 biosynthesis marker upstream of rpa3-313 mutant gene in <i>S.</i> <i>cerevisiae</i> by homologous recombination. Derived from the pRS405 vector.	This study
pDL1631	Bi-directional GAL1-10 promoter drives expression of Cdc13 ORF which possesses an N-terminal GST tag. Designed for attempted overexpression and purification of CST complex in <i>S.</i> <i>cerevisiae</i> (Ampicillin resistant) (carries URA3 biosynthesis gene). Derived from the pRS426-GALGST vector.	This study
pDL1632	Bi-directional GAL1-10 promoter drives expression of untagged Stn1 and Ten1 (Ampicillin resistant) (carries LEU2 biosynthesis gene) in <i>S. cerevisiae</i> . Derived from the pRS425-GAL vector.	This study
pDL1601	Yeast integrative plasmid used to integrate LEU2 biosynthesis marker upstream of stn1-13 mutant gene in <i>S.</i> <i>cerevisiae</i> by homologous recombination. Derived from pRS405.	This study
pDL1621	Construct used for the expression of C- terminal his-tagged Cdc13 in <i>E. coli</i> . Derived from pET22b (Novagen).	This study
pDL1622	Construct used for the expression of N- terminal his-tagged Cdc13 in <i>E. coli.</i> Derived from pET28a vector (Novagen).	This study

pDL1623	Construct used for the expression of N-	This study
	terminal his-tagged Ten1 in E. coli.	
	Derived from pET28a vector (Novagen).	
pDL1627	Plasmid containing the ORFs of the three	This study
	subunits of S. cerevisiae RPA under the	
	control of the T7 promoter, with the	
	Rpa1 ORF knocked out by a premature	
	stop codon. Derived from pDL1625.	
pDL1628	Synthetic operon containing sequence	This study
	coding for the three subunits of S.	
	cerevisiae RPA under the control of the	
	T7 promoter, with the Rpa2 ORF knocked	
	out by a premature stop codon. Derived	
	from pDL1625.	
pDL1629	Synthetic operon containing sequence	This study
	coding for the three subunits of S.	
	cerevisiae RPA under the control of the	
	T7 promoter, with the Rpa3 ORF knocked	
	out by a premature stop codon. Derived	
	from pDL1625.	

Table 8. A list of plasmids produced during this study.

2.7.3 Preparation of chemically competent E. coli by calcium chloride treatment

A single colony of Top10 chemically competent *E. coli* was inoculated in 5mL of LB nutrient broth supplemented with an appropriate antibiotic and grown overnight at 37°C. 1mL of this culture was then inoculated into 100mL of LB without antibiotics in a one litre conical flask, and grown at 37°C at 180rpm until an OD₆₀₀ of 0.6 was reached, and from this point forward all further steps were done on ice. Cells were split between 4 polypropylene tubes and each was spun in a Beckman Avanti with a JA14

rotor at 1000xG at 4°C. The supernatant was then poured off and the pellet resuspended in 5mL of ice cold 100mM MgCl₂ and then spun again for 10 mins at 4°C at 1000xG in the same rotor. The supernatant was poured off again and cells resuspended in 1mL of ice cold 100mM CaCl₂ and left on ice for 1-2 hours. At this point the contents of the four tubes were combined and 1mL of glycerol was added to give a stock of cells with 20% glycerol. After gentle swirling of the mixture, cells were aliquoted out into 60µL fractions using a pipette tip with the end cut off and immediately frozen at -80°C.

2.7.4 Transformation of chemically competent E. coli

100ng of DNA vector was added to 60μ L of competent cells and left on ice for 20 minutes. Cells were then heat shocked at 42°C for 30 seconds and replaced on ice for 5 minutes, after which 450µL of nutrient LB broth was added, the mixture was incubated at 37°C for 1 hour. During this period LB plates (with the appropriate antibiotic resistance) were left to reach room temperature in the 37°C warm room. Then 100µL of the mixture was pipetted onto an agar plate, and the lid was slid off next to a Bunsen burner, to allow the plate to dry for 10 minutes. Where transformations were less efficient the whole mixture was gently spun down at 3000rpm for 1 minute and pelleted cells resuspended in 100µL of LB before plating. Plates were then incubated overnight at 37°C and then parafilmed and refrigerated the next day.

Antibiotic	Solvent	Stock concentration (mg/mL)
Ampicillin	Water	100
Kanamycin	Water	50
Chloramphenicol	Ethanol	34
Tetracycline	Ethanol	50

2.7.5 Antibiotic stocks

Table 9. List of antibiotics used, storage conditions and solvent.

These stocks were then diluted 1/1000 in media to produce the working concentration.

2.7.6 Plasmid and construct design

DNA plasmids and constructs produced during this project were designed using Clone Manager Professional Suite 8.0 (Scientific and Educational Software, 600 Pinner Weald Way, Ste 202, Cary, NC 27513, USA) and ApE (A plasmid Editor) (freeware produced by M. Wayne Davis).

2.7.7 Restriction digest

Restriction digests were performed using either New England Biolabs or Fermentas enzymes according to the manufacturers guidelines.

2.7.8 Vector dephosphorylation

In order to prevent recircularisation of digested plasmids, restriction enzyme treated plasmids were dephosphorylated. Dephosphorylation reactions were carried out at 37°C for 1 hour by adding 1/10 volume of 10x Antarctic Phosphatase buffer (NEB) and 1µL of Antarctic Phosphatase (NEB) per 1-5ug of DNA. The Antarctic Phosphatase could then be inactivated at 65°C for 5 minutes and then DNA purified for subsequent ligation.

2.7.9 DNA purification

In order to remove extraneous salt, enzyme and buffer from restriction digested and Antarctic Phosphatase (NEB) treated plasmids, PCR products, or gel extractions, PCR purifications (QIAGEN) were performed according to the manufacturers protocol.

2.7.10 Ligation

Ligations were carried out using T4 DNA ligase (Promega) according to the manufacturer's guidelines. Three different ratios of plasmid:insert were used for ligation (1:1, 1:3, 3:1) as standard.

2.7.11 Site-directed mutagenesis

Site-directed mutagenesis was carried out using mutagenic primers which were designed using Clone Manager Professional Suite 8.0. PCR reactions were carried out according to the QuickChange protocol (Stratagene) using Velocity (HF) DNA polymerase. After isolation, mutagenized plasmids were sent for sequencing (GATC) to confirm that the desired site had been mutated.

2.8 Expression and purification of proteins in E. coli

2.8.1 Overexpression and purification of Cdc13/Stn1/Ten1 combinations

A single colony of Rosetta (DE3) pLysS transformed with the appropriate plasmids (Table 8) was inoculated into 50mL of LB and grown overnight at 37°C, after which 15mL of the saturated bacterial culture was added to 750mL of LB broth containing the relevant antibiotic. The mixture was then incubated at 37°C until an OD of 0.6 was reached. At this point the culture was induced with IPTG to a final concentration of 0.5mM and incubated for 4 hours at 37°C in a shaking incubator at 180rpm. Cultures were then split into 0.5L centrifuge pots and centrifuged in a JA10 rotor in the Beckmann Avanti at 4000rpm for 10 minutes at 4°C. The supernatant was then discarded and pellets resuspended in 15mL of HCA buffer containing 1mg/mL l of lysozyme and protease inhibitor tablets (Roche). These pellets were then frozen overnight were then thawed, treated with a spatula end of DNAse (Roche), sonicated (85%, 8x cycle) for 2 minutes, and then centrifuged at 18,000rpm in the Beckman Avanti JA20 for 40 minutes at 4°C. The supernatant containing soluble protein was then filtered through a 0.45uM filter, and mixed with 2mL of Ni-NTA resin (GE Healthcare) and incubated on ice for 1 hour on a rocker before being washed twice with 10 column volumes of HCA buffer followed by elution in 4mL of HCA buffer containing 200mM imidazole.

2.8.2 Overexpression and purification of RPA

Overexpression and purification of RPA was performed broadly in line with the procedure of Marc Wold. The synthetic operon was transformed into Rosetta pLysS

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and single colonies were used to inoculate 4 x 750mL cultures of LB in order to minimise the number of divisions cells went through while carrying the operon since it is highly toxic. Cells were grown overnight without shaking and then induced with 500mM IPTG for four hours, spun down at 4000rpm and resuspended in HI buffer containing 1mg/mL of lysozyme and protease inhibitor tablets (Roche). Cells were then frozen and the next day thawed, treated with DNAse and sonicated (85%, 8x cycle), before being spun at 18,000rpm in the Beckman Avanti JA20 for 40 minutes at 4°C. The soluble fraction was then applied to a gravity flow column containing a resin called Affi-gel blue equilibrated with HI buffer. Affi-gel blue is a resin which can make multiple interactions with biological proteins, and through targeted disruption of these interactions through modified buffer conditions desired proteins can be eluted. The column was then washed with HI buffer, HI buffer containing 800mM KCI, HI buffer containing 500mM NaSCN and then eluted in HI buffer containing 1.5M NaSCN. The peak of protein from this elute was then applied to a hydroxyapatite column equilibrated with HI buffer and washed with HI buffer and eluted with 80mM Potassium Phosphate. Eluted RPA was generally >90% pure by this point and was buffer exchanged into HI buffer, and stored in 50% glycerol at -20°C.

2.8.3 Overexpression and purification of the DNA binding domain of Cdc13

pDL1624 containing the Cdc13 DNA binding domain (Cdc13 DBD) was obtained from the lab of Deborah Wuttke. This consisted of amino acid residues 451-694 and was carried on the pET21a vector between the *Ndel* and *Xhol* restriction sites and was ampicillin resistant and expressed Cdc13 DBD with a C-terminal hexahistidine tag. We opted to express this protein in Rosetta (DE3) pLysS as this has consistently been the best expression strain in the past. Cells were induced for 4-5 hours at 22°C, harvested and then lysed in the same lysis buffer as was used for Ten1. Cells were then sonicated, centrifuged and supernatant collected and loaded onto a HiTrap chelating Nickel column (GE Healthcare), and eluted using a linear gradient of imidazole. Fractions were then visualised on a 17% SDS-PAGE gel and the protein was judged to be >95% pure and then stored in 50% glycerol and frozen at -20°C.

2.8.4 Overexpression and purification of Ten1

Ten1 was cloned into pET28a using the *Ndel* restriction site to create pDL1623 which was transformed into Rosetta (DE3) pLysS, grown up and induced overnight at 18°C. From this point forward cells were treated identically to those expressing the CST combinations in the previous section. The Ten1 produced after this purification step had high yields, so a final size exclusion chromatography step in HCA buffer was incorporated to improve the purity.

Size exclusion chromatography separated Ten1 from a major *E. coli* contaminant of approximately 60kDa, and produced protein that was >99% pure as judged by SDS-PAGE analysis. The yield was approximately 1.5mg from a 2.5L *E. coli* culture.

2.8.5 Overexpression and purification of Stn1 using a FLAG-tag

A construct (pDL1648) consisting of an N-terminally FLAG-tagged Stn1 carried on a pRSET-A vector was obtained from the lab of Victoria Lundblad. We again opted to express this protein in Rosetta (DE3) pLysS, and deviated from method suggested¹¹⁷ to facilitate easier purification. A single colony of Rosetta (DE3) pLysS transformed with pDL1648 was inoculated into 2 conical flasks containing 50mL of LB. After overnight incubation these cultures were saturated, and 15mL of saturated culture was used to inoculate 6x 750mL of LB contained in 2L conical flasks. These cultures were then grown at 37°C in a shaking incubator at 180rpm until an OD of 0.6 was reached, at which point cultures were induced with a final concentration of IPTG of 0.5mM for approximately 4 hours at 25°C. Cells were then frozen and the next day thawed, treated with DNAse and sonicated (85%, 8x cycle), before being spun at 18,000rpm in the Beckman Avanti JA20 for 40 minutes at 4°C. The supernatant was then collected and combined with 1mL of anti-FLAG M2 resin (Sigma-Aldrich) pre-equilibrated with TBS. After a 30 minute incubation period on ice, the mixture was then loaded onto a gravity flow column, washed twice with 10 column volumes of TBS and protein eluted in with five column volumes of TBS supplemented with 100ug/mL FLAG peptide (Sigma-Aldrich). Fractions were then visualised on a 17% SDS-PAGE gel and the protein was judged to be >90% pure, and then stored in 50% glycerol and frozen at -20°C.

2.8.6 Expression and purification of proteins in Spodoptera frugiperda Sf21 (Sf9) cells

The bacmid used for expression of Exo1 from Sf9 cells which are derived from ovarian tissue from *Spodoptera frugiperda* was created by transformation of pFASTbac-Exo1 into DH10bac competent cells (Invitrogen). Transformed cells were then incubated on LB plates (containing 50ug/mL kanamycin, 7ug/mL gentamycin, 10ug/mL tetracycline, 100ug/mL Bluo-gal, and 40ug/mL IPTG) for 48 hours to allow for effective blue-white colony determination. A white colony was then picked and restreaked on the same selective media to confirm the white phenotype, and then bacmid DNA extracted using the QIAGEN large construct kit.

Then Sf9 cells were transfected with the bacmid. To do this 9 x 10^5 Sf9 cells were seeded into 2mL of growth media containing 50units/mL penicillin and 50ug/mL streptomycin. These cells were allowed to attach to the bottoms of a 6 well tissue culture plate over 1 hour at 27°C. During this time 1ug of bacmid DNA was added to 100µL of Grace's medium and 6µL of Cellfectin Reagent was added to another 100µL of Grace's medium. These two mixtures were then mixed and incubated at room temperature over 20 minutes.

Sf9 cells were then washed once with 2mL of unsupplemented Grace's medium. 800µL of Grace's medium was then added to the cellfectin:bacmid mixture and added to the Sf9 cells which were then incubated at 27°C for 5 hours. The cellfectin:bacmid mixture was then removed and discarded and 2mL of complete growth media was then added to the cells. Cells were then incubated at 27°C for 72 hours. Cells were then transferred to 15mL tubes and centrifuged at 500xg for 5 minutes, and clarified supernatant collected and stored at +4°C in foil (to protect from the light) to make the P1 stock.

As this was a preliminary expression experiment we did not determine the viral titre and assumed that it was somewhere in the region of 1×10^6 to 1×10^7 pfu/mL. We then infected cells at a multiplicity of infection (MOI) of 0.1 by adding 400µL of our P1 stock to 10mL culture of Sf9 cells at a concentration of 2×10^6 cells/mL. Cells were then incubated for 48 hours at 27°C, and then centrifuged at 500xg in 15mL centrifuge tubes before removing the supernatant which forms the P2 stock. 4mL of this P2 stock is then used to infect a 100mL culture of Sf9 cells at a concentration of 2×10^6 cells/mL.

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This P3 stock is then used to infect at an MOI of 3 another 100mL culture of Sf9 cells at 2 x 10⁶ cells/mL. After a 72 hour incubation period at 27°C the cells are pelleted by centrifugation and supernatant removed. Cells were then lysed in TBS buffer, centrifuged and supernatant collected and combined with 100µL of anti-FLAG M2 resin (Sigma-Aldrich) pre-equilibrated with TBS. After a 30 minute incubation period on ice, the mixture was then loaded onto a gravity flow column, washed twice with 10 column volumes of TBS and protein eluted in with five column volumes of TBS supplemented with 100µL FLAG peptide (Sigma-Aldrich). Fractions were then visualised on a 17% SDS-PAGE gel.

2.9 Working with proteins

2.9.1 Sodium dodecyl sulphate polyacrylamide gel electrophoresis (SDS-PAGE)

Proteins were analysed on SDS-PAGE gels consisted of a separating gel and stacking gel. The separating gel contained 7-15% Design A Gel 37.5:1 acrylamide:bisacrylamide (National Diagnostics), 375mM Tris-HCl (pH8.8), 0.1% SDS, 0.05% ammonium persulphate (APS) and 0.05% N,N,N',N'-tetramethylethylenediamine (TEMED). The stacking gel contained 4% acrylamide:bisacrylamide (37.5:1), 125mM Tris-HCl (pH6.8), 0.1% SDS, 0.05% APS and 0.05% TEMED. The running buffer consisted of 25mM Tris, 250mM glycine, and 0.5% SDS. Gels were run at 30A, until the gels were fully resolved. Gels were stained in SDS-PAGE staining buffer on a platform shaker at room temperature for 30 minutes, and destained in the same solution without bromophenol blue, at room temperature overnight.

2.9.2 Protein concentration determination by Bradford Assay

The concentration of all proteins in this project was determined by performing Thermoscientific Coomasie Plus (Bradford) Protein Assays as described by the manufacturer.

2.9.3 Proteins acquired for this study

Proteins acquired for this study are listed below and frozen at -80°C.

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Protein	Details			So	urce	
Full length Cdc13	Full (untagge	length ed)	Cdc13	E. fro	Skordalakes om <i>E. coli</i>)	(prepared

Table 10. Proteins acquired for this study.

2.10 Gel shifts and exonuclease protection assays

2.10.1 Telomere protection assay

Protection assays were performed at 37°C for 5-50 minutes using 10nM of telomere or non-telomere control looped substrates. Looped oligodeoxyribonucleotides were incubated with an excess of lambda exonuclease (50U) (Fermentas) and reactions were carried out in the presence or absence of a telomere binding protein, according to the manufacturer's instructions for the times specified in the figures. Reactions were stopped with 2x stop buffer, and then loaded onto a 17 % denaturing polyacrylamide gel.

2.10.2 Electrophoretic Mobility Shift Assay (EMSA)

EMSAs were performed at room temperature for 5 minutes using 10nM of the oligodeoxyribonucleotides indicated in the figures. Oligodeoxyribonucleotides were incubated with the protein in 1x lambda exonuclease reaction buffer (Fermentas) containing 67mM Glycine-KOH pH9.4, 2.5mM MgCl₂, 0.1% triton to simulate the reaction conditions used in our telomere protection assay. Reactions were then loaded in 2x loading buffer onto a 12% native polyacrylamide gel and run for 2-6 hours at 3W per gel.

2.10.3 Cdc13-RPA competition assays

EMSAs were performed as above but with 50nM of RPA and 10nM of telomere or nontelomere control substrates. A serial dilution of Cdc13 or the DBD of Cdc13 was performed and added to produce the final concentrations indicated. Reactions were then loaded in 2x loading buffer onto a 12% native polyacrylamide gel and run for 5 hours at 3W per gel.

2.11 Recipes for yeast media

All reagents were produced by Sigma or Formedium unless indicated otherwise

2.11.1 Yeast extract, peptone, dextrose (YEPD)

1% yeast extract, 2X bactopeptone, 2% dextrose, 50mg/L adenine

To 1L: Add 10g of yeast extract and 20g of bactopeptone (+ 20g of bacto agar for solid media)to 935mL of millipore water and autoclave for 12 mins at 121°C and once cooled to ~60°C 50mL of filter-sterilised 40% dextrose and 15mL of filter-sterilised 0.5% adenine was added.

2.11.2 Yeast antibiotic stocks

Yeast selection agents	Solvent	Stock concentration (mg/mL)
G418	Water	200
CloNAT	Water	100
Hygromycin	Water	300
Thialysine	Water	50
Canavanine	Water	50

Table 11. A list of antibiotics used for yeast selection, their stock concentrations and solvents.

These stocks were then diluted 1/1000 in media to produce the working concentration.

2.11.3 Synthetic media

0.13% amino acids, 0.17% yeast nitrogen base, 0.5% ammonium sulphate or 0.1% monosodium glutamate (MSG)

To 1L: Add 1.3g of amino acid drop out powder (2.5g adenine, 1.2g argenine, 6g aspartic acid, 6g glutamic acid, 1.2g histidine, 3.6g leucine, 1.8g lysine, 1.2g methionine, 3g phenylalanine, 22.5g serine, 12g threonine, 2.4g tryptophan, 1.8g tyrosine, 9g valine, 1.2g uracil), 5g ammonium sulphate or MSG and 1.7g of yeast nitrogen base up to 500mL with Millipore water, autoclaved and then 500mL of autoclaved Millipore water was added. For solid media, add 20g agar prior to autoclaving. If G418 or CloNAT were used MSG was used as a nitrogen source as ammonium sulphate is proposed to interfere with their function ¹²⁷.

2.11.4 -Histidine/-Tryptophan/-Leucine/-Uracil

This was the same recipes as above but the appropriate amino acids/nucleotides were left out from the drop-out powder.

2.11.5 YPGLA media

This was the same recipe as for YEPD but with 0.2% dextrose, 2% lactic acid and 3% glycerol and adjusted to pH6 with concentrated NaOH.

2.11.6 YPGL media

The same recipe as YPGLA but without adenine.

2.11.7 SCGL media

This was synthetic media but with 0.2% glucose, 2% lactic acid and 3% glycerol and adjusted to pH6 with concentrated NaOH.

2.12 Yeast strains

2.12.1 Strains used in this study

All strains used in this study were either in the W303 background (*ade2-1 can1-100 trp1-1 leu2-3,112 his3-11,15 ura3 GAL+ psi+ ssd1-d2 RAD5+*) in the case of the low throughput *stn1-13* experiments and in the S288C (*SUC2+ gal2 mal2 mel flo1 flo8-1 hap1 ho bio1 bio6*) background in the case of the high throughput *stn1-13* and *rpa3-*

313 screens. Due to the fact that the W303 strains contained the *ade2-1* mutation YEPD was supplemented with adenine (50mg/L).

2.12.2 Mutant libraries

All strains found in the mutant libraries were in the S288C background. Deletion mutants were a kind gift from Charlie Boone and open reading frames (ORFs) are disrupted with the kanamycin antibiotic resistance cassette (KanMX). DAmP mutants were bought from GE Healthcare and contain the KanMX marker in the 3' UTR of essential genes. This is proposed to reduce transcript levels by 4-10 fold. Both sets of mutants are available commercially from GE Healthcare.

2.12.3 Conditional mutants used in this project

2.12.3.1 stn1-13

stn-13 is a temperature-sensitive allele of the essential telomere capping protein STN1. It is functional and shows comparable growth to wild type strains at 30°C, but leads to a capping defect at 33°C with growth decreasing with increasing temperature. This represents the semi-permissive temperature. Following shift of the temperature to 37°C cells show very poor growth and this represents the non-permissive temperature. *stn1-13* contains the following amino acid changes (T101>S, D134>V, T203>A, I209>V, S393>R, M416>T) ¹⁰⁶. Similarly to *cdc13-1, stn1-13* leads to disruption of telomere maintenance and activation of the G2/M checkpoint ⁷⁹.

2.12.3.2 rpa3-313

rpa3-313 is a temperature-sensitive allele of the essential ssDNA binding protein RPA3. It is functional and shows comparable growth to wild type strains at 30°C, but causes cell cycle arrest at 36°C. It is thought that at the non-permissive temperature *rpa3-313* leads to primarily G1 arrest. However following the shift it is thought that there is an initial increase in the number of cells arrested in G2/M phase, followed later by an
increase in G1 arrest. These cells arrest with no buds or small buds and RPA1/RPA2 is proposed to be intact but inactive ¹²⁸.

Strain	Genotype	Source
(Lydall lab strain		
collection)		
DLY8766	Matα lyp1Δ::NatMx can1Δ::STE2pr-	This study
	sp_his5 leu2::stn1-13::HPH	
DLY8767	Matα lyp1Δ::NatMx can1Δ::STE2pr-	This study
	sp_his5 leu2::stn1-13::HPH	
DLY8993	Mat a stn1-13	D. Shore
DLY8994	Mat a stn1-13	D. Shore
DLY9243	Mat a stn1-13 exo1∆::LEU2 RAD5+	This study
DLY9244	Mat a stn1-13 rad50∆::URA3 rad5-	This study
	535	
DLY9245	Matα stn1-13 exo1Δ::LEU2	This study
	rad50Δ::URA3 rad5-535	
DLY9246	Mat a stn1-13 rad9Δ::HIS3 RAD5+	This study
DLY9247	Mat a stn1-13 dot1∆::KanMX rad5-	This study
	535	
DLY9248	Mat a stn1-13 dot1∆::KanMX	This study

2.12.3 Yeast strains used in this study

	rad9∆::HIS3 rad5-535	
DLY9249	Matα stn1-13 xrs2Δ::LEU2 rad5-535	This study
DLY9253	Matα stn1-13	This study
	mre11∆::hisG::URA3::hisG rad5-535	
DLY9257	Matα stn1-13 rad50Δ::URA3 rad5-	This study
	535	
DLY9259	Mat a stn1-13 exo1∆::LEU2	This study
	rad50∆::URA3 rad5-535	
DLY9261	Matα stn1-13 dot1Δ::KanMX RAD5+	This study
DLY9262	Matα stn1-13 xrs2Δ::LEU2 RAD5+	This study
DLY9264	Mat a stn1-13 xrs2∆::LEU2	This study
	htz1∆::NatMX RAD5+	
DLY9265	Mat a stn1-13	This study
	mre11∆::hisG::URA3::hisG rad5-535	
DLY9181	Matα lyp1Δ::NatMX can1Δ::STE2pr-	This study
	sp_his5 leu2::rpa3-313::HPH	
DLY640	Mat a RAD5+	R. Rothstein
DLY1108	Mat a cdc13-1-int	D. Lydall
DLY26	Mat a ade1 arg4 aro2 his7 lys5 met4	D. Lydall
	ura2	
DLY2440	Matα ade1 arg4 aro2 his7 lys5 met4	D. Lydall
	ura2	
DLY8460	Mat a RAD5+	D. Lydall
DLY3001	Matα RAD5+	D. Lydall

DLY5442	Matα rad9Δ::HIS3	D. Lydall
DLY5441	Matα rad9Δ::HIS3	D. Lydall
DLY1273	Matα exo1Δ::LEU2 RAD5+	D. Lydall
DLY4716	Mat a htz1∆::NatMX RAD5+	D. Lydall
DLY2956	Matα htz1Δ::NatMX RAD5+	D. Lydall
DLY2551	Matα rad50Δ::hisG::URA3 RAD5+	D. Lydall
DLY1093	Mat a xrs2Δ::LEU2 RAD5+	D. Lydall
DLY1094	Matα xrs2Δ::LEU2 RAD5+	D. Lydall
DLY1331	Matα mre11Δ::hisG::URA3::hisG RAD5+	D. Lydall
DLY1412	Mat a yku70Δ::HIS3 RAD5+	D. Lydall
DLY1366	Matα yku70Δ::HIS3 RAD5+	D. Lydall
DLY1729	Matα tel1Δ::TRP1 RAD5+	D. Lydall
DLY2793	Mat a dot1∆::KanMX RAD5+	D. Lydall
DLY1726	Mat α yku70Δ::HIS3 tel1Δ::TRP1 RAD5+	D. Lydall
DLY1724	Mat α yku70Δ::HIS3 tel1Δ::TRP1 RAD5+	D. Lydall
DLY2940	Matα dot1Δ::KanMX rad9Δ::HIS3 RAD5+	D. Lydall
DLY2941	Mata dot1∆::KanMX rad9∆::HIS3 RAD5+	D. Lydall
DLY9181	Matα lyp1Δ::NatMx can1Δ::STE2pr- sp_his5 leu2::stn1-13::HPH	This study

DLY757	Mata prb1 prc1 pep4-3 leu2 trp1	D. Lydall
	ura3	

Table 12. A list of yeast strains used in this study.

2.13 Expression and purification of proteins in S. cerevisiae

2.13.1 Attempted purification of CST in S. cerevisiae

A single colony was picked and streaked out onto selective SCGL solid media and the incubated for 2 days at 30°C. The cells from this plate were then used to inoculate 150mL of selective SCGL liquid and grown overnight at 30°C in an orbital shaker. This culture was then used to seed 6 1L flasks of selective SCGL. After a day and overnight growth of the 1L culture, 1L of YPGL was added and the cells allowed to grow at 30°C for 3 hours. Cells were then induced by addition of 40g of solid galactose (2% final concentration). Following induction for 12 hours at 30°C the cells were collected by centrifugation in a Beckman Avanti JA10 at 4000G for 20 minutes. The resultant cell pellets were then resuspended in 5mL of protein extraction buffer (30mM HEPES pH7.4, 10% glycerol, 2mM EDTA, 0.02% Nonidet P-40, 2mM DTT, 300mM NaCl) supplemented with protease inhibitor tablets (Roche).

The resuspended cell pellets were then frozen at -20°C overnight, thawed and then broken open using the French Press (Constant Cell Disruption Systems) set at 40KPsi. The resulting suspension was then treated with DNAse at 37°C for 15 minutes and then clarified by centrifugation at 18,000rpm in the Beckman Avanti JA20 at 18,000rpm for 40 minutes at 4°C. The supernatant containing soluble protein was then filtered through a 0.45µM filter, and purified immediately.

2.14 S. cerevisiae genetic techniques

2.14.1 Mating, sporulation and tetrad analysis

MATa and *MATα* parental strains were streaked on top of each other on a YEPD plate using a toothpick, and left at 23°C overnight to mate. A swab of this mixture was then taken using a toothpick and streaked onto appropriate selective media, to select for growth of diploids. This was done in one of two ways: the mixture could be streaked out on double selection media and grown for 2 days at 23°C-30°C, or streaked out on one selective media, grown for 2 days at 23°C-30°C and 6 individual colonies picked and patched onto a second selective media and grown at 23°C-30°C for 2 more days.

Diploid colonies were then picked and grown up in 2mL liquid YEPD overnight at 23°C-30°C or until saturated. 1.5mL of this mixture was then discarded and the remaining 0.5mL was centrifuged in a Jouan CR4i centrifuge (Thermo Scientific) for 3 minutes at 1500rpm. The supernatant was discarded and the pellet washed twice with 5mL of sterile water before being resuspended in 2mL of 1% potassium acetate (KOAc) to allow sporulation. Cultures were then incubated at 23°C for 2-5 days and sporulation was assessed by examining the sporulation culture using phase contrast microscopy.

500µl of this sporulation culture was spun at 6000rpm in a 1.5mL microfuge tube and supernatant discarded. This pellet was then resuspended in 1mg/mL zymolyase and incubated at room temperature for 5 minutes. Spores were then examined under the microscope and once the ascus wall was partially digested, 50µL of digested spores were then spread onto a YEPD plate along a marked line. Tetrads were dissected using a tetrad microscope (Microtech) and dissected spores were allowed to germinate and form colonies at 23°C for 2-5 days.

Sporulation plates were then photographed and individual colonies replica plated onto a fresh YEPD plate. These were left to grow for 2 days at 23°C, and then replica plated onto appropriate selective media to determine genotype. Mating type was determined by mating with DLY26 (*Mata ade1 arg4 aro2 his7 lys5 met4 ura2*) and DLY2440 (*Mat alpha ade1 arg4 aro2 his7 lys5 met4 ura2*) and growing overnight at 23°C before replica plating onto -4 plates (-leu, -trp, -his, -ura) and determining mating type by complementation. Scoring for growth took place after 2-3 days of growth at 23°C.

rpa3-313 mutation was scored by replica plating onto YEPD and incubating at 36°C where any mutants would not grow. *stn1-13* mutation was scored by replica plating onto YEPD and incubating at 36°C where any mutants would grow poorly.

2.14.2 Random spore analysis

Following sporulation, cultures were centrifuged in a Jouan CR4i centrifuge (Thermo Scientific) for 3 minutes at 1500rpm. The supernatant was discarded and three washes with 5mL distilled water were performed before resuspending in 0.5mL of Zymolyase-20T solution (1mg/mL) and 10µL of B-mercaptoethanol to preferentially lyse diploid cells. This mixture was then incubated on a wheel at 30°C overnight.

Following overnight incubation, 5mL of the detergent NP-40 (1.5%) was added and then cultures were vortexed. The mixture was then spin at 3000rpm for 5 minutes and all but 1mL of the supernatant was discarded. This was then transferred to a 1.5mL microfuge tube and incubated on ice for 15 minutes, to lower the temperature in preparation for sonication.

Following incubation on ice, tetrads were sonicated for 30s at 10 microns full power and incubated on ice again. The spores were then centrifuged at 13,000rpm for 30s in a bench top microcentrifuge and the supernatant discarded. Spores were then resuspended in 1.5% NP-40 and sonicated again. Following sonication, the mixture could be checked under a phase contrast microscope, to check that the spores were fully separated. If not this step was repeated. Once successfully separated the spores were then centrifuged once more and supernatant discarded, and spores were resuspended in 1mL of water, and diluted to get 1000 spores/mL. 100µL of these spores were then plated onto 100µL of YEPD and incubated at 23°C for 2-3 days.

2.14.3 High efficiency lithium acetate transformation

2mL of liquid YEPD was inoculated with a single yeast colony and incubated on a roller overnight at 23°C or 30°C. This was then inoculated 1:50 in YEPD and incubated at the same temperature in a shaking water bath at 200rpm until it reached a cell density of approximately 2 x 10⁷ cells/mL, as determined using a haemocytometer. The culture was then harvested in a sterile 50mL polypropylene tube at 1500rpm for 3 minutes,

and then washed in 25mL of sterile water and pelleted again. The cells were then resuspended in 1mL of 1x LiAc in a microfuge tube and pelleted at 13,000rpm for 15 secs. LiAc was then aspirated and cells resuspended in 400µL of 1x LiAc, and vortexed and 50µL aliquots prepared. Cells were then pelleted, and aspirated and the transformation mix was added (240µL 50% PEG, 36µL 10x LiAc, 10µL salmon sperm DNA, 40µL of transformation DNA and 50µL water), and then vortexed. This mix was then incubated at 23°C for 30 minutes, and then heat shocked at 42°C for 20 minutes. Cells were then pelleted at 6000rpm for 15 seconds and transformation mix was removed by aspiration. Cells were then resuspended in 200µL of sterile water and plated out onto selective media.

2.14.4 One-step transformation

A single colony from a YEPD plate was resuspended in 100μ L of one-step buffer (0.2M lithium acetate, 40% PEG, 100mM DTT). 1μ L of DNA for transformation was then added along with 5.3 μ L of 10mg/mL salmon sperm DNA, and vortexed vigourously. The mixture was then incubated at 45°C for 30mins and then plated onto selective media. Transformants normally appeared within 3-5 days when grown at 23°C.

2.14.5 Spot testing

Relevant genotypes were selected and grown in liquid YEPD overnight at 23°C until they were saturated. They were then serially diluted five times on a 96 well plate. A 48 or 96 prong replica plater was then used to transfer spots of the cultures to several YEPD or other nutrient media plates grown at a variety of temperatures.

2.15 Robotics and in silico methods

2.15.1 Yeast culture conditions

The single gene deletion collection was stored at -80°C in 15% glycerol in 384 well plates ¹²⁷. Yeast strains were then transferred to complete synthetic media supplemented with G418 and grown at 30°C for 2 days.

2.15.2 Plate filling

35mL of molten agar was used to fill each SBS footprint trays (omnitrays;Nunc, Thermo Fisher Scientific) using a Perimatic GP peristaltic pump (Jencons (Scientific) Limited, Leighton Buzzard, UK). 96 well plates were filled with 200ul of liquid media or distilled water using a Wellmate plate filler with stacker (Matrix Technologies, Thermo Fisher Scientific).

2.15.3 Robotics

Solid agar-agar pinning was performed using a Biomatrix BM3-SC robot (SandP Robotics Inc., Toronto, Canada)using 1mm diameter 383 pin or 0.8mm diameter 1536 pin tools. Inoculating liquid media from solid media was carried out using the same robot using a 1mm diameter 96-pin pin tool. Spot tests were carried out using a Biomek FX robot (Beckman Coulter (UK) limited, High Wycombe, UK) using a 2mm diameter 96 pin pin tool (VandP Scientific, Inc., San Diego, CA, USA).

2.15.4 Synthetic Genetic Array (SGA) procedure

The query strains (*stn1-13* and *rpa3-313*) strains were inoculated into 3 5mL tubes of liquid YEPD and grown at 23°C overnight. Each of these cultures was then spread over 5 rectangular YEPD plates and left to grow for two days at 23°C. Three of the most even lawns were then pinned in 1536 format onto YEPD + cloNAT plates and left to grow for 2 days at 23°C. The deletion library was then transferred from 384-1536 format on fresh YEPD + G418 plates and grown for 2 days at 30°C. These deletion mutants are then pinned on top of query strains and incubated at 23°C for 12-24 hours.

The resulting mix was then pinned onto YEPD + G418/clonNAT plates and incubated at 30°C for 1-2 days. The diploids were then pinned onto enriched sporulation media containing G418, and the plates were incubated at 23°C for 5 days. Individual colonies were picked from the plate and resuspended in 10µL of water and visualised using phase contrast microscopy, to determine if sporulation had taken place.

Sporulations were then pinned onto synthetic dropout media (+MSG –His/Arg/Lys + canavanine + thiolysine for haploid selection 1 and grown for 2 days at 20°C. Then resulting colonies were then repined onto new plates of the same media for a second round of haploid selection, to select for haploids. The third stage of haploid selection involved using the same media again, but this time supplemented with G418 to select for haploids containing the gene deletion of interest. Again these plates were incubated at 20°C for 2 days. The fourth stage of haploid selection involves selecting for the query mutation combined with the gene deletion of interest on synthetic dropout media (+MSG –His/Arg/Lys/Leu + canavanine + thiolysine +G418 + hygromycin) and then these are an incubated at 20°C for 2 days. The final stage of selection involves pinning these strains onto the same plates, but this time incorporating clonNAT as well, followed by incubation at 20°C for 2 days. Synthetic interactions can at this point be determined.

2.15.5 Growth assays

To perform liquid-solid agar robotic spot tests in 384 format, colonies were inoculated into 96 well plates filled with 200µL of appropriately supplemented SD/MSG. Following growth to saturation at 20°C cells were diluted 1/100 in 200µL of water and spotted onto appropriately supplemented media and grown at a variety of different temperatures (including the semi-permissive temperature).

2.15.6 Photography

Agar plates were photographed using a spImager (SandP Robotics Inc., Toronto, Canada) with an integrated camera (Canon EOS 40D) in manual mode with a pre-set manual focus. Settings: exposure- 0.25s; aperture- F10; white balance- 3700K; ISO100; image size- large; image quality- fine; image type- .jpg.

2.15.7 Image analysis

Images were analysed using the software Colonyzer which quantified cell density from photographs. This software corrects for lighting gradients and removes spatial bias from density estimates. It is specially designed to detect low cell densities and so it can capture a wide range of culture densities from spot tests.

2.15.8 Calculating fitness

Fitness was then calculated by plotting these cell density measurements over time and fitting them to the logistic population model. A quantitative measure of fitness was then created using the numerical area under the curve (nAUC).

3. Protein preparation and *in vitro* reconstitution of the CST and RPA complexes

3.1 Background

The presence of the Cdc13-Stn1-Ten1 (CST) complex was first proposed in 2001, when it was found that growing the temperature sensitive alleles cdc13-1, stn1-13, and ten1-31 at the restrictive temperature resulted in accumulation of single stranded DNA at telomere ends, leading to activation of the G2/M checkpoint ^{79; 100; 106}. Additional coimmunoprecipitation and yeast-2-hybrid data led to the suggestion that Cdc13, Stn1 and Ten1 worked together as a complex to "cap" the telomere end ¹⁰⁶. Purification of the S. cerevisiae CST complex has yet to be achieved; however, C. glabrata CST was purified in 2013 and was found to have a Cdc13:Stn1:Ten1 stoichiometry of either 2:4:2 or 2:6:2¹⁰⁹. Cdc13 is the largest component of the CST complex with a molecular weight of 105kDa. When purified in isolation it is proposed to exist in a dimeric form ¹²⁹, and Cdc13 binds to telomere single stranded G rich DNA with high affinity and specificity ^{87; 99}. Cdc13 is proposed to be involved in the recruitment of telomerase ⁷⁶, replication of the telomere ^{97; 98} and protecting the telomere C strand from 5' to 3' exonuclease activities ^{90; 100}. Stn1 and Ten1 are two smaller subunits of the putative complex and have molecular weights of 57kDa and 18kDa respectively. Both are proposed to play important roles in telomere end protection, and Stn1 is proposed to be a negative regulator of telomerase ⁸⁰.

Replication protein A (RPA) was first discovered as an essential component required for the replication of simian virus 40 in human cells ¹¹⁸. It was later found to be an essential protein involved extensively in DNA metabolism, binding to single stranded DNA with high affinity and preventing the formation of secondary structure. It plays important roles in homologous recombination by recruiting Rad51 to single stranded DNA and triggering strand invasion ^{119; 120}. RPA consists of three components: Rpa1, 2 and 3 in descending order of molecular weight. Recruitment of RPA to single stranded DNA is proposed to activate the DNA damage response pathway leading to freezing of the cell cycle and DNA repair ⁴⁴. Recent evidence suggests there are significant structural and biochemical similarities between the putative CST complex and the RPA heterotrimer ¹¹⁷ ¹⁰⁸. Sequence similarities are particularly obvious between the OB folds of Stn1 and Rpa2 ¹¹⁷, and all three subunits make contacts with ssDNA and other proteins through the use of OB folds. Rpa2 and Rpa3, the two small subunits of RPA, bind telomere DNA weakly but specifically, similarly to Stn1 and Ten1¹¹⁷. In addition to these structural and biochemical similarities *in vivo* studies suggest that the functions of RPA and CST in *S. cerevisiae* might overlap. RPA has been proposed to be involved in promoting telomerase activity ¹²³, play an important role in lagging strand synthesis ^{118; 124; 125} and, most recently, play a potential role in telomere capping, preventing the formation of type II survivors following telomere uncapping caused by the *cdc13-1* mutation ¹²⁶.

Given these similarities between the putative CST complex and RPA, it was decided to purify the two complexes and compare their biochemical activities. However while *S. cerevisiae* RPA has been purified successfully in the past, the putative CST complex has yet to be purified from this species ^{109; 130}. Finally, previous work has shown that replacing the OB-fold of Rpa2 with the OB fold of Stn1 can rescue the viability of *rpa2* Δ strains ¹¹⁷. Therefore hybrid RPA-CST complexes, if they could be created *in vivo* by co-expression in *E. coli*, might show intermediate properties i.e. granting specificity of the RPA complex for telomere DNA or allowing CST to bind to non-telomere regions.

3.2 Expression and purification of the CST complex

Cdc13, Stn1 and Ten1 have been proposed to exist as a complex based on yeast-twohybrid and co-immunoprecipitation experiments ¹⁰⁶. Although the CST complex has been reconstituted in *C. glabrata* ¹⁰⁹, this complex has yet to be reconstituted in *S. cerevisiae* or humans. Temperature-sensitive mutants of the three "subunits" of the putative CST complex indicate that all three play a key role in telomere capping; specifically preventing the accumulation of ssDNA at the end of the chromosome^{79; 100;} ¹⁰⁶. Therefore, given the gap between genetic understanding of the role of CST components in telomere end protection and biochemical understanding of how this is achieved, we aimed to purify the three subunits in isolation and as a complex.

3.2.1 Purification of Cdc13

The Cdc13 open reading frame (ORF) was cloned into pET28a which incorporates an Nterminal hexahistidine tag (figure 9a). This tag was chosen because it is relatively small, and is not proposed to have a significant effect on the structure of native proteins ¹³¹. The construct was then used to transform Rosetta (DE3) pLysS (Novagen), which was chosen because of its propensity to express rare prokaryotic tRNAs, carried on the chloramphenicol resistant pRARE plasmid which expresses 6 rare tRNAs ¹³². Furthermore the strain is a λ DE3 lysogen expressing IPTG-inducible T7 RNA polymerase. Finally, the strain also carries the T7 lysozyme gene which enables easier breakage of cell walls and tighter control of the T7 promoter.

Transformed Rosetta *E. coli* were induced at 37°C for 4 hours and soluble protein harvested following the standard protocol (2.8.1). Soluble protein was then applied to a pre-equilibrated Ni-NTA agarose column at room temperature and protein eluted with increasing concentrations (0.2, 0.5, and 1M) OF imidazole. Assessment of the 0.2M imidazole eluate showed a band of approximately 100kDa (figure 9b), which MALDI-TOF mass spectroscopy confirmed to be Cdc13 (Appendix 1a). However, purity was very poor and Cdc13 was contaminated with several other proteins (figure 9b). A second purification step was attempted using heparin, which is a negatively charged sugar containing molecule which mimics DNA ¹³³. However, the impurities were not separated successfully, and in particular there was heavy contamination with the *E*.

coli molecular chaperone GroEL, whose identity was also confirmed by MALDI-TOF mass spectroscopy (Appendix 1b).

Since a substantial proportion of the contamination was GroEL (figure 9b), it was surmised that Cdc13 may not have folded properly, and, therefore, may have exposed hydrophobic regions to which the chaperone bound ¹³⁴. Alternatively association of this molecular chaperone could be a throw-back to a function of Cdc13 *in vivo* since its activity is modulated by the eukaryotic molecular chaperone Hsp90 ¹³⁵. In order to remove this chaperone a magnesium chloride/adenosine triphosphate (Mg-ATP) wash step, which is proposed to trigger the release of proteins by molecular chaperones, was attempted. Unfortunately this was also unsuccessful.

In the final attempt to purify Cdc13, we modified the lysis buffer composition to reduce protease degradation of our protein by including 1M urea and 1M potassium chloride ¹³⁶. The protein was then rapidly applied to a Ni-NTA agarose column to reduce the time for protease degradation to occur, and an Mg-ATP wash carried out immediately afterwards. Although the purity of Cdc13 was improved and the levels of chaperone significantly reduced, the yields of Cdc13 were exceptionally poor (figure 9c).

As purification of Cdc13 was technically very demanding we acquired the this protein from the Skordalakes laboratory (Wistar Institute, Philadelphia, USA). This lab has purified Cdc13 using similar techniques to those described above ¹²⁹. It was also decided to acquire a plasmid from the laboratory of Deborah Wuttke (University of Colorado, Boulder, USA) that encodes the DNA binding domain of Cdc13 (amino acids 451-694) and incorporates a hexahistidine tag at its C-terminus. This truncated variant is much easier to purify than the full length protein and proposed to bind similarly to DNA ¹³⁷.



Figure 9. Purification of Cdc13. A. Plasmid used to express Cdc13 in *E. coli* Rosetta pLysS *E. coli*. The antibiotic selection marker, origin of replication, promoter, terminator and relevant restriction sites are highlighted. B. Fractions of Cdc13 collected from the Ni-NTA column analysed using a 7% SDS-PAGE gel. Soluble and insoluble fractions of total cell protein and imidazole elutions are also displayed. C. Cdc13 collected from a nickel column after treatment with 1M urea, high salt buffer and MgCl₂/ATP washing. The identity of Cdc13 was determined by gel excision of the indicated protein band of the approximate molecular weight of Cdc13 (~105kDa) followed by MALDI-TOF mass spectroscopy (Appendix 1a).

3.2.2 Purification of the DNA binding domain of Cdc13

As shown in figure 10a the truncated gene, is inserted between the *Ndel* and *Xhol* restriction endonuclease sites upstream of the C-terminal hexahistidine tag and under the control of the strong T7 promoter. The protein was expressed in Rosetta pLysS, and cells were then harvested, lysed and following centrifugation supernatant prepared and loaded onto a HiTrap chelating nickel column (GE Healthcare). Fractions were eluted with a linear gradient of imidazole and visualised on a 15% SDS-PAGE gel. The protein was judged to be >95% pure (Figure 10b) and stored in 50% glycerol at -20°C.



Figure 10. Purification of the DNA binding domain (DBD) of Cdc13. A. Plasmid used to express the DNA binding domain of Cdc13 in Rosetta pLysS *E. coli*. B. Fractions collected from a Nickel column analysed on a 15% SDS-PAGE gel, during elution with imidazole.

3.2.3 Purification of Stn1

A plasmid encoding an N-terminally FLAG-tagged Stn1 carried on a pRSET-A vector was obtained from the laboratory of Victoria Lundblad (Salk Institute, California, USA) (figure 11a). Again this protein was expressed in Rosetta pLysS, with slight deviations

from the method published ¹¹⁷ to facilitate purification. Six 750ml cultures of *E. coli* were grown at 37°C to an A₆₀₀ of 0.6 and then induced by addition of 0.5mM IPTG for approximately 4 hours at 25°C. Cells were sonicated, centrifuged and the supernatant collected and mixed with anti-FLAG M2 resin. After incubation, the resin was loaded onto a column, washed and protein eluted by competition with a FLAG peptide. Fractions were then visualised on a 15% SDS-PAGE gel and the protein was judged to be >90% pure (figure 11b), and stored in 50% glycerol at -20°C.





3.2.4 Purification of Ten1

The Ten1 open reading frame was cloned into a pET28a vector to incorporate an Nterminal hexahistidine tag, giving plasmid pDL1623 (figure 12a). pDL1623 was transformed into Rosetta pLysS and induced with 0.5mM IPTG for 12 hours at 18°C. Cells were sonicated, centrifuged and supernatant collected and mixed with Ni-NTA agarose. The 200mM imidazole elute was applied to a Superdex S200 gel filtration column, and fractions visualised on a 15% SDS-PAGE gel (figure 12b). A band of 18kDa was identified, excised and identified by MALDI-TOF mass spectroscopy, confirming its identity as Ten1 (Appendix 1c).



Figure 12. Purification of Ten1. A. Plasmid used to express Ten1 in Rosetta pLysS *E. coli*. B. 15% SDS-PAGE gel showing a selection of fractions collected during gel filtration.

3.2.5 Attempted purification of the CST complex in E. coli

CST is proposed to function as a complex (Cdc13, Stn1, and Ten1) *in vivo*. Therefore an attempt was made to co-express all three proteins and directly purify the CST complex. It was hoped this would overcome the difficulties seen with Cdc13 purification and

yield a stable, soluble and active complex. It was initially decided to carry out this expression in *E. coli* as this is the best developed host for studies of this nature. To express Cdc13, Stn1 and Ten1 in *E. coli* three new plasmids were created (figures 13a-c): pDL1621 (encoding Cdc13), pDL1620 (encoding Stn1) and pDL1619 (a dual expression vector encoding Stn1 and Ten1). These plasmids were used to transform *E. coli* either individually or in a number of combinations show in table 13. This approach allows expression of all combinations of the CST proteins.

Plasmid(s)	Protein	Result
pDL1622	Cdc13	Moderate yield, poor
		purity
pDL1620	Stn1	Poor yield, poor purity
pDL1623	Ten1	Good yield, moderate
		purity
pDL1621	Cdc13	Moderate yield, poor
		purity
pDL1620 + pDL1621	Cdc13 + Stn1	Moderate yield, poor
		purity
pDL1621 + pDL1623	Cdc13 + Ten1	Moderate yield, poor
		purity
pDL1619	Stn1 + Ten1	Poor yield, poor purity
pDL1622 + pDL1619	Cdc13 + Stn1 + Ten1	Poor yield, poor purity

Table 13. Plasmids used to express different combinations of the CST subunits together. Plasmids are listed alongside the proteins that they produce and those proteins' yields and purities.

E. coli expressing different combinations of the CST complex (table 13) were grown to an OD of 0.6 and expression induced with 0.5mM IPTG over four hours. Cells were then sonicated, centrifuged and supernatant collected and mixed with Ni-NTA agarose (QIAGEN). The columns were then washed using buffer A (300mM NaCl, 50mM Tris-HCl pH8) and then eluted with buffer B (300mM NaCl, 50mM Tris-HCl pH8, 200mM imidazole). The purification procedure for Ten1 + Cdc13 was identical except soluble protein was applied to a HisTrap HP column and eluted with a linear gradient of imidazole (figure 13g). The results of the co-expressions are summarised in table 13. However, briefly, expression of Stn1 from pDL1620 was unsuccessful as Stn1 did not appear in the elute. This could be a consequence of expression from a different vector system as Stn1 was successfully purified from pDL1648 previously. One possibility was that Stn1 was being degraded by proteolysis and/or was unstable when expressed from the pET vector. However, it was hoped that co-expression of Stn1 with untagged Ten1 using a dual expression vector would help stabilise Stn1 and allow pull-down on the Ni-NTA column. Following purification and elution a band of the approximate size of Stn1 (50-75kDa) was observed (figure 13e). However MALDI-TOF mass spectroscopy confirmed that this band did not correspond to an *S. cerevisiae* protein (appendix 1e). Furthermore, no such band was detected corresponding to untagged Ten1 (~18kDa).

Co-expression and purification of Stn1 with Cdc13 using plasmids pDL1621 and pDL1620 was also attempted. Following SDS-PAGE analysis two bands of approximately 100kDa (corresponding to the size of Cdc13) and 50-75kDa (corresponding to the size of Stn1) were observed. MALDI-TOF mass spectroscopy identified the uppermost band as Cdc13 (appendix 1f) and the lower band as the molecular chaperone GroEL (appendix 1g) (figure 13f). Stn1 was not detected.

Co-expression and purification of Cdc13 and Ten1 from the plasmids pDL1621 and pDL1623 resulted in a band corresponding to Cdc13, and a band identified by MALDI-TOF mass spectroscopy as Ten1 (appendix 1h). However, another band was also observed in the 50-75kDa size range which was suspected to be the molecular chaperone GroEL (figure 13g).

Finally co-expression and purification of Cdc13, Stn1 and Ten1 from plasmids pDL1622 and pDL1619 resulted in bands corresponding to the approximate size of Cdc13, Stn1 and Ten1 following SDS-PAGE analysis of the eluate. However, MALDI-TOF mass spectroscopy indicated that the bands corresponding to the size of Stn1 and Ten1 (figure 13h) were not *S. cerevisiae* proteins (appendix 1i, j).





pDL1621 + pDL1620



pDL1622 + pDL1619



F

Figure 13. Co-expression and purification of Cdc13, Stn1 and Ten1 in *E. coli.* A. Plasmid used to express C-terminal his-tagged Cdc13 in *E. coli*. B. Plasmid used to express N-terminal his-tagged Stn1 in *E. coli*. C. Plasmid used to co-express N-terminal his-tagged Stn1 and untagged Ten1 in *E. coli*. D. Fractions taken from sequential stages of purification of N-terminal his-tagged Stn1 from *E. coli*. E. Fractions taken from sequential staged Ten1. F. Fractions taken from the 200mM elution of co-purification of N-terminal his-tagged Stn1 and Untagged Ten1. F. Fractions taken from the 200mM elution of co-purification of N-terminal his-tagged Stn1 and N-terminal his-tagged Stn1 and C-terminal his-tagged Cdc13. G. Fractions taken from an imidazole elution gradient during co-purification of C-terminal his-tagged Cdc13 and N-terminal his-tagged Ten1. H. Fractions taken from sequential stages of co-purification of N-terminal his-tagged Cdc13. N-terminal tagged Stn1 and Untagged Ten1. All fractions were visualised on 15% SDS-PAGE gels.

3.2.6 Attempted purification of the CST complex in S. cerevisiae

Since co-expression of Cdc13, Stn1 and Ten1 was unsuccessful in *E. coli* attempts were made to co-purify Cdc13, Stn1 and Ten1 from their native host, *S. cerevisiae*. The Cdc13 ORF was cloned into pRS426-GST-GAL ¹³⁸ kindly provided by Professor Peter Burgers (Washington School of Medicine, St Louis, USA) to create pDL1631. This vector incorporates an N-terminal glutathione-S-transferase(GST)-tag, and a human rhinovirus protease cleavage site, that enables removal of the GST-tag following purification. The ORF is under the control of a strong, inducible GAL promoter (figure 14a). Stn1 and Ten1 were cloned into pRS424-GAL ¹³⁸ to create pDL1632 which places untagged Stn1 and Ten1 either side of the bidirectional GAL promoter (figure 14b). These two plasmids were then sequentially transformed into *S. cerevisiae* strain DLY757 which is deficient for three major endogenous proteases.

Transformants were streaked out onto selective SCGL solid media and incubated for 2 days at 30°C. The cells from this plate were then used to inoculate liquid SCGL media and were then induced with 2% final concentration galactose for 4 hours (see 2.13). Following pelleting, lysis and collection of supernatant, the protein extract was mixed with Glutathione Sepharose 4B and then applied to a gravity-flow column. The column was washed and protein eluted using 20mM glutathione. Products of the purification were visualised on a 15% SDS-PAGE gel. Analysis of the SDS-PAGE gel revealed that bands corresponding to Cdc13 (105kDa), Stn1 (57kDa) and Ten1 (18kDa) were not present. However there was one band present with an approximate molecular weight of 30kDa (figure 14c). MALDI-TOF mass spectroscopy analysis showed this to correspond to the GST tag from *Schistosoma japonicum* (appendix 1k), which has a molecular weight of 26kDa. This presumably arises from proteolysis of the GST-Cdc13 fusion protein.



Figure 14. Co-expression and purification of Cdc13, Stn1 and Ten1 in *S. cerevisiae*. A. Plasmid used to express Cdc13 in *S. cerevisiae*. B. Plasmid used to express untagged Stn1 and Ten1 in *S.* cerevisiae. C. Fractions taken from sequential stages of co-purification of GST-tagged Cdc13 and untagged Stn1 and Ten1. Protein collected was visualised on a 15% SDS-PAGE gel.

3.2.7 In vitro reconstitution of the CST complex

Expression and co-purification of the CST complex using *S. cerevisiae* and *E. coli* was unsuccessful. However, the separately purified individual proteins were available and attempts were made to reconstitute the complex *in vitro*. FLAG-tagged Stn1 was applied to an anti-FLAG M2 resin (Sigma-Aldrich), which was washed with a buffer of pH and ionic strength similar to yeast cells (50mM Tris pH7.4, 150mM NaCl and 1mM EDTA). Purified Ten1 and Cdc13, in the same buffer, were simultaneously applied to the column which was left to stand for 5 minutes and then washed with this buffer. If Cdc13 and Ten1 form a complex with immobilised Stn1, they should be retained by the column. Stn1 (and any proteins with which it interacted) were then competitively eluted using the FLAG octapeptide. Unfortunately SDS-PAGE analysis (figure 15) revealed that only Stn1 was eluted with the FLAG octapeptide and that both Cdc13 and Ten1 ran straight through the column. Thus there is no evidence for *in vivo* formation of a CST complex from its three component proteins under these conditions.



Figure 15. *In vitro* reconstitution of the CST complex. FLAG-tagged Stn1 was expressed from *E. coli*, and the *E. coli* lysate applied to anti-FLAG M2 resin and run-through collected. The column was then washed with TBS twice (wash 1 and wash 2). A Cdc13-Ten1 mixture containing 1.5nmol Cdc13 and 1.2nmol Ten1 (shown here as Cdc13-Ten1 solution) was applied to the column containing bound Stn1 (estimated from previous purifications to be ~2.3nmol) and run through collected. The column was then washed again twice with TBS (wash 4 and wash 5) and Stn1 eluted using the FLAG octapeptide.

Protein fractions from each stage of the purification were then visualised on a 15% SDS-PAGE gel.

3.3 Attempted purification of CST-RPA hybrids

3.3.1 Similarity between RPA and CST complexes

The RPA heterotrimer and the putative CST complex have been proposed to be analogous complexes based on structural and biochemical similarities between the three subunits. These similarities largely revolve around the use of OB-folds by both proteins to contact DNA, and similarities in the crystal structures of the two smallest subunits of RPA and CST ^{108; 117}. Furthermore RPA has been proposed to play a role in telomere-specific functions including telomere extension by telomerase ¹²³, lagging strand synthesis ^{124; 125} and possibly telomere capping functions ¹²⁶. Therefore, given these biochemical and structural similarities it was decided to investigate whether the subunits of RPA and CST were interchangeable (table 14). To achieve this aim it was decided to attempt co-expression of various combinations of the RPA and CST components to determine if any hetero-complexes could be isolated.

CST complex	RPA heterotrimer
Cdc13	Rpa70
Stn1	Rpa32
Ten1	Rpa14

Table 14. Components of the CST and RPA complexes. Proteins listed in the same rows have been suggested to be homologous and therefore possibly interchangeable ¹¹⁷.

3.3.1 Purification of RPA

In order to purify RPA and carry out the co-expression experiments it was necessary to use dye-ligand affinity chromatography using cibacron blue linked to agarose (Bio-rad). This method of purification does not involve the use of tags but instead relies on the native properties of the protein being eluted. Cibacron blue forms ionic, hydrophobic and even nucleotide specific interactions with proteins, and these can be disrupted by modifying pH or ionic strength as well as using nucleotide substrates. Therefore cibacron blue binds to a very broad spectrum of proteins. By modifying buffer conditions such as increasing the levels of salts or modifying pH, the protein of interest can be eluted separately from contaminants, and so this represents a way of purifying proteins without the need for a tag. This technique has been used by the laboratory of Marc Wold to purify both human and *S. cerevisiae* RPA.

RPA was expressed from a plasmid derived from the pET11 vector acquired from the laboratory of Marc Wold (University of Iowa, USA). This plasmid carries the ORFs of the three subunits of the RPA heterotrimer (Rpa14, Rpa32 and Rpa70). All three subunits are successively arranged in the same orientation under the control of one IPTG-inducible T7 promoter and each subunit is preceded by its own Shine-Dalgarno ribosome binding site (RBS) (figure 16a).

Overexpression and purification of RPA was performed broadly in line with the procedure of Marc Wold ¹³⁹. The plasmid was transformed into *E. coli* Rosetta pLysS and single colonies were used to inoculate cultures of LB in order to minimise the number of divisions cells went through while carrying the operon since it is highly toxic. Following induction and extraction of soluble protein (see 2.8.2) the supernatant was applied to an Affi-gel blue (Biorad) column pre-equilibrated with RPA HI Buffer. After three successive washing steps RPA was eluted in HI buffer containing 1.5M sodium thiocyanate. Sodium thiocyanate was then removed following a second purification step on a hydroxyapatite column pre-equilibrate with HI buffer. RPA was then eluted with HI buffer containing 80mM potassium phosphate. Eluted RPA was >90% pure by this point (figure 16b) and was buffer exchanged into HI buffer and stored in 50% glycerol at -20°C.



Figure 16. Purification of Replication Protein A. A. Plasmid used to express RPA in Roseta pLysS *E. coli*. B. Fractions collected during the purification of wild type RPA and protein visualised on a 15% SDS-PAGE gel.

3.3.2 Purification of sub-stoichiometric RPA assemblies

In order to test whether RPA-CST hybrids could be formed site-directed mutagenesis was carried out using the Quick Change protocol to create stop codons at the start of the ORFs for each the three subunits located on the RPA synthetic operon. This resulted in the creation of three new plasmids: pDL1627 (which had a stop codon inserted near the start of the *RPA70* ORF) (figure 17a), pDL1628 (which had a stop codon inserted near the start of the *RPA32* ORF) (figure 17b) and pDL1629 (which had a stop codon inserted near the start of the *RPA32* ORF) (figure 17c).

These plasmids were then transformed into Rosetta pLysS *E. coli* individually and with their deleted members proposed CST homologue (Table 15). The purification procedure was identical to that used to purify wild type RPA, except the final hydroxyapatite purification step which was omitted.

CST component plasmid	RPA component plasmid	Proteins expressed
-	pDL1627	Rpa2 + Rpa3
-	pDL1628	Rpa1 + Rpa3
-	pDL1629	Rpa1 + Rpa2
pDL1622	pDL1627	Cdc13 + Rpa2 + Rpa3
pDL1620	pDL1628	Rpa1 + Stn1 + Rpa3
pDL1623	pDL1629	Rpa1 + Rpa2 + Ten1

Table 15. Combinations of plasmids used to create RPA-CST hybrids. The combinations of plasmids used to express two members of the RPA complex alone, and in combination with the deleted members' proposed CST homologue.

Expression of Rpa2 and Rpa3 using pDL1627 (figure 17a) expectedly resulted in the absence of Rpa1. The absence of the large subunit also resulted in substantially reduced yields of Rpa2 and Rpa3 as judged by SDS-PAGE analysis (figure 17b). Expression of Rpa1 and Rpa3 using pDL1628 (figure 17c), resulted in both the absence of Rpa2 and substantially reduced yields of Rpa1 and Rpa3 as judged by SDS-PAGE

analysis (figure 17d). Finally expression of Rpa1 and Rpa2 using pDL1629 (figure 17e), resulted in the absence of Rpa3 (figure 17f). A single band was identified at the same molecular weight as Rpa3 by SDS-PAGE but MALDI-TOF mass spectroscopy revealed that this corresponded to lysozyme, which was added during protein preparation (Appendix 1I). Interestingly, deletion of Rpa3 reduced the levels of Rpa1 and Rpa2 but the effects were less pronounced than those seen with the deletion of the other two subunits.



Figure 17. Expression and purification of the RPA heterotrimer with each of the subunits deleted by site-directed mutagenesis. Three plasmids were created by site-directed mutagenesis from pDL1625 each with one subunit of the RPA complex deleted. (A) pDL1627 which expresses Rpa2 and Rpa3 but has a stop codon inserted near the start of Rpa1 resulted in a truncated Rpa1. (B) Protein fractions collected during the expression and purification of Rpa2 and Rpa3 from pDL1627 visualised on a 15% SDS-PAGE gel. (C) pDL1628 which expresses Rpa1 and Rpa3 and has a stop codon inserted near the start of Rpa2 resulted in a truncated Rpa2. (D) Protein fractions collected during the expression and purification of Rpa1 and Rpa3 from pDL1628 visualised on a 15% SDS-PAGE gel. (E) pDL1629 which expresses Rpa1 and Rpa3 from pDL1628 visualised on a 15% SDS-PAGE gel. (E) pDL1629 which expresses Rpa1 and Rpa3. (F) Protein

fractions collected during the expression and purifications of Rpa1 and Rpa2 from pDL1629 visualised on a 15% SDS-PAGE gel.

Co-expression and purification of Cdc13, Rpa2 and Rpa3 from pDL1622 and pDL1627 dramatically reduced the levels of Rpa2 and Rpa3 produced and no Cdc13 was visible in the final elute as judged by SDS-PAGE analysis (figure 18a). Furthermore co-expression and purification of Cdc13, Rpa2 and Rpa3 from pDL1620 and pDL1628 had a substantial negative effect on the yields of Rpa1 and Rpa3 and there was no obvious band corresponding to Stn1 as judged by SDS-PAGE analysis (figure 18b). Finally co-expression of Rpa1, Rpa2 and Ten1 from pDL1623 and pDL1629 and subsequent purification resulted in a substantial reduction in the levels of Rpa1 and Rpa2 and no hybrid complex was reconstituted since Ten1 was not detected in the elute (figure 18c).



Figure 18. Purification of chimeric RPA-CST complexes. (A) Protein fractions collected after co-expression and purification of Cdc13, Rpa2 and Rpa3 from pDL1627 + pDL1622 visualised on a 15% SDS-PAGE gel. (B) Protein fractions collected after co-expression and purification of Rpa1, Stn1 and Rpa3 from pDL1628 + pDL1620 visualised on a 15% SDS-PAGE gel. (C) Protein fractions collected after co-expression and purification of Rpa1, Rpa2 and Ten1 from pDL1629 + pDL1623 visualised on a 15% SDS-PAGE gel.

3.4 Attempted purification of S. cerevisiae Exonuclease I

Attempts were also made to purify *S. cervisiae* Exo1 using an Sf9 (insect cell) expression system, as this is the only known way to produce Exo1 ¹⁴⁰. pFB-Exo1-FLAG was acquired from the lab of Prof Steve Kowalakowski (University of California, USA). This vector was then transformed into DH10 Bac cells (Invitrogen) which contain a bacculovirus shuttle vector containing a mini-att Tn7 target site and a helper plasmid which provides protein factors required for transposition (appendix 2, figure 53). Transposition between pFB-Exo1-FLAG and the bacculovirus shuttle vector (bacmid) occurs because the Exo1-FLAG ORF preceded by the polyhedrin promoter is flanked by the left and right arms of Tn7.

Transposition occurs within the Lacl gene, found on the bacmid, coding for the α peptide of LacZ, meaning that successful transposition interferes with α complementation of β -galactosidase ¹⁴¹. Therefore blue-white screening was used to
select for correct transformants. White colonies were then picked, high molecular
weight bacmid DNA isolated and then transfected into Sf9 cells allowing generation of
bacculoviruses that were used in the preliminary expression experiments.

Cells were then sonicated, centrifuged and supernatant collected and combined with pre-equilibrated anti-FLAG M2 resin (Sigma-Aldrich). After incubation, the resin was loaded onto a column, washed and protein eluted by competition with a FLAG peptide. The elute was concentrated and then run on a 15% SDS-PAGE gel, however only very faint bands were observed. A section of the gel was excised between the 100kDa and 75kDa molecular weight markers (as this was the expected size of Exo1), and sent for MALDI-TOF mas spectroscopy, but Exo1 was not detected (Appendix 1m). Given the technical difficulty of purifying this protein and the extremely poor reported yields, it was decided to use a commercial exonuclease analog, λ -exo (Thermo Scientific).
3.5 Discussion

Several proteins have been purified in house or obtained from other laboratories for use in downstream biochemical characterisation studies outlined in the next chapter. These are: the RPA heterotrimer, Cdc13, Stn1, Ten1 and the DNA binding domain of Cdc13. All except Cdc13 were purified in house. However attempts to purify the CST complex in two different expression systems, *E. coli* and *S. cerevisiae*, were unsuccessful, as well as attempts to create CST-RPA hybrids. Furthermore, purification of *S. cerevisiae* Exo1 from Sf9 insect cells was also unsuccessful.

3.5.1 Attempted purification of the CST complex

Since yeast-2-hybrid, co-immunoprecipitation and genetic studies suggest that Cdc13, Stn1 and Ten1 function together as a complex ^{79; 100; 106} it was decided to attempt to purify this complex *in vitro*. All possible combinations of Cdc13, Stn1 and Ten1 were expressed in *E. coli*, however no combination improved the yield or purity of any protein, and purification of CST as a complex failed. There are various explanations such as solubility, stability, protease degradation or simply that the complex does not exist in *S. cerevisiae*. However the most likely explanation is that the complex cannot be assembled outside of its native host, perhaps requiring eukaryotic chaperones for assembly.

Attempts were therefore made to co-express the three subunits in *S cerevisiae* in the hope that they might be assembled correctly, and form a stable complex. Cdc13 was GST-tagged, the other two subunits were untagged and all were expressed from plasmids which place the genes under the control of strong GAL inducible promoters. However, it was found that following purification, only the GST-tag remained, suggesting that Cdc13 was being proteolytically degraded and/or was unstable. Other laboratories have experienced similar difficulties in purifying the CST complex from *S*. *cerevisiae* due to an inability to reconstitute the complex or an inability to produce sufficient quantities for biochemical investigation ¹⁰⁹.

Since attempts to purify the protein from *E.coli* and *S. cerevisiae* was unsuccessful and since we had all three proteins purified individually to a good level of purity, it was

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decided to try and reconstitute the complex *in vitro*. Therefore FLAG-tagged Stn1 was bound to Anti-FLAG M2 beads and a mixture of purified Cdc13 and Ten1 in a physiological buffer was washed over the column in the hope that Cdc13 and Ten1 would bind to Stn1 and they could then be eluted as a complex. However, it was found that Cdc13 and Ten1 did not associate with FLAG-tagged Stn1. This was somewhat surprising as Stn1 and Ten1 have been purified as a subcomplex before ¹⁰⁸ and there are well documented physical interactions between Stn1 and Cdc13 ^{79; 84; 98}. One possible explanation for the lack of interaction between Stn1 and Ten1 could be the presence of the FLAG-tag at the N-terminus of the Stn1 protein which is proposed to be the location of the Ten1-interacting interpace^{117; 142}. However since the Cdc13 interacting interpace is on the C-terminus of Stn1 ⁹⁸ this would not explain the lack of interaction with Cdc13.

3.5.2 Attempted purification of CST-RPA hybrid complexes

Given the biochemical, structural and functional similarities between RPA and CST, combined with the finding that $rpa2\Delta$ strains can be rescued with a chimeric Rpa2 containing a Stn1 OB fold ¹¹⁷, it is possible that hybrid complexes of CST and RPA might exist, and have intermediate properties. However, following creation of plasmids with each of the three subunits of RPA individually knocked out by introducing an in phase stop codon, expression of the equivalent subunit in CST did not enable reconstitution of a hybrid complex. One possible explanation is use of a non-native host, thereby not allowing proper reconstitution of the complexes. The other conceivable possibility is that these complexes do not actually form *in vivo*.

3.5.3 Attempted purification of Exo1

It is unclear why attempted purification of Exo1 failed, but one possibility is that the viral stock may have contained a mixture of recombinant and non-recombinant bacmids, as we did not carry out plaque purification. Another possibility could be that the white colony picked for bacmid purification could have been a false positive, and therefore I may have been working with a non-recombinant bacmid. Finally, the conditions of the expression and purification may have been non-optimal compounding the effect of very low expected yields of Exo1.

3.5.4 Further work

The difficulties of purifying the CST complex, CST-RPA hybrid proteins and Exo1 revolve around poor/no expression and low yields and it is evident that the expression systems for these proteins need to be optimized. In order to solve this problem, an interesting avenue for further work would be to carry out high throughput protein production screening in collaboration with the Oxford Protein Production Facility (OPPF). This facility has access to the pOPIN suite of vectors ¹⁴³ which enable protein production from three different expression systems: *E. coli,* mammalian and insect cells. Furthermore they also make use of the InFusion cloning system which allows relatively high-throughput cloning into multiple expression vector types with different promoters and tags using direct recombination of PCR products ¹⁴³. This would rapidly speed up the process of purification and allow exo assays to be carried out with Exo1, and determine whether it is technically possible to purify the CST complex or CST-RPA hybrid.

4. The role of CST and RPA in telomere end protection: A biochemical approach

4.1 Background

"Telomere-end protection" is the ability of telomere-associated proteins to prevent the ends of chromosomes being recognised as DNA damage. A key aspect involves preventing access to the telomere end or modulating the activity of factors involved in DNA repair. One such factor in the model organism *S. cerevisiae* is Exonuclease I (ExoI), which is a highly processive 5' to 3' exonuclease. This enzyme is proposed to degrade the 5' recessed lagging strand at telomere ends leading to an expanding G-rich ssDNA overhang ⁸⁶. If this activity continues unimpeded, the expanding region of ssDNA recruits factors involved in DNA repair, and leads to pre-mitotic (G2/M) checkpoint activation ¹⁰⁰ and/or inappropriate repair activities such as homologous recombination (HR) ^{88; 144}, which leads to genomic instability.

The proposed CST (Cdc13-Stn1-Ten1) protein complex is proposed to play a key role in preventing resection by nucleases such as Exol ^{87; 100; 145} (figure 19a). Furthermore it is also proposed to play important roles in recruiting and regulating telomerase (Est1-Est2-Est3) to telomere ends ⁷⁶, and promoting lagging strand synthesis by pol α ^{97; 98}. Therefore the presence of the CST complex at telomere ends allows a "DSB-like" structure to persist at the chromosome terminus without processing by the DNA repair machinery or activation of the G2/M checkpoint.

Replication Protein A (RPA) is a heterotrimeric protein that plays key roles in DNA metabolism throughout the cell. Its main function is to prevent self-complementation and secondary structure formation in ssDNA ¹¹⁸. This makes it an important protein in ensuring the persistence of an unwound template for replication, and presenting ssDNA for Rad51 nucleoprotein filament formation in HR ^{45; 120}. It is therefore important in both mitosis and meiosis.

It has been proposed that CST is a telomere-specific RPA based on structural similarities and comparative gel shifts with telomeric and non-telomeric ssDNA substrates ¹¹⁷. Furthermore, RPA and CST have overlapping roles in telomere replication and the recruitment of telomerase. Therefore, based on these similarities

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we asked whether RPA and CST played overlapping roles in preventing exonuclease resection at telomere ends. To do this we reconstituted the telomere end *in vitro* and performed exonuclease protection assays in the presence and absence of members of the CST complex and the RPA heterotrimer.

4.2 Establishing a telomere exonuclease protection assay

4.2.1 Design and synthesis of dsDNA-substrates

A 90 base oligodeoxynucleotide which folds to generate 34 base-pairs of double stranded DNA, appended with a 3' single stranded extension 18 bases in length, has been used as a telomere mimic (figure 19b). The single stranded DNA, and the contiguous region in the duplex, contain multiple TG repeats, ensuring that the analogue mirrors both the structure and the sequence found at telomere ends in *S. cerevisiae* outside of G2 phase of the cell cycle ⁶⁵. Folding is facilitated by a flexible 4 thymidine linker that connects the two regions that comprise the duplex. A fluorescein-labelled thymidine is present near the extreme 3'-terminus for diagnostic purposes, enabling monitoring of both 5'-3' exonucleolysis and protein binding by gel shift analysis.

A control, which has the same overall architecture as the telomere mimic but lacks TG repeats, is derived from the Mata/Mat α mating type locus in *S. cerevisiae*, which is highly recombinogenic and, therefore, a "hot-spot" for DNA double strand breaks (figure 19c) ¹⁴⁶. This control is representative of long microhomology that is found early after initial processing of double strand breaks ¹⁴⁷. The looped substrates imitate the environment of telomeres and double strand breaks in that, *in vivo*, adjacent free ends are likely to be several Mbases removed; in the case of telomeres at the other end of the chromosome. Further the presence of only a single free end facilitates resection experiments as exonucleolysis (either 3' or 5') can only commence from a single point.



Figure 19. Structure of the budding yeast telomere. A. The binding of Cdc13, Stn1, Ten1 and RPA to the 3' single stranded DNA overhang found at telomere ends. The binding of Cdc13, Stn1 and Ten1 is proposed to render the telomere end refractory to exonuclease degradation. B. The looped oligonucleotide (telomere loop, TL) used to simulate a budding yeast telomere end. C. The looped oligonucleotide (control loop, CL) with the same structure as TL but using sequence taken from the Mata/Matα locus. X indicates a thymidine labelled with fluorescein.

4.2.2 Digestion of telomere analogues by bacteriophage λ exonuclease

Although *S. cerevisiae* Exol has been purified in the past from Sf9 cells, it is produced in miniscule amounts, and the level of homogeneity is low ¹⁴⁸. Therefore the enzyme from bacteriophage λ (λ -exo), which has a powerful 5'-3' exonuclease activity and a strong preference for 5'-phosphates, was used as a model nuclease. When TL and CL were treated with λ -exo an intermediate DNA structure is rapidly formed followed by slower degradation to much smaller oligodeoxynucleotides. Comparison of this species with some DNA standards suggested that the intermediate arose by removal of all 34 deoxynucleotides between the 5' terminus and the first (5') thymidine in the 4 thymidine linker structure (figure 20). The properties of λ -exo ^{149; 150; 151} readily explain the persistence of this prominent intermediate; when the digestion reaches the 4 thymidine linker structure the DNA becomes single-stranded, a poor substrate for the enzyme. Additionally, the processivity of λ -exo accounts for the absence of bands between the starting material and intermediate product.





Note: CL standards not shown, but same as TL but based on CL sequence

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Figure 20. Identification of the major stable intermediate produced by digestion products of TL and CL with λ -exo. A. Identification of the standards used for TL. The entire TL sequence is given and the truncated standards used identified using red bases and numbers (CL is not shown but exactly the same standards, based on the CL sequence, were used). B. Digestion of TL (10nM) with λ -exo (50 units) for the times shown and comparison of the products with the standards. C. Digestion of CL (10nM) with λ -exo (50 units) for the times shown and comparison of the products with standards. In both cases the stable intermediate runs with standard 3 and so corresponds with digestion up to the first (5') T in the T-loop.

4.3 Cdc13 protects telomeres from 5' to 3' exonuclease resection

Genetic analysis in *S. cerevisiae* suggests that the CST complex plays a key role in protecting the 5' recessed strand of the telomere from resection^{88; 106}. The ability of Cdc13, the major protein component of the complex, to inhibit degradation of the 5' recessed strand by 5'-3' exonucleases has been determined. Figure 21 shows the results obtained when TL and CL were incubated for various times with λ -exo in the presence or absence of Cdc13. In the absence of Cdc13, both loops were equally susceptible to hydrolysis by λ -exo. The gel scans indicate that the intermediate structure persisted for about a minute, before further degradation to smaller products, resulting from the tardy activity of λ -exo on single stranded DNA (figures 21a and c).

When the reaction was carried out in the presence of Cdc13, resection was strongly inhibited with TL, as evidenced by much slower conversion of the starting substrate to the intermediate and the persistence of the starting substrate at the end of digestion (270s) (figure 21b). As with the free loop, an intermediate product was initially seen, which was very stable towards further hydrolysis, assuredly by the tight binding of Cdc13 to this single stranded DNA species. The very different rates at which TL and CL are hydrolysed by λ -exo when Cdc13 is present are striking (figure 21b and d). Collectively, these results suggest that Cdc13 is able to strongly protect the 5' recessed strand at telomeres from 5' to 3' resection, and this protection is highly selective for the TG repeats associated with telomeres; a result that correlates well with the high affinity and specificity shown by Cdc13 for telomere single stranded DNA ^{87; 110}. Interestingly, Cdc13 also provides a secondary shielding effect from digestion of single stranded DNA, however this is not of physiological relevance and arises solely as a consequence of the looped substrates used in these experiments.



Figure 21. Protection of DNA ends from λ -exo by Cdc13. DNA was digested by λ -exo in the absence and presence of Cdc13 (50nM) and analysed by denaturing polyacrylamide gel electrophoresis. A. Digestion of TL without Cdc13. B. Digestion of TL in the presence of Cdc13. C. Digestion of CL without Cdc13. D. Digestion of CL in the presence of Cdc13. In all cases the figures above the gel lanes represent the time of digestion in seconds, and assays were repeated twice to ensure consistency.

4.4 The DNA binding domain (DBD) of Cdc13 protects telomeres from 5'-3' exonuclease resection

Cdc13 is a multi-domain protein with the DNA binding domain (DBD) proposed to confer high affinity for telomere DNA ^{111; 152}. Fusion of the DBD of Cdc13 to Stn1 is known to rescue the lethality of a Cdc13 null strain ¹¹⁴, suggesting that the other domains of Cdc13 are largely involved in mediating protein-protein interactions with Stn1 and Ten1.

Digestions have been conducted in the presence of the isolated DBD of Cdc13 to determine if this region is sufficient to provide protection from the λ -exo catalysed reaction. The gel patterns obtained are very similar to that seen with the full length protein (figure 22a-d). Free TL and CL are rapidly degraded by λ -exo (figure 22a and c) and no protection is afforded to CL by the DBD (figure 22d). In contrast strong shielding was provided to TL by the DBD, with its conversion to the stable intermediate being visibly slower than when the protein was absent. (figure 22b). However it is noticeable that the DBD offers less shielding to TL than does Cdc13 itself (compare figure 22b and figure 21b). Therefore the DBD of Cdc13 is capable of shielding telomeres from resection by exonucleases but the entire protein appears to be required for maximal protection.



Figure 22. Protection of DNA ends from λ -exo by the DNA binding domain of Cdc13.

DNA was digested by λ -exo in the absence and presence of the DBD (50nM) and analysed by denaturing polyacrylamide gel electrophoresis. A. Digestion of TL without the DBD. B. Digestion of TL in the presence of the DBD. C. Digestion of CL without the DBD. D. Digestion of CL in the presence of the DBD. In all cases the figures above the gel lanes represent the time of digestion in seconds, and assays were repeated twice to ensure consistency.

4.5 Cdc13 binds tightly to the telomere 3' overhang

To confirm that the shielding effect of Cdc13 and the DBD against resection by λ -exo on the TL substrate was due to a protein:DNA interaction electrophoretic mobility shift assays (EMSAs) were performed with the TL and CL substrates. The DBD bound strongly to TL but showed little evidence of binding to CL over the concentration range of DBD used in this assay (figure 23b and d). Inspection of figures 23a and b shows that the complete binding of TL is achieved by Cdc13 and DBD at concentrations of between 6 and 12nM i.e. at approximately the same concentration as the amount of TL present. This implies very tight and near stoichiometric binding of the two proteins to their TL target with a K_D value under stoichiometric binding conditions where each aliquot of protein added binds completely to the DNA ¹⁵³. Furthermore, Cdc13 showed a moderate affinity for the CL substrates (figure 23c). Collectively, these results suggest that the shielding effect of Cdc13 and the DBD against resection by λ -exo could be due to a specific and tight binding of the proteins to the 3' overhang.



Figure 23. DNA binding properties of Cdc13 and the DBD of Cdc13. The interactions of Cdc13 and the DBD with TL and CL were investigated using EMSA. A. Binding of Cdc13 to TL B. Binding of the DBD to TL. C. Binding of Cdc13 to CL. D. Binding of the DBD to CL. The numbers above the gel lanes represent protein concentration in nM. Assays were repeated twice to ensure consistency.

4.6 Interaction of Stn1 and Ten1 with TL and CL

Deletion of any component of the CST complex is lethal in *S. cerevisiae*, and genetic experiments using temperature sensitive mutations have demonstrated that interference with their activities results in lability at telomere ends ^{79; 100; 154}. ChIP analysis has indicated that Stn1 and Ten1 associate with telomere DNA ¹¹⁶, although this is not an unequivocal test of a direct DNA-protein interaction as Stn1 or Ten1 could bind via a "sandwich" contact mediated by a second telomere binding protein. Therefore, the ability of Stn1 and Ten1 to bind to the telomere mimic and afford protection from 5'-3' exonuclease activity was directly tested. To determine if Stn1 and Ten1 bound to the TL and CL substrates gel shifts were performed. Neither Stn1 nor Ten1 caused a shift in the mobility of the TL or CL substrates (figure 24a-d) at any of the concentrations used in this assay.



Figure 24. DNA binding properties of Ten1 and Stn1. The interactions of Ten1 and Stn1 with TL and CL were investigated using EMSA. A. Binding of Ten1 to TL B. Binding of Ten1 to CL. C. Binding of Stn1 to TL. D. Binding of Stn1 to CL. The numbers above the gel lanes represent protein concentration in μ M. Assays were repeated twice to ensure consistency.

4.7 Ten1 and Stn1 bind to G-rich single stranded DNA

However since both proteins are proposed to be telomere binding proteins, a further shift with a 50bp single stranded oligonucleotide was carried out using the sequences taken from the 3' overhang of the telomere and control loop (figure 25a). It was found that Ten1 showed very weak binding to both the 50bp G-rich telomere single stranded oligonucleotide (T) (figure 25b) T and the non-telomere control (C) substrate and little specificity (figure 25c). Stn1, on the other hand was found to show a weak but specific affinity for the T substrate which was not recapitulated with the C substrate (figure 25d, e). This data may also tentatively suggest that Stn1 and Ten1 bind with higher affinity to longer telomere overhangs, however this would need to be directly tested, by generating a loop with a 50bp overhang region. Furthermore, if nM levels of Cdc13 but μ M levels of Stn1 and Ten1 are required to fully bind the telomere analogue, clearly almost all of the binding of the CST complex to telomeres will be mediated by Cdc13.

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HEX-TGGCACGCGGACAAAATGCAGCACGGAATATGGGACTACTTCGCGCAACA



Figure 25. Binding of Ten1 and Stn1 to single stranded DNA. The interactions of Ten1 and Stn1 with a 50bp G-rich telomere single stranded oligonucleotide (T) and non-telomere sequence control (C) were investigated using EMSA. A. The sequence of the T and C oligonucleotides. B. Binding of Ten1 to T. C. Binding of Ten1 to C. D. Binding of Stn1 to T. E. Binding of Stn1 to C. The numbers above the gel lanes represent protein concentration in μM. Assays were repeated twice to ensure consistency.

4.8 Investigation into whether Stn1 and Ten1 protect TL and CL substrates from resection

Figure 26 shows the results obtained when TL and CL were incubated for various times with λ -exo in the presence or absence of Stn1 or Ten1. In contrast to Cdc13 (figure 26a), in the presence or absence of these two proteins, both the telomere and control loops were hydrolysed by λ -exo at similar rates (figure 26b-d). Given that Stn1 and Ten1 have been proposed to exist as a subcomplex, it was decided to carry out the assay in the presence of both proteins. However, again in the presence and absence of the mixture of Stn1 and Ten1, both TL and CL were hydrolysed by λ -exo at similar rates (figure 26e). Collectively these results suggest that Stn1 and Ten1 alone or in combination do not protect the TL and CL substrates from resection by λ -exo. However given that Stn1 and Ten1 do not bind the looped oligonucleotides, this result was not unexpected.

One possible function of Stn1 and Ten1 is to enhance the ability of Cdc13 to protect telomere ends from resection, rather than directly protecting the telomere end themselves. To test this possibility different combinations of Cdc13, Stn1 and Ten1 were mixed, and their ability to protect telomere ends determined. However, all combinations of Cdc13 with Stn1 and Ten1 offered no enhancement of protection above that provided by Cdc13 alone (figure 27a-d).







Figure 26. Protection of DNA ends from λ -exo by Stn1 and Ten1. DNA was digested by λ -exo in the absence and presence of Stn1 (150nM) or Ten1 (50nM) and analysed by denaturing polyacrylamide gel electrophoresis. A. Digestion of TL with Cdc13. B. Digestion of TL in the absence of any protein. C. Digestion of TL in the presence of Stn1. D. Digestion of TL in the presence of Ten1. E. Digestion of TL in the presence of Stn1 and Ten1. In all cases the figures above the gel lanes represent the time of digestion in minutes, and assays were repeated twice to ensure consistency.



Figure 27. Protection of DNA ends from λ -exo by combinations of Cdc13, Stn1 and Ten1 mixed *in vitro*. DNA was digested by λ -exo in the absence and presence of 50nM of Cdc13, 150nM Stn1 and 50nM Ten1. A. Digestion of TL with Cdc13. B. Digestion of TL in the presence of Cdc13 and Stn1. C. Digestion of TL in the presence of Cdc13 and Ten1. D. Digestion of TL in the presence of Cdc13, Stn1 and Ten1. In all cases the figures above the gel lanes represent the time of digestion in minutes, and assays were repeated twice to ensure consistency.

4.9 RPA affords protection to telomeres and telomere-like structures from 5'-3' exonuclease catalysed resection.

RPA, the eukaryotic single stranded DNA binding protein, plays a key role in the processing of double strand breaks as it coats single stranded DNA, generated as intermediates during repair. RPA also has roles in helping to unwind DNA as well as recruiting checkpoint proteins ^{155,28}. *In vitro*, using *S. cerevisiae* proteins, RPA has been shown to inhibit resection at double strand breaks; intriguingly the opposite is observed with human systems ^{29,156}. To investigate the influence of RPA on resection at telomeres, the hydrolysis of TL and CL by λ -exo has been investigated in its presence.

As found previously both loops were rapidly degraded in the absence of added protein (figures 28a and c). In the presence of RPA both TL and CL were protected from degradation (figures 28b and d). However, unusually while a stable intermediate was formed during the digestion of TL, this was not the case for CL, for reasons that, at present, remain obscure (figure 28d). There also appears to be a difference in the degree of protection that RPA affords the telomere and control loops. The biggest difference between the behaviour of Cdc13 and RPA in these assays is the ability of RPA to protect the control looped substrate (figure 28d) for which Cdc13 offers no protection (figure 21d). Thus Cdc13 is specific in protecting telomeres against exonuclease-catalysed reactions whereas RPA shields all sequences including those that arise a consequence of double strand breaks. Furthermore RPA appears to offer more protection to TL (figure 28b) than the isolated DBD of Cdc13 (figure 22b), although any differences between Cdc13 and RPA are less marked (compare figures 21b and 28b).





Figure 28. Protection of DNA ends from λ -exo by RPA. DNA was digested by λ -exo in the absence and presence of RPA. A. Digestion of TL in the absence of RPA. B. Digestion of TL in the presence of RPA. C. Digestion of CL in the absence of RPA. D. Digestion of CL in the presence of RPA. In all cases the figures above the gel lanes represent the time of digestion in seconds, and assays were repeated twice to ensure consistency.

4.10 RPA binds tightly to 3' overhang structures

To confirm that the shielding effect of RPA against resection by λ -exo on the TL and CL substrates was due to a protein:DNA interaction EMSAs were performed. RPA bound strongly to TL and CL over the concentration range of RPA used in this assay. Furthermore 10nM TL and CL was bound by ~10nM RPA; suggesting sub-10nM K_D values, as found previously with Cdc13 (figures 29a and b). Collectively, these results suggest that the shielding effect of RPA against resection by λ -exo was due to a specific and tight binding of the proteins to the 3' overhang of the TL and CL substrates.



Figure 29. DNA binding properties of RPA. The interactions of RPA with TL and CL were investigated using EMSA. A. Binding of RPA to TL B. Binding of RPA to CL. The numbers above the gel lanes represent protein concentration in nM, and assays were repeated twice to ensure consistency.
4.11 RPA and Cdc13 specifically protect TL substrates from resection over extended time points

RPA appeared to offer marginally more protection to TL than CL over the 270s time points (compare figure 28b and figure 28d), and therefore like Cdc13 may offer specific protection to telomere ends. Therefore any selectivity was investigated by extending the hydrolysis time to 50 minutes. A λ -exo digestion of TL, carried out for longer times in the presence of Cdc13 or RPA, confirmed that the two proteins offered about similar protection (compare figures 30b and 30d). Expectedly, Cdc13 offered no protection to the CL substrate (figure 30a), however surprisingly, the level of protection provided by RPA to CL was poor (figure 30c) compared to its protection of TL (figure 30d) over the extended time points.



Figure 30. Protection of DNA ends from λ -exo by RPA and Cdc13 over extended time points. DNA was digested by λ -exo in the absence and presence of 50nM RPA or Cdc13. A. Digestion of CL in the presence of Cdc13. B. Digestion of TL in the presence of Cdc13. C. Digestion of CL in the presence of RPA. D. Digestion of TL in the presence of RPA. In all cases the figures above the gel lanes represent the time of digestion in minutes, and assays were repeated twice to ensure consistency.

4.12 The DNA binding domain of Cdc13 is able to out-compete RPA for binding to telomere ends

CST is proposed to be involved in inhibiting activation of the G2/M checkpoint whereas RPA is proposed to contribute to it. To investigate whether Cdc13 can outcompete RPA at telomere ends a competition assay was carried out with DBD and RPA. DBD was chosen for this assay rather than Cdc13 because there was a large size difference compared to RPA meaning the DNA-DBD and DNA-RPA bands were well separated during EMSA analysis.

Firstly, an RPA-TL or RPA-CL complex was created using 50nM RPA and 10nM of either TL or CL. DBD was then added in incrementally increasing concentrations (figure 31). When DBD and RPA were present at equimolar concentrations with the TL substrate, there was a significantly higher proportion of the DBD-TL complex formed than the RPA-TL complex (figure 31a). In addition when DBD was present at half the concentration of RPA the proportion of DBD-TL and RPA-TL complexes was roughly the same. In contrast, when DBD and RPA were mixed with the CL substrate, none of the concentrations of DBD used resulted in the formation of the DBD-CL complex, and only the RPA-CL complex was formed (figure 31c).

The inverse experiment was also carried out by creating a DBD-TL complex using 50nM DBD and 10nM of TL (figure 31). RPA was then added in incrementally increasing concentrations. When DBD and RPA were present at equimolar concentrations both the RPA-TL and DBD-TL complexes were formed although more of the DBD-TL complex was present (figure 31b). As expected mixing CL (10nM) with DBD (50nM) did not result in a protein-DNA complex and addition of RPA to the mixture resulted in formation of an RPA-CL complex (figure 31d).



Figure 31. DNA binding competition between Cdc13 DBD and RPA. The relative affinities of Cdc13 DBD and RPA for nucleic acids were probed by challenging an initial protein-DNA complex with a competitor protein. Analysis was by EMSA. A. Challenging a TL-RPA complex with Cdc13 DBD. B. Challenging a TL-Cdc13 DBD complex with RPA. C. Challenging a CL-RPA complex with Cdc13 DBD. D. Challenging a CL-Cdc13 DBD complex with RPA. In all cases the 10nM nucleic acid and 50nM of the initially added protein were used. The figures above the gel lanes represent the concentration of the added challenger protein in nM.

4.13 Discussion

Both RPA and the putative CST complex are DNA binding proteins with high affinity for single stranded DNA ^{87; 118; 152}. CST is proposed to bind to telomere G-rich 3' overhangs with high affinity and specificity ^{112; 137}, whereas RPA is non-specific ¹¹⁸. Furthermore, recent work has highlighted the structural, biochemical and functional overlap between the two proteins ^{108; 117}. Both proteins bind to the 3' overhang of telomeres and appear to have similar structures and overlapping functions, and all three subunits of CST are proposed to be involved in protecting the 5' recessed strand from resection^{79; 100; 106}. To probe the relative contributions of CST and RPA in preventing telomere resection, a telomere protection assay was established using looped oligonucleotides resembling a telomere end and a 5' to 3' nuclease processed double strand break. It was hoped that by carrying out *in vitro* reactions with an Exonuclease I analog, λ -exo, the degree of protection provided by RPA and the subunits of CST could be determined.

4.13.1 Cdc13 but not Stn1 or Ten1 protect telomere ends from resection by λ -exo

Cdc13 recruits telomerase to telomere ends ⁷⁶ to add telomere repeats to the 3' overhang, and is also proposed to be involved in tethering lagging strand synthesis to telomerase activity to ensure complete replication of the telomere end ⁹⁷. Cdc13 is also proposed to protect the 5' recessed strand of telomere from resection by powerful 5' to 3' exonucleases including Exo1 ^{86; 100}, although the biochemical mechanism of this protection has yet to be determined. Stn1 is proposed to act as a negative regulator of telomerase activity by competing with the telomerase subunit Est1 for binding to Cdc13 ⁷⁹. Furthermore Stn1 also interacts with aspects of the lagging strand machinery ⁹⁸. The role of Ten1 is poorly understood. However temperature-sensitive mutations of Cdc13, Stn1 and Ten1 lead to the accumulation of single stranded DNA at the telomere suggesting that they important in telomere end protection ^{79; 100; 106}. The generation of single stranded DNA leads to activation of the G2/M checkpoint to allow this "damage" to be repaired.

While the role of Cdc13 is protecting the telomere from exonuclease resection has been demonstrated using genetic techniques, it has not yet been demonstrated

biochemically. We therefore tested its ability to protect looped oligonucleotides resembling telomere ends. Interestingly, it was found that Cdc13 strongly inhibited resection on the 5' recessed strand of the telomere substrate but not the non-telomere control. This "specificity" is likely granted by the specific binding of Cdc13 to the G-rich 3' overhang found on the telomere but not the non-telomere control. Furthermore the isolated DNA binding domain of Cdc13 is able to provide protection from resection at telomeres but the full length protein is noticeably more efficient.

However, these experiments support the notion that the DBD is the core of the Cdc13 protein that is surrounded by other OB folds which mediate protein-protein interactions and enable Cdc13 to act as a nucleating centre for the formation of the telomere cap ¹¹⁰. Furthermore, crystal structures of the DNA binding domain suggest it possesses an extended β 2- β 3 loop which enables the high affinity binding to telomere DNA ¹¹¹. The simplest explanation for the observed inhibition of resection is steric hindrance; following binding of Cdc13 access of exonucleases to the 5' recessed strand is suppressed. It is likely that Cdc13 (924 amino acids), which considerably larger than the single OB fold of around 200 amino acids that forms the DBD, provides a much greater degree of steric hindrance explaining is enhanced potency.

In contrast to Cdc13; Stn1 and Ten1 show poor affinity for ssDNA and do not inhibit 5' to 3' resection at telomere ends. Furthermore the affinity demonstrated by Stn1 and Ten1 for telomere ssDNA is much weaker than the affinity shown by Cdc13 for non-telomere ssDNA. It is therefore unlikely that Stn1 and Ten1 bind telomere ends independently of Cdc13. However, since overexpression of Stn1 and Ten1 can rescue the lethality of *cdc13* Δ cells ¹⁴², this begs the question of how these proteins are able to influence telomere capping in the absence of Cdc13. One possibility is that they are recruited to telomere ends by other high affinity ssDNA binding proteins to help modulate telomerase extension and C-strand synthesis ¹¹⁶.

4.13.2 RPA protects telomere substrates from resection by λ -exo

In addition to the well-established roles of RPA in DNA replication and repair ¹¹⁸, it is also becoming increasingly evident that RPA plays key roles in telomere homeostasis. It is proposed to be involved in telomere replication ^{124; 125} and providing access to

telomerase ¹²³, functions also classically associated with Cdc13. It has also been suggested that the purported CST complex is an RPA-like complex, based on structural similarities ¹⁰⁸ and comparative gel shifts ¹¹⁷. Given their similarities, we investigated whether RPA could modulate nuclease resection on the 5' recessed strand at telomeres, similarly to Cdc13.

It was found that RPA inhibited resection of the 5' recessed strand on both the telomere loop and the non-telomere control, and was more effective at inhibiting resection at the telomere than the DNA binding domain of Cdc13, but similar to the full-length Cdc13. Therefore, for both RPA and Cdc13 the specificity of binding translates into specificity of protection. While these experiments are not quantitative, inspection of the gels shows that Cdc13 and RPA differ very little in their ability to protect telomeres from resection by nucleases.

4.13.3 Cdc13 outcompetes the RPA heterotrimer for binding to telomere ssDNA

Both Cdc13 and RPA bind to ssDNA at the telomere, however they have contrasting cellular consequences. While the binding of Cdc13 leads to recruitment of telomere-specific factors, maintenance of the telomere end and continued cell division, binding of RPA leads to recruitment of the DNA damage checkpoint machinery leading to cell cycle arrest ⁴⁴. Therefore we investigated whether Cdc13 could out-compete RPA at telomere ends *in vitro*, and perhaps thereby define the chromosome end as being distinct from a double strand break.

Expectedly the DBD of Cdc13 was unable to out-compete RPA for binding to the nontelomere control, since *in vivo* this might lead to the establishment of telomeres at chromosomal locations other than the chromosome end. Furthermore when RPA and DBD were present at equimolar concentrations with the telomere loop, the DBD-TL complex was dominant and only a two-fold excess of RPA was required for equal binding. Thus although Cdc13 binds more strongly to telomeres than RPA ^{112; 157}, the ratio required for equal binding to the telomere loop is rather small. This suggests that the predominance of Cdc13 at telomere ends cannot fully be explained by its affinity for telomere G-overhangs alone, particularly since RPA is superabundant in the cell ¹⁵⁸.

4.14 Further work

This chapter has demonstrated using biochemical techniques that both Cdc13 and the RPA heterotrimer can inhibit resection of the 5' recessed strand of telomere loop substrates, by binding tightly to the 3' overhang. Furthermore, this study was not able to demonstrate using *in vitro* techniques that Stn1 and Ten1 play a role inhibiting resection at telomere ends.

Given that I was not able to demonstrate that Stn1 and Ten1 are able to inhibit resection at telomere ends using this *in vitro* assay, and that genetic work suggests strongly that *STN1* and *TEN1* play key roles in inhibiting the generation of single stranded DNA at the telomere end ^{79; 106}, further experimental work will be necessary to determine the biochemical nature of this protection. It is possible that the two proteins do not exhibit strong binding activities on their own, and therefore reconstitution of the CST complex or Stn1-Ten1 subcomplex may be illuminating. However my attempts to reconstitute this complex *in vitro* were unsuccessful and so further work will be dependent on successful purification of these proposed complexes.

Furthermore, since the EMSAs carried out in this study were carried out using 10nM DNA, this precludes the quantitative determination of the binding constant (K_D). Therefore more sensitive techniques such as 32P labelling could be used to accurately determine K_D values for RPA and Cdc13 binding to the telomere loop substrate and non-telomere control.

Finally since all of the assays above were carried out with a commercially available exonuclease, λ -exo, it would be interesting to repeat these experiments using *S. cerevisiae* Exo1, which unfortunately was not available for these experiments because of the technical difficulty in purifying it. Comparing the protection assays carried out with λ -exo with those carried out with Exo1 may allow identification of specific protein-protein interactions that influence resection at telomeres and elsewhere in the genome.

5. The role of Cdc13 and Stn1 in telomere end protection: A genetic investigation

5.1 Background

The proposed CST complex (Cdc13-Stn1-Ten1) plays a key role in telomere end protection. Thermolabile mutations in any of the "subunits" demonstrated that when their function is disrupted there is accumulation of ssDNA at the telomere end and activation of the Rad9 and Mec3-dependent G2/M checkpoints ^{79; 83; 106}. Furthermore, yeast-2-hybrid data which showed that the three subunits interacted was used as further evidence that they behaved as a complex ¹⁰⁶; however such a complex has yet to be reconstituted biochemically ¹⁰⁹.

Cdc13 has been extensively biochemically and genetically characterised and is known to bind *S. cerevisiae* telomere G-overhangs with high affinity and specificity through its third OB fold (the DNA binding domain) ^{110; 152}. Genetic work has demonstrated that ssDNA accumulates in *cdc13-1* mutants as a result of exonuclease activity, in particular Exo1 ⁸⁶. Furthermore, it has been found that *cdc13-1* mutants can continue to divide at non-permissive temperatures if aspects of the checkpoint machinery are removed ⁸³. However, these cells continue to accumulate ssDNA, and eventually telomeres become critically short and cells undergo programmed cell death ⁸⁸.

The two smaller "subunits" are, however, comparatively understudied. The larger of these two subunits, Stn1, plays roles in three major telomere maintenance pathways. Firstly, similarly to Cdc13 and Ten1, Stn1 is proposed to play an integral role in telomere capping, preventing the telomere from being recognised and processed as a double strand break. Secondly, Stn1, similarly to Cdc13, is a negative regulator of telomerase, since disruption of its function leads to telomere overextension ⁷⁹. Finally, Stn1 is proposed to be involved in promoting efficient lagging strand synthesis at the telomere in humans ¹⁵⁹, and interacts with the regulatory subunit of DNA polymerase α , Pol12 ⁹⁸.

Not only is Stn1 proposed to make a contribution to telomere capping alongside Cdc13, it is also proposed to cap telomeres independently. Overexpression of the N-terminal region of Stn1 and its partner Ten1 allows cells to grow in the absence of

Cdc13 ¹⁴². Furthermore deletion of aspects of the DNA damage machinery, and the nonsense-mediated mRNA decay pathway can allow growth of *cdc13* Δ cells; however similar interventions cannot rescue *stn1* Δ or *ten1* Δ cells, leading to the suggestion that the two smaller subunits of the CST complex could be more important than Cdc13 ¹¹⁶.

Moreover, given that the biochemical data from Chapter 3 and 4 of this thesis seemed to suggest that Stn1 binds to telomere overhangs poorly, and does not protect telomeres from 5' to 3' resection *in vitro*, I wanted to determine if *STN1* played an analogous role to *CDC13* at telomeres. To do this SGA and QFA was carried out using the thermolabile mutation *stn1-13* and comparisons made with the genetic interactions demonstrated by *cdc13-1*. It was also hoped that such comparisons would help to identify new genes involved in telomere capping.

5.2 Carrying out the *stn1-13* SGA and QFA

5.2.1 Creation of the stn1-13 strain

It was decided to carry out a genetic screen with a temperature-sensitive allele of *STN1*, which one of the two smaller subunits of the putative CST complex. This subunit was chosen for investigation because biochemical investigation of the three subunits of CST had suggested that Stn1 and Ten1 were not capable of directly protecting telomere ends from resection. Therefore it was thought that genetic investigation might further elucidate how Stn1 is able to inhibit the generation of single stranded DNA at telomeres. Out of the two subunits *STN1* was chosen because a temperature-sensitive allele of this gene was available, *stn1-13⁷⁹*, and attempts to create a temperature-sensitive *TEN1* allele had failed.

In order to create a strain that was suitable for the SGA and QFA process it was necessary to incorporate a *LEU2* marker upstream of *stn1-13*. To do this a ~500bp region between *RAD55* and *SED1* upstream of *STN1* containing a natural unique restriction site (*BmgBI*) was amplified by PCR using high fidelity Phusion polymerase using primers M3501 and M3502 which incorporated *BamHI* restriction sites at their 5' ends. This PCR product had A-overhangs added by post-PCR treatment with Taq polymerase, and was subsequently cloned into PCR4-TOPO (Life Technologies). The PCR product was then subcloned into the *BamHI* site of the yeast integrative vector pDL1241 which contained the *LEU2* marker to create pDL1600. Successful TOPO and sub-cloning was confirmed by dropping out the cassette from the constructs using *BamHI*. Following successful cloning, pDL1601 was linearized using BmgBI corresponding to the natural unique restriction site (figure 32a). This linearized plasmid was then purified using silica membrane columns (QIAGEN).

Following successful purification of the linearized pDL1601, it was transformed into a *stn1-13* strain taken from the temperature sensitive library using the Lithium Acetate transformation protocol (see 2.14.3). Successful integration of the construct was confirmed by growth on –leucine. Subsequently one of these transformants was transformed using a hygromycin marker (*HPH*) flanked with its promoter and terminator regions cut out from pDL1045 using the restriction enzymes *Bglll* and *Sacl*

(figure 32b). Since the promoter and terminator regions are identical to those used by the *G418* marker located downstream of *stn1-13* in this strain, homologous recombination replaced the *G418* marker with *HPH* following transformation.

Finally, to confirm that the *HPH* and *LEU2* markers had integrated in the correct genome locations (immediately down and upstream of *stn1-13* respectively) and to create the final strain for our SGA and QFA; the transformed strain was mated with DLY7329. Diploids were then sporulated and tetrads analysed by random spore analysis (figure 32c). Correct integration of the selective markers was confirmed by cosegregation of temperature-sensitivity, growth on –leucine and HPH media. Spore analysis confirmed that 38/40 germinated haploids that were temperature sensitive also grew on –LEU and HPH suggesting strongly that they were linked to *stn1-13*. An appropriate strain (DLY8766) was then picked to carry out the screen.

5.2.2 Synthetic Genetic Array (SGA)

DLY8766 was mated in 1536 format with the deletion mutant array (DMA), diploids selected and sporulated and five stages of meiotic progeny selection were carried out to create $stn1-13 xxx\Delta$ mutants (figure 33). The fitness of these strains was then determined and plotted against the fitness of the DMA grown at the same temperature in the absence of the ts allele. Significant deviations from the line of best fit are judged to be interactions, with those points above the line being fitter than the equivalent strain from the DMA and those below the line being less fit (figure 34).

Generally during SGA mutants containing deletions of genes in very close proximity to query gene (in this case *STN1*) are difficult to make, because it is very unlikely that a recombination event during meiosis would be able to separate such closely linked genes. This means that these final strains often appear sicker than you would expect them to be, and so appear below the SGA line of best fit, or are dead. This can be used as an extra level of quality control to ensure that the strain is correct.

The *stn1-13* SGA showed that *RAD55, SED1* and *RRP8* (which are all in close physical proximity to *STN1* on chromosome 4) demonstrated strong synthetic sick phenotypes (figure 34). This confirmed that *STN1* was genetically linked to its selection markers, and so was suitable for QFA.



Figure 32. Creating a *stn1-13* **strain suitable for the SGA and QFA process.** A. A vector diagram of pDL1600 illustrating relevant restriction sites, yeast and bacterial selective markers and the origin of replication. B. A vector diagram of pDL1241 illustrating relevant restriction sites, yeast and bacterial selective markers and the origin of replication. C. A tetrad dissection sheet showing how colonies taken from 12 randomly selected spores grown on a range of selective media and after mating with strains DLY26 and DLY2440 to test for mating type.



Figure 33. The SGA workflow. Lawns of stn1-13 were created and pinned in 1536 format and grown for 2 days at 20°C on YPD supplemented with NatMX (YPD N). These colonies were then pinned on top of deletion library strains in 1536 format on YPD. The guery and library strains were then left for 12-24 hours to mate at 23°C and diploids were selected by growing the strains on YPD supplemented with the antibiotics G418 and NatMX. Following diploid selection, diploids were sporulated on minimal sporulation media (ESM) supplemented with G418 over 5 days at 23°C. Following sporulation, spores were transferred to synthetic media containing dextrose supplemented with canavanine and thiolysine (SDM rhk CT) and grown for 2 days at 20°C. After two days these strains were then transferred to another SDM rhk CT plate and then grown again for 2 days at 20°C. Strains were then transferred to synthetic media supplemented with canavanine, thiolysine and G418 (SDM rhk CTG) and grown for another 2 days at 20°C. Strains were then transferred to synthetic media supplemented with canavanine, thiolysine, G418, HPHMX but without leucine and grown for another 2 days at 20°C. Finally strains were then transferred to synthetic media supplemented with canavanine, thiolysine, G418, HPHMX and NatMX but without leucine and grown for a final 2 days at 20°C. These stages are illustrated in the figure (picture provided by Eva Holstein 2014).



Fitness (F) of yfg Δ ura 3Δ at 20°C (nAUC)

Figure 34. Fitness of *stn1-13* **strains at 20°C.** *stn1-13* strains with different gene deletions/knockdowns were grown at 20°C over the course of several days. Coloured points located above the grey line of best fit represent gene deletions or knockdowns that suppress the temperature-sensitivity of *stn1-13* strains. Coloured points located below the grey line of best fit represent gene deletions or knockdowns that enhance the temperature-sensitivity of *stn1-13* strains. Out of the highlighted genes *STN1* is part of the DAmP collection as it is an essential gene, while *RRP8*, *RAD55* and *SED1* are non-essential and therefore gene deletions.

5.2.3 Quantitative fitness analysis (QFA)

stn1-13 xxx strains were grown to saturation in liquid media and then spotted in 384 format onto solid media. The fitness of these strains was then determined by analysis of sequential photographs of spotted yeast. The standard way of calculating the fitness during quantitative fitness analysis is using the generalized logistic model; however an important aspect of this model is that it assumes that the inoculum density of each spot is the same. However, due to a technical problem during the course of these experiments the Beckman robot developed an error whereby it inoculated slightly different quantities of yeast into each of the four quadrants of each 384 plate (which is sourced from four 96-well plates). Therefore it was decided to use the numerical area under the curve (nAUC) to calculate fitness as this is a model-free way of calculating fitness and therefore is less susceptible to this technical noise.

In order to ensure that no plates had been incorrectly labelled, inoculated or photographed pairwise fitness comparisons were made between replicates of a single master plate grown on the same media and under the same conditions. Figure 35b shows a histogram of the correlation coefficients obtained from each pairwise comparison. However notably although the majority had correlations above 0.5 a significant minority were below 0.5 suggesting there was significant variability between replicates which may have been caused by inaccurate spotting by the robot. Furthermore, in order to ensure that all yeast were behaving similarly on all plates (which could be affected by variations in growth conditions such as temperature or media preparation) box plots were used to compare growth of all replicates of a given master plate with all the other master plate replicates (figure 35a). Interestingly the box plots indicated that there was significant variability between the plates. Notably, however this variability followed a pattern from the first plate to the last, suggesting that it could be a technical artefact such as a pinning error. Growth curves of each spot (which are used to calculate fitness) were also visually inspected to ensure the data fitted the logistic growth model well as deviations could indicate issues with photography or image analysis. The data of each curve appeared to fit the growth curves well.

The fitness of the query strains ($stn1-13 xxx\Delta$) was then plotted against the fitness of the corresponding strain from the DMA (the control strains). Significant deviations from the line of best fit were judged to be interactions, with those points above the line indicating gene deletions that suppress the temperature-sensitive phenotype of ts-allele and those points below the line indicating gene deletion that enhance this phenotype.





A

В

Figure 35. Variations in fitness between replicates and plates. A. Box plots indicating the distribution of fitnesses across all replicates of a given master plate. Notches in the boxes are an indication of significance of difference, with overlapping notches indicating no significant difference. B. A histogram of pairwise fitness comparisons between all replicates of a single plate grown under the same conditions. Values above 0.5 are considered an acceptable correlation.

5.3 *exo1* Δ is a suppressor of the *stn1-13* temperature-sensitive phentotype

When telomeres are uncapped using the temperature-sensitive mutant *cdc13-1* it is thought that ssDNA is generated at the telomere-end by the action of three nuclease activities: Exo1, and two hypothetical nucleases: ExoX and ExoY. Genetic experiments established that ExoX has an activity dependent on Rad24 and the 9-1-1 complex, and ExoY could generate ssDNA in the absence of Exo1 and ExoX close to the telomere end ⁹⁰. In the *cdc13-1* genome-wide screen *rad24* Δ , *rad17* Δ , *ddc1* Δ and *exo1* Δ were identified as suppressors of *cdc13-1* ³⁷. Furthermore, *pif1* Δ , which codes for a 5' to 3' DNA helicase, was also found to be a suppressor of the *cdc13-1* phenotype and was proposed to have characteristics of ExoY since it is not dependent on Rad24/9-1-1 and it is proposed to be involved in resection close to the telomere end ⁸⁹.

exo1 Δ was identified as a strong suppressor of temperature-sensitivity in the *stn1-13* screen as judged by the large spot size of *stn1-13 exo1* Δ strains (figure 36a) and the larger area under most of the *exo1* Δ growth curves (figure 36b, c) as compared to the *stn1-13 his3* Δ strains. This resulted in the positioning of *exo1* Δ above the grey cloud of non-interacting genes in the *stn1-13* fitness plot (figure 36d). Furthermore, when *stn1-13 exo1* Δ mutants were generated in an alternative genetic background (W303) and spot tested they grew more strongly than *stn1-13* strains at 36°C (figure 36e). A similar suppression of the temperature sensitivity of *cdc13-1* was seen when *EXO1* was deleted at 27°C (figure 36f) and 30°C (figure 36g). This suggests that ssDNA generation in these mutants is might be partially dependent on Exo1.

The *stn1-13* screen also identified 2 members of the 9-1-1 complex (*RAD17, DDC1*) and the clamp loader (*RAD24*) as non-interacting genes, as judged by the comparable growth between *stn1-13* strains deleted for these genes and *stn1-13 his3* Δ strains (figure 36a). Furthermore there was a comparable area under the curve between most of 3 deletion strains and the *stn1-13 his3* Δ controls (figure 36b, c). This resulted in the positioning of *rad17* Δ , *rad24* Δ and *ddc1* Δ within the grey cloud of non-interacting genes (figure 36d). This is in stark contrast to the phenotype of *cdc13-1* strains deleted for these three genes which are substantially fitter than *cdc13-1 his3* Δ strains and located above the grey cloud of non-interacting genes at both 27°C (figure 36f) and

30°C (figure 36g). This suggests that generation of single stranded DNA at the telomeres of *stn1-13* mutants may be 9-1-1 and Rad24-independent.





stn1-13 rad17[~]





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J000244_033_029 Treatment: 33 Medium: SDM_rhlk_CTGNH Plate: 15

IIS3	HIS3	HIS3	[™] → K=0.0889	ні б К=0.0882	HIS3	HIS3
	r=8.8 g=0.0011 v=0.213 MDR=7.49 MDP=6.57 AUC=0.377 DT=3.2	r=11.1 g=0.0011 v=0.15 MDR=7.11 MDP=6.18 AUC=0.283 DT=3.38	<pre>r=12.7 g=0.0011 v=0.1 MDR=6.08 MDP=6.34 AUC=0.283 DT=3.94</pre>	$\begin{array}{c} r=11.3 \\ g=0.0011 \\ v=0.1 \\ MDR=5.4 \\ MDP=6.33 \\ AUC=0.263 \\ DT=4.44 \end{array}$	$\begin{array}{c} r = 10.6 \\ g = 0.0011 \\ v = 0.1 \\ MDR = 5.14 \\ MDP = 6.43 \\ AUC = 0.269 \\ DT = 4.67 \end{array}$	r=9.49 g=0.0011 v=0.1 MDR=4.57 MDP=6.37 AUC=0.239 DT=5.26
³ 4 ⁵ 6 ⁷ IIS3	$\begin{array}{c} 0 & 1 & 2 & 3 & 4 & 5 & 6 & 7 \\ & & & RAD24 \\ & & & \\$	$\int_{K=0.102}^{0} \int_{r=11.6}^{1} \frac{1}{2} \int_{K=0.102}^{3} \int_{R^{02C}}^{4} \int_{R^{02C}}^{5} \int_{R^{02C}}^{6} \int_{R^{02C}}^{7} \int_{R^{02C}}^{8} \int_{R^$	$\sum_{n=0}^{0} \sum_{k=0.0921}^{1} \sum_{r=6.04}^{2} \sum_{k=0}^{3} \sum_{k=0}^{4} \sum_{r=0}^{7} \sum_{k=0}^{7} \sum_{r=0}^{7} \sum_{k=0}^{7} \sum_{r=0}^{7} \sum_{k=0}^{7} \sum_{r=0}^{7} \sum_{k=0}^{7} \sum_{r=0}^{7} \sum_{k=0}^{7} \sum_{r=0}^{7} \sum_{k=0}^{7} \sum_{r=0}^{7} \sum_{r=0}^$	0 1 2 3 4 5 6 RAD52 [™] [™] [™] [™] [™] [™] [™] [™]	$ \begin{array}{cccccccccccccccccccccccccccccccccccc$	0 1 2 3 4 MRE1 K=0.025 r=0.605
	g=9e-04 v=0.73 MDR=6.91 MDP=6.79 AUC=0.387 DT=3.47	g=9e-04 v=0.166 MDR=8.62 MDP=6.82 AUC=0.374 DT=2.78	<pre> g=9e-04 v=0.361 MDR=6.84 MDP=6.68 AUC=0.335 DT=3.51 DT=3</pre>	<pre></pre>	General and a second se	g=0.0011 v=0.75 MDR=0.763 MDP=4.51 AUC=0.017 DT=25
³ ⁴ ⁵ ⁶ ⁷ R269C ⁶	0 1 2 3 4 5 6 7 PGM2 K=0.0831 r=4.15	$\begin{array}{c} 0 & 1 & 2 & 3 & 4 & 5 & 6 & 7 \\ \mathbf{PRM4} \\ \mathbf{F} = 0.0775 \\ \mathbf{F} = 3.76 \end{array}$	$\mathbf{PHB2}^{0} = \begin{bmatrix} K=0.131 \\ r=4.58 \end{bmatrix} \mathbf{K} = \begin{bmatrix} K=0.131 \\ r=$	$ \begin{array}{c} 0 & 1 & 2 & 3 & 4 & 5 & 6 \\ & & & \mathbf{PTK1} \\ & & & \\ & & & \\ & & & \\ & & & \\ & & & \\ & & & \\ & & & \\ & & & \\ & & & \\ & & & \\ & & & \\ & & & \\ & & & \\ & & & \\ \end{array} \right) $	7 0 1 2 3 4 5 6 7 MRE11 K=0.025 r=0.373	$\mathbf{R}^{0} = \begin{bmatrix} 0 & 1 & 2 & 3 & 4 \\ \mathbf{R}^{0} & \mathbf{R}^{2} & \mathbf{R}^{3} & \mathbf{R}^{4} \\ \mathbf{R}^{0} & \mathbf{R}^{0} & \mathbf{R}^{2} & \mathbf{R}^{2} \\ \mathbf{R}^{0} & \mathbf{R}^{0} & \mathbf{R}^{0} & \mathbf{R}^{0} \\ \mathbf{R}^{0} & \mathbf{R}^{0} & \mathbf{R}^{0} & \mathbf{R}^{0} \\ \mathbf{R}^{0} & \mathbf{R}^{0} & \mathbf{R}^{0} & \mathbf{R}^{0} \\ \mathbf{R}^{0} & \mathbf{R}^{0} $
	g=9e-04 v=0.755 MDR=5.73 MDP=6.53 AUC=0.31 DT=4.19	g=0.00103 v=0.832 MDR=5.23 MDP=6.24 AUC=0.287 DT=4.59	<pre> g=9e-04 v=0.925 MDR=6.51 MDP=7.18 AUC=0.504 DT=3.6 </pre>	- g=9e-04 v=0.652 - MDR=5.74 MDP=6.41 - AUC=0.283 DT=4.18	g=0.0011 v=0.75 MDR=0.47 MDP=4.51 - AUC=0.00784 DT=25	- g=9e-04 v=1.08 - MDR=6.1 MDP=6.72 - AUC=0.368 DT=3.93
³ 262W ⁶	$\sum_{K=0.0727}^{0} \sum_{r=3.5}^{1} \sum_{K=0.0727}^{2} \sum_{r=3.5}^{3} \sum_{r=1}^{4} \sum_{r=1}^{5} \sum_{r=1}^{6} \sum_{r=1}^{7} \sum_$	$\int_{K=0.101}^{0} \int_{r=4.46}^{1} \frac{1}{4} \int_{K=0.101}^{2} \frac{3}{4} \int_{K=0.101}^{4} \frac{5}{7} \int_{K=0.101}^{6} \frac{1}{7} \int_{K=0.101}^{1} \frac{1}{7} \int_{K=0.101}^{$	$ECM5 = \frac{1}{1} + \frac{1}{1}$	$ECM5 = \frac{1}{1} + \frac{1}{1}$	$\begin{bmatrix} 7 & 0 & 1 & 2 & 3 & 4 & 5 & 6 & 7 \\ MNT4 \\ \hline & & & \\ & & & & \\ & & & & \\ & & & \\ & & & & \\ & & & & \\ & & & & \\ & & & & \\ & & & & \\ & & & & \\ & & & & \\ & & & & \\ & & & & \\ & & & & \\ & & & & \\ & & & & \\ & & & & \\ & & & & \\ & & & & \\ & & & & \\ & & & & \\ & & & & & \\ & & & & \\ & & & & \\$	$\begin{bmatrix} 0 & 1 & 2 & 3 & 4 \\ 0 & 1 & 2 & 3 & 4 \\ NMD \\ \hline NMD \\ \hline K=0.171 \\ r=4.74 \end{bmatrix}$
	g=0.001 v=1 MDR=4.95 MDP=6.18 AUC=0.27 DT=4.85	g=9e-04 v=0.706 MDR=6.14 MDP=6.81 - AUC=0.378 DT=3.91	$ \begin{array}{c} g = 9e - 04 \\ v = 0.765 \\ MDR = 5.82 \\ MDP = 6.64 \\ - AUC = 0.335 \\ DT = 4.12 \end{array} $	g=9e-04 v=0.708 MDR=6.02 MDP=6.86 - AUC=0.387 DT=3.99	$ \begin{array}{c} g = 9e - 04 \\ v = 0.803 \\ MDR = 5.42 \\ MDP = 6.55 \\ - AUC = 0.309 \\ DT = 4.43 \end{array} $	$ \begin{array}{c} & g=9e-04 \\ & v=0.99 \\ & MDR=6.78 \\ & MDP=7.57 \\ & AUC=9.662 \\ & DT=3.54 \end{array} $
³ 130 ⁴ ⁵ ⁶ ⁷	0 1 2 3 4 5 6 7 TOS3 K=0.0814 r=4 12		$\begin{bmatrix} 0 & 1 & 2 & 3 & 4 & 5 & 6 & 7 \\ RAD50 \\ RE0.025 \\ r=2.9 \end{bmatrix}$	0 1 2 3 4 5 6 RAD9 K=0.0253 r=5.9	7 0 1 2 3 4 5 6 7 MNT4	K=0.0258
	g=9e-04 v=0.746 MDR=5.68 MDP=6.5 AUC=0.302 DT=4.23	g=9e-04 v=0.798 MDR=5.33 MDP=6.35 - AUC=0.271 DT-4.5	$\begin{array}{c} = & g=9e-04 \\ = & v=0.75 \\ = & MDR=3.73 \\ = & AUC=0.0874 \\ = & DT=6.43 \end{array}$	g=0.0011 v=0.1 MDR=2.06 MDP=4.52 - AUC=0.0497 DT=11.6	g=0.00106 v=0.826 MDR=5.18 MDP=6.28 AUC=0.303 DT=4.63	$\begin{array}{c} g=9e-04\\ v=1.39\\ MDR=3.87\\ MDP=4.84\\ - \\ AUC=0.0964\\ DT=6.21\end{array}$
3 4 5 6 7 TK1	0 1 2 3 4 5 6 7 YDR269C K=0.0848 r=2 80	0 1 2 3 4 5 6 7 RAD17 K=0.115 r=4.58	$ \begin{array}{c} & 0 & 1 & 2 & 3 & 4 & 5 & 6 & 7 \\ & & & & & & & & \\ & & & & & & & \\ & & & &$	$ \begin{array}{c} 0 & 1 & 2 & 3 & 4 & 5 & 6 \\ & & & & & & \\ & & & & & & \\ & & & & &$	7 0 1 2 3 4 5 6 7 ZRT3 $K=0.0806$ $r=2.60$ $K=0.0806$	$\sum_{k=0.0962}^{0} K=0.0962$
	$ \begin{array}{c} & = 3.69 \\ g = 9e - 04 \\ v = 0.628 \\ MDR = 5.2 \\ MDP = 6.56 \\ AUC = 0.298 \\ DT = 4.64 \end{array} $	$ \begin{array}{c} & = 4.38 \\ g = 9e - 04 \\ v = 0.979 \\ \hline MDR = 6.53 \\ MDP = 6.99 \\ - & AUC = 0.446 \\ \hline DT = 0.246 \\ \hline DT $	General and a second se	$\begin{array}{c} & = -4.03 \\ g = 9e - 04 \\ v = 0.583 \\ - & MDR = 6.17 \\ MDP = 6.76 \\ - & AUC = 0.359 \\ \hline \end{array}$	$\begin{array}{c} = & 1-3.09 \\ = & g=0.0011 \\ = & v=0.796 \\ = & MDR=5.09 \\ MDP=6.2 \\ = & AUC=0.294 \\ = & DT=4.70 \\ \end{array}$	$\begin{array}{c} & = -4.13 \\ g = 0.0011 \\ v = 0.698 \\ - & MDR = 5.65 \\ MDP = 6.45 \\ - & AUC = 0.355 \end{array}$
ۍ ۲				0		
³ ⁴ ⁵ ⁶ ⁷ ⁶ ⁷ ⁸ ^{807C06}	0 1 2 3 4 5 6 7 K=0.0782	0 1 2 3 4 5 6 7 ALD3 K=0.0915 K=0.0915	0 1 2 3 4 5 6 7 PHB2 ⁸ ^ο K=0.0795 ^{κο7C09}	0 1 2 3 4 5 6 ESBP6 K=0.0625	$\mathbf{EST1}^{100} = \mathbf{K} = 0.053$	0 1 2 3 4 MNT K=0.0819
	<pre></pre>	$ \begin{array}{c} r = 4.76 \\ g = 9e - 04 \\ v = 0.576 \\ MDR = 6.28 \\ MDP = 6.67 \\ - & AUC = 0.339 \end{array} $	$\begin{array}{c} & F=4.31 \\ g=9e-04 \\ v=0.633 \\ \hline MDR=5.77 \\ MDP=6.46 \\ - & AUC=0.292 \end{array}$	$\begin{array}{c} = 3.48 \\ g=0.00108 \\ v=0.87 \\ - & MDR=4.82 \\ MDP=5.85 \\ - & AUC=0.23 \end{array}$	<pre></pre>	$ \begin{array}{c} r = 4.38 \\ g = 9e - 04 \\ v = 0.65 \\ MDR = 5.89 \\ MDP = 6.51 \\ - & AUC = 0.303 \end{array} $
		DT=3.82	9 DT=4.16	0 _ DT=4.98	€ _ DT=5	
³ ⁴ ⁵ ⁶ ⁷ HS3	O 1 2 3 4 5 6 7 YDR269C κ=0.0715 K=0.0715	0 1 2 3 4 5 6 7 STD1 K=0.106	о 1 2 3 4 5 6 7 REH1 «В К=0.0804	0 1 2 3 4 5 6 CHK1 K=0.102	$\begin{bmatrix} 7 & 0 & 1 & 2 & 3 & 4 & 5 & 6 & 7 \\ EXO1 & EXO1 & & & & \\ \hline & & & & & & \\ \hline & & & & & &$	о 1 2 3 4 RCF К=0.0951
	$ \begin{array}{c} r=3.26 \\ g=0.0011 \\ v=0.803 \\ MDR=4.48 \\ MDP=6.02 \\ - & AUC=0.252 \end{array} $	$ \begin{array}{c} r = 4.55 \\ g = 0.0011 \\ v = 0.639 \\ MDR = 6.12 \\ MDP = 6.59 \\ - & AUC = 0.395 \end{array} $	$ \begin{array}{c} & r=3.97 \\ g=0.0011 \\ v=0.721 \\ \hline MDR=5.39 \\ MDP=6.19 \\ AUC=0.297 \end{array} $	$ \begin{array}{c} r = 4.84 \\ g = 9e - 04 \\ v = 0.827 \\ MDR = 6.79 \\ MDP = 6.83 \\ - & AUC = 0.4 \end{array} $	r=4.4 g=9e-04 v=0.888 MDR=6.24 MDP=7.17 AUC=0.492	$ \begin{array}{c} r=4.12 \\ g=0.0011 \\ v=0.739 \\ MDR=5.66 \\ MDP=6.43 \\ - & AUC=0.353 \end{array} $
******* ÷		DT=3.92	00 DT=4.45	0 0 0 0 0 0 0 0 0 0 0 0 0 0 0 0 0 0 0	DT=3.85	0 DT=4.24
³ ⁴ ⁵ ⁶ ⁷ PK3	O 1 2 3 4 5 6 7 YGL217C κ=0.0551	0 1 2 3 4 5 6 7 RAD50 K=0.0269	$ \begin{array}{cccccccccccccccccccccccccccccccccccc$	0 1 2 3 4 5 6 PNG1 K=0.0843	7 0 1 2 3 4 5 6 7 HAP4 5 6 7 6 7 HAP4 5 6 7 6 7 6 6 7 6 6 7 6 6 7 6 6 7 6 6 7 6 6 7 6 6 7 6 6 7 6 6 7 6 6 7 6	0 1 2 3 4 LYS1 K=0.001
	r=3.85 g=9e-04 v=0.73 MDR=5.2 MDP=5.94 AUC=0.203	$ \begin{array}{c c} r=2.58 \\ g=9e-04 \\ v=1.42 \\ MDR=3.68 \\ MDP=4.9 \\ - & AUC=0.0991 \end{array} $	r=3.8 g=0.0011 v=0.792 MDR=5.25 MDP=6.2 AUC=0.298	$ \begin{array}{c} r=3.81 \\ g=0.0011 \\ v=0.787 \\ MDR=5.26 \\ MDP=6.26 \\ AUC=0.31 \end{array} $	$ \begin{array}{c} r=3.11 \\ g=0.000961 \\ v=1.08 \\ MDR=4.4 \\ MDP=5.87 \\ - & AUC=0.205 \end{array} $	r=0 g=0.001 v=1 MDR=0 MDP=0 AUC=0
	DT=4.62	DT=6.53	0 DT=4.58	0 0 0 0 0 0 0 0 0 0 0 0 0 0 0 0 0 0 0	€ _ DT=5.46	OT=25
³ ⁴ ⁵ ⁶ ⁷ 0\$3	0 1 2 3 4 5 6 7 HAT1 K=0.0812	0 1 2 3 4 5 6 7 ARA1 K=0.106	0 1 2 3 4 5 6 7 K NS1 [№] [№] K=0.0836	0 1 2 3 4 5 6 HIS3 K=0.103	7 0 1 2 3 4 5 6 7 PHB2 ^{C10} K=0.0781	0 1 2 3 4 HIS3
	$ \begin{array}{c} r = 4.09 \\ g = 9e - 04 \\ v = 0.729 \\ MDR = 5.62 \\ MDP = 6.5 \\ - \\ AUC = 0.3 \end{array} $	$ \begin{array}{c} r=4.72 \\ g=9e-04 \\ v=0.592 \\ MDR=6.3 \\ MDP=6.87 \\ - & AUC=0.389 \end{array} $	$ \begin{array}{c} & r=3.64 \\ g=0.001 \\ v=0.904 \\ MDR=5.12 \\ MDP=6.38 \\ - \\ AUC=0.307 \end{array} $	$\begin{array}{c} & r=4.42 \\ g=0.0011 \\ v=0.655 \\ - & MDR=5.96 \\ MDP=6.56 \\ - & AUC=0.384 \end{array}$	$ \begin{array}{c} & r=4 \\ g=9e-04 \\ v=0.721 \\ MDR=5.47 \\ MDP=6.44 \\ - \\ AUC=0.286 \end{array} $	$ \begin{array}{c} r=4.19 \\ g=0.0011 \\ v=0.713 \\ MDR=5.74 \\ MDP=6.56 \\ - & AUC=0.383 \end{array} $
		DT=3.81	9. DT=4.69	0 DT=4.02	- 9: - DT=4.39	DT=4.18
³ ⁴ ⁵ ⁶ ⁷ PS6	о 1 2 3 4 5 6 7 PRM4 К=0.0836	0 1 2 3 4 5 6 7 RAD24 K=0.0918	о 1 2 3 4 5 6 7 RAD52 «В К=0.0253 К=0.0253	0 1 2 3 4 5 6 HIS3 K=0.0706	$\begin{bmatrix} 7 & 0 & 1 & 2 & 3 & 4 & 5 & 6 & 7 \\ KHA1 \\ K=0.0743 \end{bmatrix} K=0.0743$	O 1 2 3 4 ARA K=0.0838
	$ \begin{array}{c} r = 4.07 \\ g = 9e - 04 \\ v = 0.716 \\ MDR = 5.58 \\ MDP = 6.54 \\ - \\ AUC = 0.307 \end{array} $	$ \begin{array}{c} r=4.13 \\ g=9e-04 \\ v=1.18 \\ MDR=5.91 \\ MDP=6.67 \\ - \\ AUC=0.357 \end{array} $	$ \begin{array}{c} & r=0 \\ g=0.0011 \\ v=0.1 \\ MDR=0 \\ MDP=4.52 \\ - \\ AUC=2.6e-18 \end{array} $	$\begin{array}{c} r=3.57\\ g=0.00101\\ v=0.896\\ MDR=4.99\\ MDP=6.13\\ - & AUC=0.26\end{array}$	$\begin{array}{c} r=3.33 \\ g=0.00103 \\ v=0.859 \\ MDR=4.64 \\ MDP=6.17 \\ - \\ AUC=0.265 \end{array}$	r=3.88 g=9e-04 v=0.835 MDR=5.42 MDP=6.54 - AUC=0.309
		$\mathbf{D} = \begin{bmatrix} \mathbf{D} \mathbf{I} = 4.06 \\ \mathbf{D} \end{bmatrix}$	0. D1=25	0		
³ ⁴ ⁵ ⁶ ⁷ L109C _{R12C06}	$ \begin{array}{c} 0 & 1 & 2 & 3 & 4 & 5 & 6 & 7 \\ YDL109C \\ K=0.0934 \\ $	0 1 2 3 4 5 6 7 HAT1 K=0.0967	HIS3 = K=0.078	0 1 2 3 4 5 6 ECM5 K=0.0957	$ \begin{array}{cccccccccccccccccccccccccccccccccccc$	о 1 ² ³ ⁴ YPK К=0.0978
	$ \begin{array}{c} & 1-4.27 \\ g=9e-04 \\ v=0.695 \\ MDR=5.85 \\ MDP=6.7 \\ - \\ AUC=0.345 \end{array} $	$ \begin{array}{c} & = -4.29 \\ g = 9e - 04 \\ v = 0.722 \\ MDR = 5.92 \\ MDP = 6.75 \\ - & AUC = 0.359 \\ DT = 4.00 \end{array} $	$ \begin{array}{c} & = 3.83 \\ g = 9e - 04 \\ v = 0.836 \\ MDR = 5.38 \\ MDP = 6.44 \\ - \\ AUC = 0.289 \\ DT = 4.46 \end{array} $	$\begin{array}{c} & = & 1 = 3.30 \\ g = 0.00106 \\ v = 0.824 \\ \hline & MDR = 5.52 \\ MDP = 6.5 \\ - & AUC = 0.356 \\ \hline & DT = 4.05 \end{array}$	$\begin{array}{c} & -4.21 \\ g = 9e - 04 \\ v = 0.757 \\ \hline MDR = 5.83 \\ MDP = 6.66 \\ - AUC = 0.339 \\ \hline DT = 4.44 \end{array}$	$\begin{array}{c} & = -4.08 \\ g = 9e - 04 \\ v = 0.804 \\ - & MDR = 5.71 \\ MDP = 6.76 \\ - & AUC = 0.363 \end{array}$
3 4 5 6 7 LB2	0 1 2 3 4 5 6 7 K=0.08 K=0.08	0 1 2 3 4 5 6 7 NMD2 K=0.135 K=0.135	0 1 2 3 4 5 6 7 GPH1 ¹⁰ ¹⁰ ¹⁰ ¹⁰ ¹¹ ¹² ¹² ¹³ ¹⁵ ¹⁵ ¹⁵ ¹⁵ ¹⁵ ¹⁵ ¹⁵ ¹⁵ ¹⁵	0 1 2 3 4 5 6 EMI5 K=0.0482	$ \begin{array}{cccccccccccccccccccccccccccccccccccc$	R = 0.0771
	$ \begin{array}{c} & = 3.03 \\ & = 9e - 04 \\ & v = 1.05 \\ & MDR = 5.17 \\ & MDP = 6.47 \\ & AUC = 0.298 \\ & DT = 4.04 \end{array} $	- g=9e-04 v=1.22 MDR=6.12 MDP=7.23 - AUC=0.521	$ \begin{array}{c} & = 3.62 \\ & = 9e - 04 \\ & v = 0.914 \\ & MDR = 5.39 \\ & MDP = 6.57 \\ & - \\ & AUC = 0.318 \end{array} $	$\begin{array}{c} & = 3.1 \\$	$ \begin{array}{c} = & 1=3.47 \\ = & g=0.001 \\ v=1 \\ MDR=4.91 \\ MDP=6.31 \\ = & AUC=0.291 \\ DT=4.02 \end{array} $	$\begin{array}{c} = 3.67 \\ g = 9e - 04 \\ v = 0.929 \\ \hline MDR = 5.18 \\ MDP = 6.42 \\ - & AUC = 0.285 \\ \hline DT = 4.04 \end{array}$
			0. DT=4.46	0 DT=5.4	€ - 0	
3 4 5 6 7 HB2 R14C06	0 1 2 3 4 5 6 7 RAD17 K=0.101	0 1 2 3 4 5 6 7 LYP1 K=0.0375	$ \begin{array}{c} 0 & 1 & 2 & 3 & 4 & 5 & 6 & 7 \\ YDR262W \\ K=0.0796 \\ K=0.0796 \end{array} $ R14C09	0 1 2 3 4 5 6 FIT2 ^{R14C} ^{R14C}	$ \begin{array}{cccccccccccccccccccccccccccccccccccc$	ο 1 2 3 4 PET1 κ=0.0816
	$ \begin{array}{c} & F=4.29 \\ g=9e-04 \\ v=1.09 \\ MDR=6.14 \\ MDP=6.81 \\ - \\ AUC=0.392 \end{array} $	$ \begin{array}{c} F=0.237 \\ g=9e-04 \\ v=1.5 \\ MDR=0.34 \\ MDP=5.38 \\ - \\ AUC=0.00409 \end{array} $	$\begin{array}{c} & F=3.74 \\ g=9e-04 \\ v=0.919 \\ MDR=5.28 \\ MDP=6.47 \\ - \\ AUC=0.295 \end{array}$	$\begin{array}{c} & = 4.24 \\ g = 9e - 04 \\ v = 0.789 \\ MDR = 5.91 \\ MDP = 6.71 \\ - \\ AUC = 0.353 \end{array}$	$\begin{array}{c} = 3.87 \\ g = 9e - 04 \\ v = 0.869 \\ MDR = 5.43 \\ MDP = 6.45 \\ - \\ AUC = 0.292 \end{array}$	$\begin{array}{c} = 3.79 \\ g=0.00103 \\ v=0.894 \\ MDR=5.31 \\ MDP=6.31 \\ - \\ AUC=0.305 \end{array}$
		DT=25	0 DT=4.55	0 DT=4.06	- 0; - DT=4.42	0 DT=4.52
³ ⁴ ⁵ ⁶ ⁷ R269C _{R15C06}	0 1 2 3 4 5 6 7 FIT2 K=0.0967	0 1 2 3 4 5 6 7 ALD3 K=0.099	0 1 2 3 4 5 6 7 STD1 K=0.0981	0 1 2 3 4 5 6 ESBP6 K=0.0973	7 0 1 2 3 4 5 6 7 GPH1 K=0.106	о 1 ² ³ ⁴ РЯМ К=0.0966
	$\begin{array}{c ccccccccccccccccccccccccccccccccccc$	$ \begin{array}{c c} & r=4.71 \\ g=0.0011 \\ v=0.571 \\ & MDR=6.15 \\ & MDP=6.49 \\ & AUC=0.367 \end{array} $	<pre> r=5.56 g=9e-04 v=0.421 MDR=6.72 MDP=6.77 AUC=0.358 r=5.56 g=9e-04 v=0.421 NDR=6.72 MDP=6.77 AUC=0.358 r=5.56 r=5.56 r=5.</pre>	<pre>r=5.96 g=9e-04 v=0.367 MDR=6.84 MDP=6.76 AUC=0.352</pre>	$ \begin{array}{c} & r = 5.86 \\ g = 9e - 04 \\ v = 0.366 \\ MDR = 6.78 \\ MDP = 6.89 \\ - \\ AUC = 0.382 \end{array} $	r=5.48 g=9e-04 v=0.402 MDR=6.52 MDP=6.75 - AUC=0.347
		DT=3.9	00 - DT=3.57	9 _ DT=3.51	DT=3.54	0 DT=3.68
3 4 5 6 7 IIS3	0 1 2 3 4 5 6 7 HIS3 K=0.113	0 1 2 3 4 5 6 7 HIS3 K=0.116	0 1 2 3 4 5 6 7 HIS3 K=0.11 κ ^{R16C09}	0 1 2 3 4 5 6 HIS3 K=0.116	7 0 1 2 3 4 5 6 7 HIS3 4 5 6 7 4 5 6 7 4 5 6 7 4 5 6 7 5 5 6 7 5 5 6 7 5 5 5 6 7 5 5 5 5 5 5 5 5 5 5 5 5 5	0 1 2 3 4 HIS3 Κ=0.109
	$ \begin{array}{c} r = 17 \\ g = 0.0011 \\ v = 0.1 \\ MDR = 8.55 \\ MDP = 6.68 \\ AUC = 0.406 \end{array} $	$ \begin{array}{c c} r=17 \\ g=0.0011 \\ v=0.1 \\ MDR=8.57 \\ MDP=6.73 \\ AUC=0.418 \end{array} $	<pre>r=15.9 g=0.0011 v=0.1 MDR=7.92 MDP=6.64 AUC=0.385</pre>	<pre>r=14.9 g=0.0011 v=0.108 MDR=8.02 MDP=6.72 AUC=0.408</pre>	$ \begin{array}{c} r = 13.7 \\ g = 9e - 04 \\ v = 0.118 \\ MDR = 8.1 \\ MDP = 6.97 \\ - \\ AUC = 0.394 \end{array} $	r=13.8 g=9e-04 v=0.112 MDR=7.83 MDP=6.91 AUC=0.373
		DT=2.8	010 DT=3.03	07 - DT=2.99	DT=2.96	0 0 0 0 0 0 0 0 0 0 0 0 0 0 0 0 0 0 0



Time since inoculation (days)

HIS3	HIS3	HIS3	HIS3	HIS3	HIS3
o K=0.121 r=16.8 g=0.0011 v=0.1	5 K=0.124 r=14.8 g=0.0011 v=0.11	 → K=0.125 r=9.51 g=0.0011 v=0.184 	 → K=0.121 r=9.19 g=9e-04 v=0.184 	O K=0.131 r=9.09 g=9e-04 O v=0.184	K=0.142 r=6.32 g=9e-04 v=0.263
N – MDR=8.54 MDP=6.78 – AUC=0.432 DT=2.81	MDR=8.18 MDP=6.82 - AUC=0.437 DT=2.93	MDR=7.58 MDP=6.83 - AUC=0.439 DT=3.17	MDR=7.51 MDP=7.07 - AUC=0.416 DT=3.2	MDR=7.52 MDP=7.18 AUC=0.449 DT=3.19	 MDR=6.47 MDP=7.3 AUC=0.466 DT=3.71
ЕМІ5 К=0.0698 К=0.0698	YMR206W K=0.105	ARA1 K=0.0964	ZRT3 K=0.105	EXO1	HIS3
r=4.63 g=0.0011 v=0.588 MDR=5.96	$ \begin{array}{c} r = 5.17 \\ g = 0.0011 \\ v = 0.523 \\ MDR = 6.63 \end{array} $	r=4.38 g=0.0011 v=0.679 MDR=5.94	$ \begin{array}{c} r = 4.37 \\ g = 0.0011 \\ v = 0.706 \\ MDR = 5.97 \end{array} $	r=4.41 g=9e-04 v=0.919 MDR=6.27	r=4.99 g=9e-04 v=0.447 MDR=6.26
MDP=5.99 AUC=0.263 DT=4.03	MDP=6.58 - AUC=0.394 DT=3.62	MDP=6.45 - AUC=0.36 DT=4.04	MDP=6.58 - AUC=0.394 DT=4.02	MDP=7.23 AUC=0.512 DT=3.8	MDP=7.11 AUC=0.436 DT=3.83
	$3 \ \ \ \ \ \ \ \ \ \ \ \ \ $				
REH1	YPK3 K=0.0886	K=0.0837	₩NT4 K=0.0782	ECM5 K=0.0893	HIS3 K=0.108
g=0.00109 v=0.815 MDR=5.51	g=0.00108 v=0.835 MDR=5.49	g=0.001 v=1 MDR=5.39	$\begin{array}{c} & = 3.91 \\ g = 0.00106 \\ v = 0.841 \\ HDR = 5.43 \end{array}$	- 1=4.54 g=9e-04 v=0.605 MDR=6.05	$ \begin{array}{c} $
MDP=6.24 - AUC=0.31 DT=4.36	MDP=6.36 – AUC=0.331 DT=4.37	MDP=6.38 - AUC=0.316 DT=4.45	MDP=6.21 - AUC=0.294 DT=4.42	MDP=6.63 AUC=0.329 DT=3.97	MDP=6.91 - AUC=0.375 DT=3.89
		$\begin{array}{c} 0 \\ 0 \\ 0 \\ 0 \\ 0 \\ 0 \\ 0 \\ 0 \\ 0 \\ 0 $	$ \begin{array}{c} 0 \\ 0 \\ 0 \\ 1 \\ 2 \\ 3 \\ 4 \\ 5 \\ 6 \\ 7 \\ \hline C \\ $		
کالکا ۳۳4C19 کې ۲=4.35	ССССО К=0.108 г=3.96	СЭДГО К=0.0742 r=4.28	© K=0.0665 r=3.75	۳۵۵۵۵۵ ۲=4.47	К=0.127 r=5.91
g=0.0011 v=0.712 MDR=5.94 MDP=6.44	g=0.00106 v=0.882 MDR=5.58 MDP=6.67	g=9e-04 v=0.729 MDR=5.86 MDP=6.37	g=0.001 v=1 MDR=5.29 MDP=6.05	g=9e-04 v=0.602 MDR=5.92 MDP=6.52	g=9e-04 v=0.338 MDR=6.72 MDP=7.15
AUC=0.359 DT=4.04	AUC=0.402 DT=4.3	- AUC=0.279 DT=4.1	AUC=0.253 DT=4.54	AUC=0.304 DT=4.05	AUC=0.444 DT=3.57
$\begin{array}{c ccccccccccccccccccccccccccccccccccc$	0 1 2 3 4 5 6 7 FIT2	0 1 2 3 4 5 6 7 CHK1	° 1 2 3 4 5 6 7 7RT3	° 1 2 3 4 5 6 7 FSBP6	0 1 2 3 4 5 6 7 HIS3
$ \begin{array}{c} & & \\ & \\ & \\ & \\ & \\ & \\ & \\ & \\ & \\ $	K=0.089 r=4.21 g=0.0011	$\begin{bmatrix} 0 \\ 0 \\ 0 \\ - \end{bmatrix} = \begin{bmatrix} K = 0.0866 \\ r = 4.65 \\ q = 9e - 04 \end{bmatrix}$	$\begin{bmatrix} 0 & & \\ 0 $		$ \begin{array}{c} $
No v=1 NDR=4.73 MDP=5.13 MDP=5.13 MUC=0.124	v = 0.696 MDR = 5.71 MDP = 6.34 AUC = 0.321	v=0.834 MDR=6.51 MDP=6.59	$ \begin{array}{c} & & \\ & & \\ & & \\ & & \\ & \\ & & \\ & \\ $	0 0 0 0 0 0 0 0 0 0 0 0 0 0	V=0.308 MDR=7.09 MDP=7.13 AUC=0.445
DT=5.07	DT=4.2	0 - DT=3.69	0 - DT=4.36	0 DT=4.06	DT=3.38
REH1	PET130	FIT2	EST1	RCF1	0 1 2 3 4 5 6 7 HIS3
	$\begin{cases} K=0.0902 \\ r=4.29 \\ g=0.0011 \\ v=0.672 \end{cases}$	$\begin{array}{c} & & \\$	$ \begin{array}{c} $	K=0.0749 r=3.75 g=0.00109 K=0.782	$ \begin{bmatrix} K=0.135 \\ r=6.56 \\ g=9e-04 \\ r=0.202 \end{bmatrix} $
No v=0.001 0 MDR=5.81 0 MDP=6.4 0 AUC=0.344	V = 0.078 MDR=5.79 MDP=6.36 AUC=0.336	$ \begin{array}{c} 0 \\ 0 \\ 0 \\ 0 \\ 0 \\ 0 \\ 0 \\ 0 \\ 0 \\ 0 $	NOR = 0.852 MDR=5.25 MDP=5.95 AUC=0.255	No v=0.789 No MDR=5.15 MDP=6.1 NO AUC=0.276 AUC=0.276 No	 v=0.298 MDR=7.09 MDP=7.23 AUC=0.472
0 - DT=4.13	DT=4.14	O DT=4.12	DT=4.57	0 - DT=4.66	DT=3.38
$\mathbf{R}^{\circ} = \mathbf{R}^{\circ} \mathbf{R}^{\circ}$	GPH1 GPH1 6 7	$\mathbf{R}^{0} = \mathbf{R}^{0} \mathbf{R}^{1} \mathbf{R}^{2} \mathbf{R}^{3} \mathbf{R}^{4} \mathbf{S}^{5} \mathbf{R}^{6} \mathbf{R}^{7}$	$\mathbf{LYP1}^{0 1 2} \mathbf{LYP1}^{4 5 6 7}$	e e Sta e	0 1 2 3 4 5 6 7 HIS3
\vec{O} K=0.109 r=4.57 g=9e-04 v=1.01	K=0.0956 r=4.32 g=0.0011 v=0.655	$ \begin{array}{c c} $	$ \begin{array}{c} $	K=0.0544 K=0.0544 r=3.37 g=0.001 v=1	$ \begin{array}{c ccccccccccccccccccccccccccccccccccc$
MDR=6.52 MDP=6.92 AUC=0.426	MDR=5.8 MDP=6.44 - AUC=0.353	MDR=3.91 MDP=4.8 AUC=0.0915	MDR=0 MDP=0 AUC=0	MDR=4.73 MDP=5.76 AUC=0.202	- MDR=6.86 MDP=7.12 - AUC=0.436
		Q D1=6.14	0 0 -		
FIT2	FIT2	PHB2	$CLB2^{\text{ROBC22}}$	2 ^m K=0.0798	HIS3
r=4.12 g=0.0011 v=0.692	$ \begin{array}{c} r = 4.59 \\ g = 9e - 04 \\ v = 0.652 \end{array} $	r = 4.04 g = 9e - 04 v = 0.736	$ \begin{array}{c} $	r=4.24 g=9e-04 v=1.08	$ \begin{array}{c} r = 6.02 \\ g = 9e - 04 \\ v = 0.339 \end{array} $
MDR=5.58 MDP=6.39 AUC=0.34 DT=4.3	5 MDR=6.23 MDP=6.78 AUC=0.368 DT=3.85	MDR=5.56 MDP=6.57 AUC=0.314 DT=4.32	MDR=4.51 MDP=5.04 AUC=0.129 DT=5.32	MDR=6.04 MDP=6.47 AUC=0.312 DT=3.97	MDR=6.89 MDP=7.23 AUC=0.474 DT=3.48
	$\begin{bmatrix} 3 \\ - \end{bmatrix} \\ \begin{bmatrix} - \\ - \\ - \end{bmatrix} \\ 0 \\ 1 \\ 2 \\ 3 \\ 4 \\ 5 \\ 6 \\ 7 \\ \end{bmatrix}$				
PUF6	NMD2 K=0.155	LYS14 K=0.0361	PGM2 K=0.0914	NMD2 K=0.157 K=0.157	HIS3 K=0.142
r=4.09 g=9e-04 v=0.795 MDR=5.73	$ \begin{array}{c} r = 4.56 \\ g = 9e - 04 \\ v = 1.03 \\ MDR = 6.54 \end{array} $	$ \begin{array}{c c} r=6.96e-18 \\ g=9e-04 \\ v=0.954 \\ c & MDR=9.62e-18 \end{array} $	r=4.18 g=9e-04 v=0.743 MDR=5.77	$ \begin{array}{c c} & r=4.63 \\ & g=9e-04 \\ & v=0.942 \\ & MDR=6.61 \end{array} $	r=5.67 g=9e-04 v=0.372 MDR=6.75
MDP=6.94 - AUC=0.406 DT=4.19	MDP=7.43 AUC=0 DT=307	MDP=5.33 - AUC=0 DT=25	MDP=6.67 - AUC=0.339 DT=4.16	← MDP=7.45 AUC=0 DT=3,03 Q	MDP=7.3 AUC=0.497 DT=3.55
	$3 \rightarrow 2 \qquad 3 \qquad 4 \qquad 5 \qquad 6 \qquad 7$	$ \begin{array}{c} 0 \\ 0 \\ 0 \\ 0 \\ 0 \\ 0 \\ 0 \\ 0 \\ 0 \\ 0 $	$\begin{array}{c} 0 \\ 0 \\ 0 \\ 0 \\ 0 \\ 0 \\ 0 \\ 0 \\ 0 \\ 0 $		
YGL21/C K=0.0589 r=3.46	K=0.0814 r=3.85	PEI130 K=0.0853 r=3.9	PUF6 K=0.0891 r=3.48	ESBP6 K=0.0849 r=3.77	HIS3 K=0.118 r=4.73
g=0.001 v=1 MDR=4.86	$ \begin{array}{c} g = 9e - 04 \\ v = 0.846 \\ MDR = 5.39 \\ MDD = 0.5 $	$ \begin{array}{c} & = & = & = & = & = & = & = & = & = & $	$\begin{array}{c} g = 9e - 04 \\ v = 1.09 \\ 0 \end{array} \\ MDR = 4.97 \\ MDR = 0.02 \\ \end{array}$	g=0.00103 v=0.875 MDR=5.28	g=9e-04 v=0.462 MDR=5.97
AUC=0.22 DT=4.93	AUC=0.301 DT=4.45	AUC=0.315 DT=4.4	AUC=0.326 DT=4.83	AUC=0.315 DT=4.55	AUC=0.41 DT=4.02
$ \begin{array}{cccccccccccccccccccccccccccccccccccc$	$\begin{array}{c} & & & \\ & & & & \\ & & & \\ & & & \\ & & & \\ & & & & \\ & & & \\ & & & \\ & & & \\ & & & \\ & & & & \\ & & & \\ & & & \\ &$	0 1 2 3 4 5 6 7 FMIS	o 1 2 3 4 5 6 7 IVD1	0 1 2 3 4 5 6 7 HIS2	0 1 2 3 4 5 6 7 HIG2
$\begin{bmatrix} & & & & & \\ & & & & \\ & & & & \\ & & & \\ & & & \\ & & & & \\ & & & \\ & & & & \\ $	$\begin{bmatrix} K = 0.0713 \\ r = 3.1 \\ r = 0.04 \end{bmatrix}$	$\begin{bmatrix} K = 0.0563 \\ r = 3.15 \\ r = 0.04 \end{bmatrix}$	$\begin{bmatrix} \mathbf{K} \\ \mathbf{K} \end{bmatrix} \begin{bmatrix} \mathbf{K} \\ \mathbf{K} \end{bmatrix} \end{bmatrix} \begin{bmatrix} \mathbf{K} \\ \mathbf{K} \end{bmatrix} \begin{bmatrix} \mathbf{K} \\ \mathbf{K} \end{bmatrix} \begin{bmatrix} \mathbf{K} \\ \mathbf{K} \end{bmatrix} \end{bmatrix} \begin{bmatrix} \mathbf{K} \\ \mathbf{K} \end{bmatrix} \begin{bmatrix} \mathbf{K} \\ \mathbf{K} \end{bmatrix} \begin{bmatrix} \mathbf{K} \\ \mathbf{K} \end{bmatrix} \end{bmatrix} \begin{bmatrix} \mathbf{K} \\ \mathbf{K} \end{bmatrix} \begin{bmatrix} \mathbf{K} \\ \mathbf{K} \end{bmatrix} \end{bmatrix} \begin{bmatrix} \mathbf{K} \\ \mathbf{K} \end{bmatrix} \begin{bmatrix} \mathbf{K} \\ \mathbf{K} \end{bmatrix} \end{bmatrix} \begin{bmatrix} \mathbf{K} \\ \mathbf{K} \end{bmatrix} \begin{bmatrix} \mathbf{K} \\ \mathbf{K} \end{bmatrix} \end{bmatrix} \begin{bmatrix} \mathbf{K} \\ \mathbf{K} \end{bmatrix} \begin{bmatrix} \mathbf{K} \\ \mathbf{K} \end{bmatrix} \end{bmatrix} \begin{bmatrix} \mathbf{K} \\ \mathbf{K} \end{bmatrix} \begin{bmatrix} \mathbf{K} \\ \mathbf{K} \end{bmatrix} \end{bmatrix} \end{bmatrix} \begin{bmatrix} \mathbf{K} \\ \mathbf{K} \end{bmatrix} \end{bmatrix} \begin{bmatrix} \mathbf{K} \\ \mathbf{K} \end{bmatrix} \end{bmatrix} \begin{bmatrix} \mathbf{K} \\ \mathbf{K} \end{bmatrix} \end{bmatrix} \end{bmatrix} \begin{bmatrix} \mathbf{K} \\ \mathbf{K} \end{bmatrix} \end{bmatrix} \begin{bmatrix} \mathbf{K} \\ \mathbf{K} \end{bmatrix} \end{bmatrix} \begin{bmatrix} \mathbf{K} \\ \mathbf{K} \end{bmatrix} \end{bmatrix} \end{bmatrix} \begin{bmatrix} \mathbf{K} \\ \mathbf{K} \end{bmatrix} \end{bmatrix} \begin{bmatrix} \mathbf{K} \\ \mathbf{K} \end{bmatrix} \end{bmatrix} \end{bmatrix} \begin{bmatrix} \mathbf{K} \\ \mathbf{K} \end{bmatrix} \end{bmatrix} \begin{bmatrix} \mathbf{K} \\ \mathbf{K} \end{bmatrix} \end{bmatrix} \end{bmatrix} \begin{bmatrix} \mathbf{K} \\ \mathbf{K} \end{bmatrix} \end{bmatrix} \end{bmatrix} \begin{bmatrix} \mathbf{K} \\ \mathbf{K} $	$\begin{bmatrix} & & & & \\ & & & & \\ & & & \\ & & & \\ & & & & \\ & & & \\ & & & & \\ & & & & \\ & & & & \\ & & & & \\ & & & & \\ & & & & \\ & & & & \\ & & & & \\ & & & & \\ & & & & \\ & & & & \\ & & & & \\ & & & & \\ & & & & \\ & & & & $	K=0.124 r=5.33 r=00.04
0 0 0 0 0 0 0 0 0 0 0 0 0 0	g=9e-04 v=1.03 MDR=4.4 MDP=6.31	g=9e-04 v=1.36 MDR=4.52 MDP=5.97	0 0 0 0 0 0 0 0 0 0 0 0 0 0 0 0 0 0 0	0 0 0 0 0 0 0 0 0 0 0 0 0 0	g=9e-04 v=0.417 MDR=6.54 MDP=7.1
AUC=0.327 DT=4.35	AUC=0.252 DT=5.46	AUC=0.21 DT=5.31	AUC=0 DT=25	Q - AUC=0.306 DT=4.7	AUC=0.439 DT=3.67
ARA1	0 1 2 3 4 5 6 7 YPK3	$\mathbf{y}^{0} = \mathbf{y}^{1} \mathbf{y}^{2} \mathbf{y}^{3} \mathbf{y}^{4} \mathbf{z}^{5} \mathbf{z}^{6} \mathbf{z}^{7}$	0 1 2 3 4 5 6 7 HAP4	0 1 2 3 4 5 6 7 KNS1	0 1 2 3 4 5 6 7 HIS3
$ \vec{K} = 0.0816 $ $ \vec{K} = 0.0816 $ $ \vec{r} = 3.61 $ $ \vec{g} = 9e - 04 $ $ \vec{K} = 0.082 $	$ \begin{array}{c} $	$ \begin{array}{c} $	$ \begin{array}{c} & & \\ & & $	K=0.0888 r=3.52 g=9e-04 r=4.04	$ \begin{array}{c} $
No v=0.982 NDR=5.11 MDP=6.5 AUC=0.301	V=0.961 MDR=5.15 MDP=6.59 AUC=0.319	$ \begin{array}{c} 0 \\ 0 \\ 0 \\ - \\ 0 \\ - \\ 0 \\ - \\ 0 \\ 0 \\$	$ \begin{array}{c} & V = 1.47 \\ MDR = 3.78 \\ MDP = 5.59 \\ - \\ AUC = 0.156 \end{array} $	No v=1.04 No MDR=5.02 MDP=6.62 NO AUC=0.325 AUC=0.325 No	v=0.543 MDR=5.23 MDP=6.8 - AUC=0.341
9 DT=4.69 0 0 0	DT=4.66	9 - DT=4.76	DT=6.34	€ DT=4.78	DT=4.59
$\mathbf{K} = 0 + $	$HAP4^{5} = 67$	$\sum_{K=0.070}^{1} \sum_{K=0.070}^{2} \sum_{K=0.070}^{3} \sum_{K=0.070}^{4} \sum_{K=0.070}^{5} \sum_{K=0.070}^{6} \sum_{K=0.070}^{7} \sum_{K=0.070}^{1} \sum_{K=0.070}^{2} \sum_{K=0.070}^{3} \sum_{K=0.070}^{4} \sum_{K=0.070}^{5} \sum_{K=0.070}^{6} \sum_{K=0.070}^{7} \sum_{K=0.070}^{1} \sum_{K=0.070}^{7} \sum_{K=0.070}^$	$\mathbf{TSA1}^{0} = \mathbf{K}_{-0.062}^{1} \mathbf{F}_{-0.062}^{2} \mathbf{TSA1}^{4} \mathbf{TSA1}^{5} \mathbf{K}_{-0.062}^{6} \mathbf{TSA1}^{13C22}$		U 1 2 3 4 5 6 7 HIS3 K=0.14
c r=3.07 g=9e-04 v=1.06	$ \begin{array}{c} r = 0 \\ r = 0 \\ g = 0.0011 \\ v = 0.1 \end{array} $	r = 3.6 g = 9e - 04 v = 0.986	$ \begin{array}{c} $	r=4.04 g=0.0011 v=0.1	$ \begin{array}{c} r = 0.14 \\ r = 4.29 \\ g = 9e - 04 \\ v = 0.55 \end{array} $
O MDR=4.36 O MDP=6.16 AUC=0.229 DT=5.51	ы мрк=0 MDP=4.52 AUC=2.6e-18 DT=25	о MDR=5.1 MDP=6.46 – AUC=0.292 DT=4.71	O MDR=4.38 MDP=6.13 AUC=0.227 DT=5.48	MDR=1.41 NDP=4.52 AUC=0.0325 DT=17	MDR=5.72 MDP=7.28 AUC=0.482 DT=4.19
				0.1	
	$B_{3} = \begin{bmatrix} & & & & & & & & & & & & \\ & & & & & &$				
к=0.131 К=0.131	YNL011C K=0.0751	WDL109C K=0.0783	KNS1 K=0.0755	MRE11 ² К=0.0375	HIS3 K=0.101
$ \begin{array}{c c} r = 4.14 \\ g = 9e - 04 \\ v = 1.51 \\ MDR = 5.96 \end{array} $	r=3.49 g=9e-04 v=0.984 MDR=4.94	r=3.52 g=9e-04 v=0.987 MDR=4.99	$ \begin{array}{c} r = 3.3 \\ g = 9e - 04 \\ v = 1.06 \\ MDR = 4.7 \end{array} $	r=0.123 g=9e-04 v=1.5 MDR=0.177	r=3.64 g=9e-04 v=0.573 MDR=4.82
MDP=7.18 AUC=0.508 DT=4.0	MDP=6.38 - AUC=0.275 DT=4.86	MDP=6.44 - AUC=0.287 DT=4.81	MDP=6.39 - AUC=0.274 DT=5.1	MDP=5.38 - AUC=0.00171 DT=25	MDP=6.81 AUC=0.334 DT=4.98
				0.0	
	$3 - \frac{1}{2} - \frac{3}{4} - \frac{4}{5} - \frac{5}{6} - \frac{7}{7}$	0 - 1 - 2 - 3 - 4 - 5 - 6 - 7	$\begin{bmatrix} 0 & 1 & 2 & 3 & 4 & 5 & 6 & 7 \\ & & & & & & & & & & & & \\ \end{bmatrix}$	$\begin{array}{c} 0 \\ 0 \\ 1 \\ 2 \\ 3 \\ 4 \\ 5 \\ 6 \\ 7 \\ 7 \\ 7 \\ 7 \\ 7 \\ 7 \\ 7 \\ 7 \\ 7$	
LYS14	EXO1	EYP1 [∞] K=0.001 r=0 ^{R15C21}	YNL011C K=0.0798 r=3.44	RAD50 K=0.0253 r-5 71	HIS3 K=0.11 r-3.71
- g=9e-04 0 v=0.257 MDR=6.27	$ \begin{array}{cccccccccccccccccccccccccccccccccccc$	- g=0.001 y=1 MDR=0	$\begin{array}{c} - & - & - \\ - & g = 0.000985 \\ 0 & - & V = 0.894 \\ 0 & - & MDR = 4.83 \end{array}$	- g=0.0011 02 - MDR=2	- g=9e-04 v=0.71 MDR=5.12
MDP=6.2 AUC=0.231 DT=3.83	MDP=7.22 AUC=0.518 DT=3.9	0 MDP=0 AUC=0 DT=25	MDP=6.34 AUC=0.288 DT=4.97	MDP=4.52 AUC=0.0481 DT=12	MDP=6.93 AUC=0.383 DT=4.69
	$3 \xrightarrow{-1} \times \times$	$\begin{array}{c} 0 \\ 0 \\ 0 \\ 0 \\ 1 \\ 2 \\ 0 \\ 1 \\ 0 \\ 1 \\ 0 \\ 0 \\ 0 \\ 0 \\ 0 \\ 0$	$0 - \frac{1}{2} + $	$0.0 \\ 0.0 $	$\begin{array}{c} - & & \\ & &$
HIS3 K=0.0975 r=4.95	HIS3 K=0.0915 r=4.54	₩ HIS3 K=0.107 r=4.62	HIS3 K=0.0815 r=3.95	ні53 К=0.104 r=3.79	HIS3 K=0.116 r=3.79
g=0.0011 v=0.449 MDR=6.02 MDP=6.47	g=9e-04 v=0.532 MDR=5.87 MDP=6.67	G G G G G G G G G G G G G G	<pre>g=9e-04 v=0.591 MDR=5.2 MDP=6 5</pre>	g=0.0011 v=0.713 MDR=5.19 MDP=6.56	g=9e-04 v=0.626 MDR=5.13 MDP=7.01
- AUC=0.35 DT=3.99	$= \begin{array}{c} 100 F = 0.07 \\ AUC = 0.328 \\ DT = 4.09 \end{array}$	AUC=0.387 DT=3.93	AUC=0.285 DT=4.61	AUC=0.37 DT=4.62	AUC=0.395 DT=4.67
	0 1 2 3 4 5 6 7				0 1 2 3 4 5 6 7





0.8

0.6

0.4

0.2

0.0

0.0

0.2

Fitness (F) of yfg Δ cdc13-1 at 30°C (MDPxMDR)







0.4

Fitness (F) of yfg∆ ura3∆ at 30°C (MDPxMDR)

RAD17

EXO1

RAD24

0.6

0.8

Figure 36. Fitness of stn1-13 strains with aspects of the resection machinery deleted grown at 33°C. A. A photograph of one replicate of plate 15 harbouring EXO1, RAD24 and RAD17 deletion mutants after growth at 30°C over 4 days. B. Representative growth curves taken from the same plate. HIS3 deletion mutants were used as a noninteracting control for comparison. C. All growth curves taken from 1/8 replicates of plate 15 harbouring the EXO1, RAD17, and RAD24 deletion mutants. D. stn1-13 strains with different gene deletions of aspects of the DNA damage response were grown at 33°C over the course of several days and fitness plotted. Coloured points located above the grey line of best fit represent gene deletions that suppress the temperaturesensitivity of stn1-13 strains. Coloured points located below the grey line of best fit represent gene deletions that enhance the temperature-sensitivity of stn1-13 strains. E. Spot tests showing the effect of deletion of RAD17 and RAD24, alone and together, and EXO1 on the fitness of W303 stn1-13 strains grown at the indicated temperatures. F. cdc13-1 strains with different gene deletions of aspects of the DNA damage response were grown at 27°C over the course of several days and fitness plotted (data taken from Addinall et al 2011). G. cdc13-1 strains with different gene deletions/knockdowns of aspects of the DNA damage response were grown at 30°C over the course of several days and fitness plotted (data taken from Addinall et al 2011).

5.4 *rad9* Δ is an enhancer of the *stn1-13* temperature-sensitive phenotype

Telomere uncapping in *cdc13-1* mutants at the restrictive temperature leads to the accumulation of ssDNA, which is turn activates the G2/M checkpoint, to prevent cells going through mitosis with long ssDNA overhangs which would lead to genetic instability. *RAD9* is part of the signal transduction cascade that leads to freezing of the cell cycle ⁸². It acts as a sensor ¹⁶⁰, stimulating Mec1 phosphorylation of the downstream effector kinases Chk1 and Rad53 ^{161; 162; 163}. It plays important roles in activating the G1/S, intra-S and G2/M checkpoints ^{164; 165; 166} in response to DNA damage at the telomere and elsewhere in the genome.

The *stn1-13* screen identified *rad9* Δ as a strong enhancer of the *stn1-13* temperaturesensitive phenotype based on the observation that *stn1-13 rad9* Δ strains grew weakly at 33°C compared to *stn1-13 his3* Δ strains (figure 37a). Furthermore the growth curves of *stn1-13 rad9* Δ strains were very shallow with a very low area under the curve compare to *stn1-13 his3* Δ strains (figure 37b). This led to the positioning of *rad9* Δ below the grey cloud of non-interacting genes close to 0 fitness in the *stn1-13* fitness plot (figure 37c). This phenotype was recapitulated in the W303 background (figure 37d) suggesting that this phenotype is reflecting a true genetic interaction. Interestingly, this phenotype was the opposite of that observed when *cdc13-1 rad9* Δ cells went through the SGA and QFA process. In these screens *cdc13-1 rad9* Δ strains were substantially fitter than the *stn1-13 his3* Δ control and positioned above the cloud of non-interacting genes when grown at 27°C (figure 37e), in roughly the same area as *stn1-13 exo1* Δ strains.

The *stn1-13* screen also identified *dot1* Δ as a strong enhancer of the *stn1-13* temperature-sensitivity phenotype. This is based on the observation that *stn1-13 dot1* Δ strains had a very weak growth curve with a very low area under the curve compared to *stn1-13 his3* Δ controls (appendix 3, plate 4). This led to the positioning of *dot1* Δ at the bottom of the fitness plot, near *rad9* Δ (figure 37c). However, this phenotype was not recapitulated in the W303 background (figure 37d), suggesting it could be an artefact of the S288C background. Furthermore if *RAD9* and *DOT1* were simultaneously deleted in the *stn1-13* background it led to a very strong fitness defect (figure 37d). These results are particularly interesting because Dot1 is involved in

methylating residue K79 of histone H3 ¹⁶⁷ which is important in Rad9 checkpoint activation and Rad9-mediated inhibition of ssDNA generation ¹⁶⁸. However, it will be important to resolve why *dot1* Δ is behaving differently in the two genetic backgrounds. However, the fact that simultaneous deletion of *DOT1* and *RAD9* made these *stn1-13* mutants much sicker than the single deletions, could suggest that *DOT1* has *RAD9*-independent function which could impinge on telomere capping.

Finally deletion of *CHK1* in the *stn1-13* QFA had no effect on the fitness of *stn1-13* strains at 33°C as judged by the similar growth of *stn1-13 chk1* Δ strains and *stn1-13 his3* Δ controls and the similar area under their respective growth curves (figure 37a, b). This led to the positioning of *chk1* Δ in the centre of the grey cloud of non-interacting genes (figure 37c). This is in contrast to what was seen in the *cdc13-1* QFAs since *chk1* Δ was identified as a strong suppressor at both 27°C and 30°C (figure 37e, f). Since both Stn1 and Cdc13 are classically proposed to form a heterotrimeric complex which protects the telomere end, it will be interesting to further investigate how *stn1-13* influences checkpoint activation.











Α





0.8



Ε

Figure 37. Fitness of *stn1-13* strains with *RAD9*, *DOT1* and *CHK1* deleted grown at 33°C. A. A photograph of one replicate of plate 15 harbouring *RAD9* and *CHK1* deletion mutants after growth at 33°C over 4 days. B. Representative growth curves taken from the same plate. *HIS3* deletion mutants were used as a non-interacting control for comparison. C. *stn1-13* strains with *RAD9*, *DOT1* and *CHK1* deleted were grown at 33°C over the course of several days and fitness plotted. D. Spot tests showing the effect of deletion of *RAD9* and *DOT1* alone and together on the fitness of W303 *stn1-13* strains grown at the indicated temperature. E. *cdc13-1* strains with *RAD9*, *DOT1* and *CHK1* deleted were grown at 27°C over the course of several days and fitness plotted days and fitness plotted (data taken from Addinall et al 2011). F. *cdc13-1* strains with *RAD9*, *DOT1* and *CHK1* deleted were grown at 30°C over the course of several days and fitness plotted (data taken from Addinall et al 2011).

5.5 mre11 Δ , rad50 Δ , xrs2 Δ , yku70 Δ , yku80 Δ and ten1 Δ are enhancers of the stn1-13 temperature-sensitive phenotype

Both *cdc13-1* and *yku70Δ/yku80*Δ mutants cause a temperature-dependent disruption of telomere capping and accumulation of single strand DNA at the telomere end followed by activation of the G2/M checkpoint. In both cases, deletion of *EXO1* can suppress the temperature-sensitive phenotype of these strains by limiting the amount of ssDNA generated. This led to the suggestion that the Ku heterodimer is involved in capping the telomere end alongside the putative CST complex ⁸⁶. Furthermore, deletion of *YKU70* or *YKU80* in the *cdc13-1* background makes these mutants sicker ³⁷, again supporting the notion that these proteins are involved in capping. Similarly to the Ku heterodimer, deletion of any component of the MRX complex in the *cdc13-1* background makes these mutants sicker ¹⁰⁵. This discovery was somewhat surprising since MRX contributes to nuclease resection at double strand breaks ¹⁴⁰, but at telomeres it appears to be carrying out a telomere capping function.

The *stn1-13* screen identified deletion all three members of the MRX complex as enhancers of the *stn1-13* temperature-sensitivity phenotype based on the weak growth of *stn1-13 mre11* Δ and *stn1-13 rad50* Δ strains at 33°C (figure 38a) and the low area under the curve values for their growth curves and that of *stn1-13 xrs2* Δ as compared to *stn1-13 his3* Δ control strains (figure 38b, appendix 3, plate 15). This results in the positioning of *mre11* Δ , *rad50* Δ and *xrs2* Δ low on the fitness plot below the line of best fit (figure 38c). However *mre11* Δ is very close to the line of best fit because oddly the *STN1 mre11* Δ strain which is used for comparison was also dead, which is probably a technical artefact as *mre11* Δ is a not a lethal deletion. These results were recapitulated in the W303 background (figure 38d). These results correlate well with the results in the *cdc13-1* background, where *mre11* Δ , *rad50* Δ and *xrs2* Δ are located close to each other and beneath the line of best fit a 27°C and 30°C (figure 38e, f). This suggests that the MRX complex contributes to the vitality of *stn1-13* and *cdc13-1* strains grown at the semi-permissive temperature.

The *stn1-13* screen also identified the two members of the Ku heterodimer (*YKU70* and *YKU80*) as having no interaction with the *stn1-13* temperature sensitive phenotype. This is based on the comparable growth of the *stn1-13* yku70 Δ and *stn1-13* yku80 Δ
strains with that of the *stn1-13 his3* Δ control strain as judged from the area under their respective growth curves (appendix 3, plate 10, 11). This results in the placement of *YKU70* and *YKU80* close to the line of best fit in the grey cloud of non-interacting genes (figure 38c). However, when it was attempted to recapitulate this result in the W303 background, it was found that the *stn1-13 yku70* Δ and *stn1-13 yku80* Δ strains were substantially sicker than *stn1-13* when grown at 36°C (figure 38d). This suggested that the Ku heterodimer is an enhancer of the *stn1-13* temperature sensitive phenotype. This is similar to its effect in the *cdc13-1* background where it is a strong enhancer of the *cdc13-1* temperature-sensitive phenotype (figure 38e, f).

Finally this screen identified $ten1\Delta$ as a strong enhancer of the stn1-13 temperaturesensitive phenotype. This is based on the reduced area under the curve observed in the growth curve of stn1-13 strains with a DAmP allele of *TEN1* compared to stn1-13 $his3\Delta$ controls (appendix 3, plate 17). This resulted in the placement of *TEN1* below the cloud of non-interacting genes in the fitness plot (figure 38c). The same was not true in the cdc13-1 30°C screen where *TEN1* was found in the cloud of non-interacting genes (figure 38f). The strong synthetic sick interaction between stn1-13 and *TEN1* combined with evidence that the two exist as a subcomplex suggests that they may function together in the cell, perhaps independently of *CDC13*.



Α





cdc13-1 @ 27°C







163

F

Figure 38. Fitness of *stn1-13* **strains with aspects of the telomere capping machinery disrupted grown at 33°C.** A. A photograph of one replicate of plate 15 harbouring *MRE11* and *RAD50* deletion mutants after growth at 33°C over 4 days. B. Representative growth curves taken from the same plate. *HIS3* deletion mutants were used as a non-interacting control for comparison. C. *stn1-13* strains with *MRE11*, *RAD50, XRS2, YKU70, YKU80* and *TEN1* deleted/knocked down were grown at 33°C over the course of several days and fitness plotted. D. Spot tests showing the effect of deletion of *MRE11, RAD50, XRS2* and *YKU70* on the fitness of W303 *stn1-13* strains grown at the indicated temperatures. E. *cdc13-1* strains with *MRE11, RAD50, XRS2, YKU70, YKU80* and *TEN1* deleted/knocked down were grown at 27°C over the course of several days and fitness plotted (data taken from Addinall et al 2011). F. *cdc13-1* strains with *MRE11, RAD50, XRS2, YKU70, YKU80* and *TEN1* deleted/knocked down were grown at 30°C over the course of several days and fitness plotted (data taken from Addinall et al 2011).

5.6 Deletion of aspects of the nonsense-mediated mRNA decay pathway suppresses the *stn1-13* temperature-sensitive phenotype

Nonsense-mediated mRNA decay (NMD) is a surveillance pathway and is found in all eukaryotes. It functions to remove those mRNA transcripts where there is a premature termination codon ¹⁶⁹. However, its involvement in the cell extends further than this, and it has been proposed to play an important role in telomere homeostasis ¹⁷⁰. Interestingly deletion of aspects of NMD can lead to the up-regulation of various telomere-related genes including the Ku heterodimer, telomerase, and the two small subunits of the putative "CST complex", Stn1 and Ten1. Furthermore it has also been proposed to regulate the levels of telomere-associated RNA (TERRA) ^{171; 172; 173}.

It was recently discovered that deletion of aspects of the NMD pathway in the *cdc13-1* genetic background allowed cells to grow at the non-permissive temperature, similarly to interventions involving Exo1 and aspects of the DNA damage response (DDR) pathway ¹⁷⁴. It has also been reported that *cdc13-1 nmd2* Δ *exo1* Δ or *cdc13-1 nmd2* Δ *rad24* Δ can completely bypass the need for *CDC13*, but similar genetic interventions could not bypass the need for *STN1* and *TEN1*, leading to the suggestion that Stn1 and Ten1 could be more important at telomeres than Cdc13 ¹¹⁶.

In the *stn1-13* screen we found that if *stn1-13* was combined with deletions of the NMD pathway (*UPF3, NMD2, KEM1, EBS1* and *NAM7*) there was a suppression of temperature-sensitivity. This is based on the higher area under the curve observed in most of the growth curves of these strains compared to *stn1-13 his3* strains (appendix 3, plates 3, 5, 6, 10, 15). This results in the placement of the NMD deletion strains above the cloud of non-interacting genes, as strong suppressors (figure 39a). This result was recapitulated in the W303 background when *KEM1* which codes for a 5' to 3' exonuclease involved in mRNA decay, and implicated in telomere capping, was deleted, as evidenced by strong growth of *stn1-13 kem1* strains compared to *stn1-13* strains at 36°C (figure 39b). This result is very similar to that found in *cdc13-1* where the NMD genes were located above the grey cloud of non-interacting genes at 27°C and 30°C (figure 39c, d). Furthermore all five of these NMD genes were amongst the top 10 genetic interaction scores for suppressors in both the *cdc13-1* deletion of aspects of

the NMD pathway in the *stn1-13* background allows growth at the restrictive temperature.







cdc13-1 @ 30°C



Figure 39. Fitness of *stn1-13* **strains with aspects of the nonsense mediated mRNA decay pathway deleted.** A. *stn1-13* strains with *UPF3, NMD2, KEM1, EBS1* and *NAM7* deleted were grown at 33°C over the course of several days and fitness plotted. B. Spot tests showing the effect of deletion of *KEM1* on the fitness of W303 *stn1-13* strains grown at the indicated temperatures. C. *cdc13-1* strains with *UPF3, NMD2, KEM1, EBS1* and *NAM7* deleted were grown at 27°C over the course of several days and fitness plotted (data taken from Addinall et al 2011). D. *cdc13-1* strains with *UPF3, NMD2, KEM1, EBS1* and *IAM7* deleted were grown at 30°C over the course of several days and fitness plotted (data taken from Addinall et al 2011).

5.7 Lagging strand synthesis is compromised in stn1-13 mutants

Lagging strand synthesis is carried out primarily by two polymerases: pol α and pol δ ¹⁷⁵. Pol α consists of four subunits, of which 3 are enzymatically active *in vitro* ⁹⁸. Its role in lagging strand synthesis is primarily synthesis of the small ~10 nucleotide RNA primers and initiation of lagging strand replication. However, due to its poor fidelity, because of its lack of proofreading exonuclease activity, pol δ is proposed to complete lagging strand synthesis.

The *stn1-13* screen identified *PRI1* and *PRI2* (component of DNA polymerase α) DAmP alleles as enhancers of the *stn1-13* temperature-sensitive phenotype. This is judged by the low area under curve displayed by the *stn1-13 pri1* DAmP alleles and *stn1-13 pri2* DAmP alleles compared to the *stn1-13 his3* Δ control strains (appendix 3, plate 17). This resulted in the placement of *PRI1* and *PRI2* below the cloud of non-interacting genes (figure 40a). When the same DAmP alleles were introduced to the *cdc13-1* background they had less of a pronounced effect with *pri2* being located in the cloud of non-interacting genes, and *pri1* was a weaker enhancer (figure 40b, c). However interestingly when DAmP alleles of *POL1* or *POL12* were introduced which are the catalytic subunit of DNA polymerase α and the β -subunit of the α -primase complex respectively, they had little impact on the fitness of *stn1-13* strains (figure 40a).

Furthermore the *stn1-13* screen also identified *pol32* Δ as a strong enhancer of the *stn1-13* mutation, with the *stn1-13 pol32* Δ strain having a very shallow growth curve, resulting a fitness close to 0 (appendix 3, plate 7). The resulted in the positioning of *pol32* Δ near the bottom of the *stn1-13* fitness plot, below the cloud of non-interacting genes (figure 40a). This was similar to its positioning in the *cdc13-1* screen both 30°C and 27°C (figure 40b, c). Identification of *pol32* Δ as an enhancer in the *stn1-13* screen was recapitulated in the W303 background, with *stn1-13 pol32* Δ strains growing much more weakly than *stn1-13* strains at 36°C (figure 40d). Finally as Pol32 is proposed to be involved in the creation of long flaps during okazaki fragment processing with Pif1, I wondered if *PIF1* might also be identified as an enhancer of the *stn1-13* temperature-sensitive phenotype. The W303 spot test of *stn1-13 pif1* confirmed this hypothesis, with *stn1-13 pif1* Δ strains growing more weakly than *stn1-13* provide the provide the strains growing more weakly than *stn1-13* provide the strains growing more weakly than *stn1-13* provide the provide the provide the strains grow

these results suggest that lagging strand synthesis may be compromised in *stn1-13* mutants.



Ε

150

100

50

c

0

Fitness (F) of yfg∆ cdc13-1 at 27°C (MDPxMDR)

В



		30oC	36oC
	Wild type (DLY8460) Wild type (DLY3001) stn1-13 (DLY8994) stn1-13 (DLY8993)		
D	stn1-13 pol32Δ (DLY9455) stn1-13 pol32Δ (DLY9455)	●●●●● ●●●●●	•••





Figure 40. Fitness of *stn1-13* strains with aspects of the lagging strand machinery deleted. A. *stn1-13* strains with *POL1, POL3, POL12, PRI1* and *PRI2* knocked down or *POL32* deleted were grown at 33°C over the course of several days and fitness plotted. B. *cdc13-1* strains with *POL1, POL3, POL12, PRI1* and *PRI2* knocked down or *POL32* deleted were grown at 27°C over the course of several days and fitness plotted (data taken from Addinall et al 2011). C. *cdc13-1* strains with *POL1, POL3, POL12, PRI1* and *PRI2, POL3, POL12, PRI1* and *PRI2* knocked down or *POL32* deleted were grown at 30°C over the course of several days and fitness plotted (data taken from Addinall et al 2011). D. Spot test showing the effect of deletion of *POL32* on the fitness of W303 *stn1-13* strains grown at the indicated temperatures. E. Spot test showing the effect of deletion of *PIF1* on the fitness of W303 *stn1-13* strains grown at the indicated temperatures.

5.8 *RAP1* DAmP, *rif1* Δ , *and rif2* Δ are mild enhancers of the *stn1-13* temperaturesensitive phenotype

An integral aspect of telomere homeostasis is the maintenance of telomere length by telomerase. In *S. cerevisiae* telomerase consists of three subunits: Est1, Est2 and Est3 which are recruited to telomeres by the Cdc13-Est1 interaction ^{176; 177}. It is important that extension of the G-overhang by telomerase is carefully coordinated with fill-in synthesis of the C-rich strand by the lagging strand machinery. Currently there are proposed to be two major mechanisms of negative regulation of telomerase in *S. cerevisiae*. Firstly recruitment of telomerase is proposed to be regulated by modulation of the interaction between Est1 and Cdc13 by Stn1 which competes with Est1 for binding ⁸⁰. Secondly, promotion of lagging strand synthesis produces a shorter 3' overhang structure which is refractory to telomerase activity ⁹⁸. This negative regulation is counteracted by positive regulation by Cdc13 when telomeres become too short; however this mechanism is poorly understood. However one possibility is that Cdc13 could be phosphorylated by Tel1 enhancing recruitment of telomerase ¹⁷⁸, however this has since been disputed ¹⁷⁹. Regardless of the mechanism it is now known to be regulated in part by a Rap1-Rif1-Rif2 protein counting mechanism ¹⁸⁰.

The *stn1-13* screen identified all three members of the Rap1-Rif1-Rif2 complex (*RAP1* DAmP, *rif1* Δ , and *rif2* Δ) as enhancers of the *stn1-13* temperature-sensitive phenotype. This is based on the lower area under the curve observed in these three mutants compared to the *stn1-13 his3* Δ control (appendix 3, plates 2, 10, 18). This resulted in the positioning of DAmP Rap1, *rif1* Δ and *rif2* Δ together beneath the grey cloud of non-interacting genes (figure 41a). This is in contrast to the *cdc13-1* screen carried out at 27°C where *rif2* Δ was a suppressor of the *cdc13-1* temperature-sensitive phenotype and *rif1* Δ was an enhancer (figure 41b). Furthermore, it is also in contrast to the *cdc13-1* screen identified in the grey cloud of non-interacting genes (figure 41b). Furthermore, it is also in contrast to the *cdc13-1* screen identified in the grey cloud of non-interacting genes (figure 41b). Furthermore, it is also in contrast to the *cdc13-1* screen identified in the grey cloud of non-interacting genes (figure 41b).

Furthermore, the *stn1-13* screen identified *tel1* Δ as being a slight suppressor of the *stn1-13* temperature-sensitive phenotype, although this is not obviously clear from the area under the *stn1-13 tel1* Δ growth curves as compared to *stn1-13 his3* Δ (appendix 3, plate 1). However, the fitness plot placed *tel1* Δ above the cloud of non-interacting

genes suggesting that it either has no-interaction or a slight suppressive effect (figure 41a). This was recapitulated in the W303 background with *stn1-13 tel1* Δ strains growing largely similarly to *stn1-13* strains, however there was no evidence of suppression (figure 41d). In addition we found that if *TEL1* was deleted in the *stn1-13 yku70* Δ background it had an additive enhancement effect on the temperature-sensitivity of these strains causing weaker growth than *stn1-13 tel1* Δ or *stn1-13 yku70* Δ (figure 41d).

Finally the *stn1-13* screen identified *est1* Δ and *est3* Δ (two components of telomerase) as having no effect on the temperature-sensitivity of *stn1-13* (figure 41a). However the same deletion mutants in the *cdc13-1* background at 27°C had a strong negative effect on fitness (figure 41b, c). These results suggest that telomerase is less important to the vitality of *stn1-13* than *cdc13-1* strains.



stn1-13 @ 30°C





Figure 41. Fitness of *stn1-13* strains with genes involved in telomerase activity deleted. A. *stn1-13* strains with *RAP1* knocked down or *RIF1*, *RIF2*, *EST1*, *EST3*, *TEL1* deleted were grown at 33°C over the course of several days and fitness plotted. B. *cdc13-1* strains with *RAP1* knocked down or *RIF1*, *RIF2*, *EST1*, *EST3*, *TEL1* deleted were grown at 27°C over the course of several days and fitness plotted (data taken from Addinall et al 2011). C. *cdc13-1* strains with *RAP1* knocked down or *RIP1* knocked down or *RIF1*, *RIF2*, *EST1*, *EST3*, *TEL1* deleted were grown at 30°C over the course of several days and fitness plotted (data taken from Addinall et al 2011). D. Spot tests showing the effect of deletion of *YKU70* and *TEL1* alone and together, on the fitness of W303 *stn1-13* strains grown at the indicated temperature.

5.9 Common suppressors and enhancers in the *cdc13-1* and *stn1-13* screen

In order to make a more global comparison of the suppressors and enhancers from the *cdc13-1* and *stn1-13* screens the top 100 suppressors and top 100 enhancers of *stn1-13* and *cdc13-1* mutations as measured by genetic interaction strength (GIS) were listed (Appendix 5) and common genes identified. 10 common suppressors and 10 common enhancers were identified (table 16) (figure 42).

Common enhancers	Common suppressors	
mak31∆ (Maintenance of Killer 31) (Non-	$doa1\Delta$ (Involved in ubiquitin-mediated	
catalytic subunit of the NatC	protein degradation)	
acetyltransferase complex)		
$mak3\Delta$ (Maintenance of Killer 3) (Catalytic	ebs1 ^Δ (Involved in translation inhibition	
subunit of the NatC acetyltransferase)	and nonsense mediated decay)	
<i>hsc82</i> Δ (Cytoplasmic chaperone)	<i>kem1</i> Δ (<i>xrn1</i> Δ) (5' to 3' exoribonuclease)	
<i>hpr5</i> Δ (<i>srs2</i> Δ) (Helicase and ATPase)mak3	$cbc2\Delta$ (Cap binding complex 2) (Involved	
	in export of mRNA from nucleus. Has a	
	potential telomere maintenance function)	
<i>mak10</i> Δ (Maintenance of Killer 10) (Non-	bts1∆ (Geranylgeranyl diphosphate	
catalytic subunit of the NatC	synthase)	
acetyltransferase complex)		
<i>pph3</i> ∆ (Catalytic subunit of the protein	<i>ubc4</i> ∆ (Ubiquitin conjugating enzyme.	
phosphatase PP4 complex)	Key part of the anaphase promoting	
	complex)	
<i>bck1</i> Δ (MAPKKK acting in the protein	<i>exo1</i> ∆ (5' to 3' exonuclease and flap	
kinase C signalling pathway)	endonuclease)	
$pol32\Delta$ (Third subunit of DNA polymerase	<i>nmd2</i> ∆ (Protein involved in the nonsense-	
delta)	mediated decay pathway)	
<i>bub2</i> ∆ (Mitotic exit network regulator)	<i>upf</i> 3∆ (Protein involved in the nonsense-	
	mediated decay pathway)	
<i>cin8</i> ∆ (Kinesin motor protein)	<i>rpn4</i> ∆ (Transcription factor that promotes	
	expression of proteasome genes)	

Table 16. Common suppressors and enhancers in the *cdc13-1* **and** *stn1-13* **screens.** The top 100 genetic interaction scores for *cdc13-1* and *stn1-13* suppressors at 30°C were compared and 10 overlapping suppressors and enhancers were identified and are listed in the table above.





cdc13-1 @ 30°C



Fitness (F) of yfg∆ ura3∆ at 30°C (MDPxMDR)

Figure 42. Fitness of *stn1-13* strains grown at 33°C highlighting the common suppressors and enhancers identified in the *rpa3-313* and *cdc13-1* screens. *stn1-13*, and *cdc13-1* strains were grown at the indicated temperatures over the course of several days and fitness plotted. A. A *stn1-13* fitness plot showing the positioning of the common suppressors and enhancers. B. A *cdc13-1* fitness plot showing the positioning of the common suppressors and enhancers when *cdc13-1* was grown at 27°C (data taken from Addinall et al 2011). C. A *cdc13-1* fitness plot showing the solution of the common suppressors and enhancers when *cdc13-1* was grown at 30°C (data taken from Addinall et al 2011).

5.10 Common enhancers

5.10.1 Deletion of any member of N-terminal acetylation complex C enhances the stn1-13 and cdc13-1 phenotype

N-terminal acetylation involves the transfer of an acetyl group to the α amino group of the first amino acid, and is one of the most common chemical modifications of proteins. Classically, it is thought the N-terminal acetylation prevents the degradation of proteins potentially by preventing the N-terminal ubiquitination of proteins and therefore their targeting for degradation. However, interestingly N-terminal acetylation can also target proteins for ubiquitin-mediated degradation as well ¹⁸¹. It is likely that this chemical modification of proteins is part of a protein quality control mechanism to degrade misfolded proteins, and affect *in vivo* protein stoichiometry ¹⁸². Furthermore the NatC complex has previously been identified as being involved in regulating telomere length ¹⁸³. Interestingly all three components of the NatC acetyltransferase complex (*mak31*Δ, *mak3*Δ, *mak10*Δ) were identified as enhancers of the *cdc13-1* and *stn1-13* mutations (figures 42a-c, table 16). This suggests strongly that the NatC complex plays an important role in cell survival in the absence of proper capping.

5.10.2 Hsc82 deletion enhances the stn1-13 and cdc13-1 phenotype

Hsc82 is one of two isoforms of the Hsp90 molecular chaperone that share 97% sequence identity and its activity is required to fold difficult-to-fold proteins into their nascent configurations, and refold denatured proteins ¹⁸⁴. Interestingly, partial loss of function alleles of *STN1* and *CDC13* can be bypassed by overexpression of Hsp90 proteins, which also leads to telomere shortening ¹⁸⁵. Furthermore Hsp82 has been implicated in modifying the interaction of Cdc13 with DNA, to facilitate the transition between capped and telomerase-extendible telomeres, by altering the interaction of Cdc13 with telomere DNA ¹³⁵. Interestingly both the *cdc13-1* and *stn1-13* screens identified *hsc82* as a strong enhancer of the temperature-sensitive phenotype (figures 42a-c, table 16). This supports previous findings that *HSC82* can compensate for partial loss of telomere capping.

5.10.3 Srs2 is an important helicase in maintaining the vitality of stn1-13 *and* cdc13-1 *strains*

The major role of *SRS2* is to inhibit the repair of DNA lesions by homologous recombination. It is thought that one mechanism by which *SRS2* inhibits homologous recombination is by interfering with the interaction between Rad51 and ssDNA ¹⁸⁶. Therefore deletion of *SRS2* leads to an excessive recombination phenotype. Interestingly *SRS2* interacts physically with Mre11 and Sgs1 and is proposed to be phosphorylated by Cdk1 ¹⁸⁷. Our *stn1-13* screen and the *cdc13-1* screen independently identified *SRS2* deletion as an enhancer of the temperature sensitive phenotype (figure 42a-c, table 16). This suggests that *SRS2* plays an important role in maintaining the integrity of *S. cerevisiae* telomeres.

5.10.4 Pph3 promotes growth in the presence of the cdc13-1 and stn1-13 mutations

Rad53 phosphorylation plays an important role in establishing the G2/M checkpoint following *cdc13-1* mediated uncapping ¹⁸⁸. However, Rad53 dephosphorylation is also important since it allows cells to exit from this cell cycle checkpoint. Interestingly we identified the 1 essential subunit of the protein phosphatase PP4 complex as being important for the fitness of *cdc13-1* and *stn1-13* strains (figure 42a-c, table 16). This is particularly interesting in light of the fact that the PP4 complex is proposed to dephosphorylate Rad53 and prevent replication fork restart failure and late origin firing ¹⁸⁹.

5.10.5 Deletion of BCK1 inhibits growth in the presence of the cdc13-1 and stn1-13 mutations

BCK1 is part of the protein kinase C signalling pathway, which is important in a wide variety of cellular processes. *BCK1* deletion in wild type cells results in a temperature-sensitive cell lysis phenotype which is suppressed by osmotic stabilising agents ¹⁹⁰. It is possible that the temperature-related cell lysis effect may exacerbate temperature-sensitive mutations, therefore rendering the exacerbation of the temperature-sensitive growth of *cdc13-1* and *stn1-13* mutants an artefact (figure 42a-c, table 16).

5.10.6 Bfa1-Bub2 complex and CIN8 deletion compromise growth of cdc13-1 and stn1-13 strains

Bfa1-Bub2 is a complex involved in regulating the spindle checkpoint ¹⁹¹. In the presence of damaged spindles or lagging chromosomes the anaphase promoting complex (APC) is inhibited by two pathways involving Cdc20 and Cdh1. APC^{CDH1} is regulated by a second pathway that depends on Bfa1-Bub2 GTPase activity on Tem1, leading to accumulation of GDP bound Tem1 and inhibition of the downstream anaphase promoting complex preventing cytokinesis ¹⁹². Interestingly our screen identified the Bfa1-Bub2 complex as important for the vitality of both *stn1-13* and *cdc13-1* strains. Furthermore deletion of CIN8, which is a kinesin motor protein important for chromosome segregation ¹⁹³ enhanced the temperature-sensitivity of both strains (figure 42a-c, table 16). This lends further evidence to the idea that *stn1-13* and *cdc13-1* strains are particularly sensitive to deletion of aspects of the cellular machinery involved in disassembly of the mitotic spindle.

5.11 Common suppressors

5.11.1 doa1*\Delta* is a suppressor in the stn1-13 and cdc13-1 backgrounds

DOA1 is involved in ubiquitin-mediated protein degradation and promoting efficient non-homologous end joining ^{194; 195}. However, notably while *STN1 doa1* Δ grew normally in the *cdc13-1* screen at 27°C (figure 42b), at 30°C *STN1 doa1* Δ appeared to be very sick (figure 42c), and in the *stn1-13* screen *STN1 doa1* Δ was dead (figure 42a). Therefore it is possible that *doa1* Δ strains have a temperature-sensitive phenotype, or this strain is an artefact. However, when combined with the temperature-sensitive mutation *stn1-13* and *cdc13-1* these strains were substantially fitter (figure 42a-c, table 16).

5.11.2 Deletion of UBC4 improves the fitness of stn1-13 and cdc13-1 strains

UBC4 is a ubiquitin-conjugating enzyme which is a key part of the anaphase promoting complex (APC) in *S. cerevisiae* ^{196; 197; 198}. Its role is to ubiquitinate and therefore mark for degradation various proteins involved in inhibiting cytokinesis. Interestingly it is

also involved in degrading histone H3 ¹⁹⁹ and regulating levels of DNA polymerase α ²⁰⁰. The *stn1-13* and *cdc13-1* screen both picked up *ubc4* Δ as a strong suppressor of temperature-sensitivity in this background (figure 42a-c, table 16). This is particularly interesting in light of the role of *CDC13* and *STN1* in lagging strand synthesis. It is also interesting since we identified deletion of the genes coding for the Bfa1-Bub2 complex, which is important in inhibiting cytokinesis, as strong enhancers. Collectively these results suggest an important role for the spindle checkpoint in maintaining fitness in the *stn1-13* background.

5.11.3 CBC2 is involved in maintaining telomeres in the cdc13-1 *and* stn1-13 *background*

Cbc2 is part of the 5' RNA-capping complex Cbc1-Cbc2 which plays an important role in mRNA biogenesis ²⁰¹. Cbc1-Cbc2 have also been proposed to form a nuclear NMD-independent pathway of mRNA decay. Furthermore Cbc2 and Npl3 have been proposed to perform a telomere capping function ²⁰². Interestingly we identified *cbc2* Δ as a strong suppressor of both the *stn1-13* and *cdc13-1* mutations suggesting it may play an important role in telomere maintenance, similar to NMD (figures 42a-c, table 16).

5.11.4 bts1\(\Delta\) suppresses stn1-13 and cdc13-1 mutant temperature sensitivity

BTS1 is involved in protein prenylation which is a covalent lipid modification made to proteins, to allow hydrophilic proteins to associate with cell membranes. Notably disruption of this gene renders cells cold-sensitive, as this gene is involved in the prenylation of a number of different proteins in the cell ²⁰³. Deletion of this gene in the *stn1-13* and *cdc13-1* background was a strong suppressor of the temperature-sensitivity of *cdc13-1* and *stn1-13* (figure 42a-c, table 16).

5.11.5 rpn4∆ suppresses stn1-13 and cdc13-1 mutant temperature-sensitivity

RPN4 is a transcription factor that affects the expression of proteasome genes. Interestingly, when mutations are introduced into this gene or it is deleted, normally short lived proteins which are degraded because of the "N-end pathway" involving ubiquitin, persist for longer. Furthermore deletion of this gene in the conditional lethal

mutant *sec63-101*, rescues this phenotype. It is thought that this is achieved by stabilization of the partially functional Sec63-101 protein from degradation ²⁰⁴. In both the *cdc13-1* and *stn1-13* screens *rpn4* Δ was identified as a strong suppressor suggesting it may be performing a similar function in these temperature-sensitive backgrounds (figures 42a-c, table 16).

5.12 Discussion

The biochemical results from the first two chapters of this thesis suggested to us that Stn1 binds to telomere DNA poorly, and only when there are extended 3' overhangs. We therefore decided to carry out a genome-wide screen with a temperature-sensitive allele of *STN1*, *stn1-13*, to determine if *STN1* played an analogous role to *CDC13* at telomere ends.

5.12.1 Telomere uncapping in stn1-13 *triggers different resection pathways to* cdc13-1

Our genetic screen identified *exo1* Δ as being a strong suppressor in both the *cdc13-1* and *stn1-13* backgrounds. Furthermore deletion of genes coding for both the MRX complex and Ku heterodimer made *cdc13-1* and *stn1-13* mutants sicker. Taken together this data suggests that similarly to *cdc13-1* ^{86; 100}, *stn1-13* plays an integral role in protecting the telomere ends from resection. However, interestingly while deletion genes coding for the 9-1-1 complex and Pif1 caused strong suppression of temperature sensitivity in the *cdc13-1* background ^{37; 89}, the same interventions with the 9-1-1 complex had no genetic interaction and *pif1* Δ was an enhancer in the *stn1-13* strain. This may indicate that while resection in the *cdc13-1* background is dependent on the combined activity of Exo1, ExoX and ExoY ⁹⁰, resection in the *stn1-13* background could be solely dependent on Exo1.

5.12.2 Lagging strand synthesis is compromised in stn1-13 mutants

Our screen also identified 3/4 members of the lagging machinery as enhancers of the *stn1-13* mutation. Furthermore deletion of *POL32* which is part of pol δ resulted in strong enhancement of temperature sensitivity. This suggested that lagging strand synthesis in *stn1-13* mutants was compromised. This is particularly interesting since Stn1 is proposed to be involved in promoting lagging strand synthesis and when overexpressed is recruited to non-telomeric sites by Pol12¹¹⁵.

5.12.3 Deletion of the G2/M checkpoint compromises the fitness of stn1-13 strains

Notably we also found that if *RAD9* was deleted in the *stn1-13* background cells were substantially sicker at the semi-permissive temperature. This suggests that the G2/M checkpoint is important for maintaining the viability of *stn1-13* strains in contrast to *cdc13-1*, where the phenotype is the opposite. This phenotype has been observed in *stn1* temperature-sensitive mutants before and it has been suggested that such strains require a transient checkpoint delay to complete telomere replication and/or repair damage induced by the mutant Stn1²⁰⁵.

5.12.4 A model for telomere uncapping in stn1-13 mutants

It has been demonstrated previously that some *stn1* temperature-sensitive mutants accumulate ssDNA as internal gaps rather than at telomere ends, and these gaps are generated by impaired lagging strand synthesis ²⁰⁵. We speculate that when *stn1-13* strains are grown at the non-permissive temperature lagging strand synthesis is compromised leading the accumulation of single strand DNA gaps. These regions of ssDNA are further expanded by the activity of Exo1 which has been shown to process stalled replication forks ²⁰⁶. Interestingly, this model might also explain why deletion of the G2/M checkpoint makes these mutants sicker, since they do not have time to repair the damage inflicted by Exo1 at internal regions of the telomere.

5.12.5 Inhibition of cytokinesis improves the fitness of stn1-13 and cdc13-1 strains

Interestingly we found that deletion of genes involved in the inhibition of the anaphase promoting complex as part the spindle checkpoint enhanced the fitness defect exhibited by *stn1-13* and *cdc13-1* strains. Conversely, deletion of genes involved in the removal of inhibition promoted growth. Collectively these results suggest that early activation of the anaphase promoting complex, through deregulation of the spindle checkpoint leads to a fitness defect in telomere capping defective strains. One potential explanation for this fitness defect is that incompletely replicated and/or repaired telomeres are segregated during mitosis and this leads to genomic instability and cell death.

5.12.6 Nonsense-mediated mRNA decay compromises the stability of uncapped telomeres

Similarly to the *cdc13-1* screen ³⁷, our screen identified deletion of the 6 components of the nonsense mediated mRNA decay pathway as strong suppressors of the *stn1-13* mutation. Previous work with *cdc13-1* has suggested that deletion of aspects of the NMD pathway rescues the temperature-sensitivity of this strain by altering the stoichiometry of the three CST components ¹¹⁶. Our screen suggests that deletion of NMD in the *stn1-13* background has a similar suppressive effect suggesting that altering the stoichiometry of CST or increasing the levels of other telomere associated proteins, can rescue the temperature-sensitivity of this strain.

5.12.7 An important role for the N-terminal acetylation complex in maintaining the vitality of stn1-13 and cdc13-1 strains

The genome-wide screens for *cdc13-1*³⁷ and *stn1-13* independently identified all three members of the N-terminal acetylation complex as being strong enhancers of the temperature-sensitive phenotype. The major role of this complex is modify the N-terminus of proteins to prevent their ubiquitination. It is proposed to play an important role in protein quality control and altering the stoichiometry of certain protein complexes ^{181; 182}. This is particularly interesting in light of the proposed role for NMD in altering the stoichiometry of the CST complex ¹¹⁶. Furthermore the role of this complex in regulating telomere length raises the possibility that deletion of members of the NatC complex in the *stn1-13* and *cdc13-1* background may reduce the levels of telomere associated proteins and/or alter the stoichiometry of the CST complex leading to a synthetic phenotype.

5.13 Future work

This genome-wide screen has revealed that two members of the putative CST complex: Cdc13 and Stn1, behave somewhat differently from each other. It has previously been shown that when *stn1-13* cells are grown at the restrictive temperature they accumulate single stranded DNA and activate the G2/M checkpoint similarly to *cdc13-*1. Our screen has confirmed that deletion of a major 5' to 3' exonuclease promotes the growth of *cdc13-1* strains, and this is most likely due to the accumulation of single stranded DNA. An interesting avenue for further work would be to use quantitative amplification of single stranded DNA (QAOS) to determine if the levels of ssDNA generated in *stn1-13 exo1* Δ mutants are lower than those found in *stn1-13* mutants grown at the semi-permissive temperature.

Furthermore we speculated that single stranded DNA accumulates at internal regions of the telomere in *stn1-13* mutants, as a consequence of defective lagging strand replication and degradation by Exo1. It would therefore be interesting to determine what the single stranded DNA looks like in these strains. One way of testing this would be to arrest the cells in G1 phase with α factor, prepare genomic DNA and treat some with the commercially available Exo1 (which digests DNA from the 3' end of the telomere) and Mung Bean nuclease (a single strand endonuclease) ²⁰⁵. The remaining genomic DNA could then be treated with *Xhol* and restriction fragments analysed, and single stranded DNA detected by InGel assay. If the single strand DNA in *stn1-13* mutants was present as internal gaps, we would expect the signal to dissipate following treatment with Mung bean nuclease, whereas if it was present as terminal extensions if would disappear following treatment with Exo1. This assay could then be repeated with *stn1-13 rad9*\Delta strains and *stn1-13* strains with aspects of the lagging strand synthesis knocked down.

Finally it would also be interesting to follow up our finding that the N-terminal acetylation complex plays a role in maintaining to vitality of *stn1-13* and *cdc13-1* strains grown at semi-permissive temperatures. One possibility would be overexpress this complex and see if it could recapitulate the phenotype shown by *cdc13-1 nmd* Δ and *stn1-13 nmd* Δ strains. Further experiments such as chromatin

immunoprecipitation and western blots could then be used to determine if the stoichiometry of the CST complex is altered similarly to NMD and/or if the levels of certain telomere associated proteins are altered.

6. The role of RPA in telomere end protection: A genetic investigation

6.1 Background

Replication protein A is a ubiquitous single stranded DNA binding protein found in all eukaryotes ¹¹⁸. It binds to single stranded DNA with high affinity and plays an important role in preventing the formation of secondary structure. Furthermore RPA is also proposed to play an integral role in homologous recombination both in response to DNA damage and during meiosis by recruiting Rad51 to single stranded DNA encouraging strand invasion and homologous recombination ^{119; 120}. RPA is also proposed to be involved in activating the cell cycle checkpoint by recruiting the ATM/ATR checkpoint machinery ⁴⁴. Therefore RPA plays multiple roles in important DNA transactions in eukaryotes.

Recently it has been found that there are structural similarities between RPA and the telomere capping proteins Cdc13, Stn1 and Ten1¹⁰⁸. Although the existence of Cdc13, Stn1 and Ten1 as a heterotrimeric complex (CST) has yet to be proven conclusively biochemically, genetic evidence suggest they cooperate to prevent the telomere end being processed as a double strand break ^{79; 100; 106}. Interestingly both RPA and at least one member of the putative "CST" complex have strong affinity for single stranded DNA, albeit CST binds with strong specificity to telomere ssDNA ⁹⁹. Furthermore the interactions of RPA and CST with both other proteins and DNA is mediated by OB folds ¹¹⁷. Interestingly it has also been noted that there are significant structural similarities between the two small subunits of the two "complexes" ¹⁰⁸, however evidence for structural similarities between the large subunits is less convincing ¹²².

In addition to these structural similarities it is also becoming increasingly evident that RPA and CST share functionality. For instance RPA is known to promote telomerase activity in *S. cerevisiae* in conjunction with the Ku heterodimer, Est1 and Cdc13¹²³. It also plays an important role in promoting lagging strand synthesis and interacts with both Mcm10 and Pol12^{118; 124}. Furthermore this promotion of lagging strand synthesis has been reconstituted biochemically using mammalian telomere repeats ¹²⁵.

Finally as well as being enriched at telomeres particularly in late S-phase, recent work has suggested that RPA could play a capping role, similarly to the CST complex. It was found that if Rpa1 was mutated in the context of the *cdc13-1* mutation, type II survivors were generated, leading the authors to propose that telomere damage continued to accumulate if Rpa1 was mutated, suggesting it is protecting the telomere end ¹²⁶.

RPA plays an integral role in DNA metabolism in all eukaryotes and increasingly appears to play important telomere specific roles such as involvement in telomerase activity and telomere capping. Furthermore there are clear structural and biochemical similarities between the small subunits of the RPA and CST complexes. Therefore given these functional and structural similarities, and the suggestion that RPA could be performing a capping function, I wondered to what extent the genetic interaction profiles of temperature-sensitive mutations of known capping proteins (*cdc13-1, stn1-13* and *yku70* Δ) overlapped with the genetic interaction profile of a temperature-sensitive allele of RPA, *rpa3-313*. I hope this will yield new insights into what role, if any, RPA might be playing at telomere ends.

6.2 Carrying out the rpa3-313 SGA and QFA

6.2.1 Creation of the rpa3-313 strain

It was decided to carry out a genetic screen with a temperature-sensitive allele of the small subunit of RPA. This particular allele was chosen because it exhibited a tight temperature-sensitive phenotype at 36°C, making it highly amenable to the SGA and QFA process (figure 43a and c). Notably *rpa1-m2* was judged to be an inappropriate strain because colonies were heterogeneous and temperature-sensitivity was poor (figure 43b and c). A similar procedure used to create an *rpa3-313* strain suitable for the SGA and QFA process as had been for the *stn1-13* strain. Briefly, a *LEU2* marker carried on an integrative vector (pDL1600) (figure 44a) was inserted in between *rpa3-313* and *CPS1*. A transformant was then recovered on –LEU and secondarily transformed with the HPH marker cut out from pDL1241 (figure 44b) with restriction enzymes *Bgll1* and *Sac1* which recombined in place of the G418 marker located immediately downstream of *rpa3-313*.

To confirm that these two markers had integrated correctly the final strain was mated with DLY7329, tetrads manually dissected and the germination of spores and growth of colonies analysed on selective media. Correct integration of the selective markers was confirmed by co-segregation of temperature-sensitivity, growth on –leucine and HPH media. Spore analysis (figure 44c and d) confirmed that in 46/49 of dissected haploids the LEU and HPH markers were linked with *rpa3-313* suggesting strongly that they had integrated correctly. An appropriate strain (DLY9181) was then picked to carry out the screen.

DLY9181 was then mated with the DMA and SGA performed. Interestingly in the *rpa3-313* SGA only *kre9* Δ (which was in close proximity to the *HPH* marker, downstream of the *rpa3-313* ORF) appeared to demonstrate synthetic sickness (figure 45). The other gene in close proximity to the marker-linked *rpa3-313* was *CPS1* which is located just upstream of the *LEU2* marker. The fitness of *rpa3-313* cps1 Δ strains was actually slightly higher than the line of best fit (figure 45). One possible explanation for this result could be the greater distance between *CPS1* and the *LEU2* marker both as a consequence of its positioning on the genome, and the insertion of the whole pRS405

vector between the *CPS1* and *LEU2* loci. However, since there was evidence of linkage with *KRE9* and spore analysis had suggested both markers were strongly linked with the temperature sensitive phenotype, it was decided to continue with the screen.

QFA was then performed and correlation coefficients between replicates of the same plates, and variation of fitness between plates was then analysed. Variation between replicates of the same plates was judged to be acceptable since the correlation coefficients were mostly above 0.5 (figure 46b). However there was significant variation between fitness of different master plates as judged from the box plots (figure 46a). Notably, however this variability followed a pattern from the first plate to the last, suggesting that it could be a technical artefact such as a pinning error. Growth curves from each plate were also inspected and the majority of colonies demonstrated growth which fitted the logistic growth model well. The fitness of the query strains ($rpa3-313 xxx\Delta$) was then plotted against the fitness of the corresponding strain from the DMA and results analysed.





Figure 43. Testing the suitability of Replication Protein A temperature-sensitive strains for the SGA and QFA process. A. This plot shows how the fitness (maximal doubling rate, MDR) of an *rpa3-313* strain changes with temperature from 20°C - 38°C. B. Another plot showing how the fitness (MDR) of an *rpa1-M2* strain changes over the same temperature range. C. A serial dilution of a wild type strain (DLY8460), *cdc13-1* strain (DLY1108), two *rpa1-M2* strains taken from different positions in the same collection, and the *rpa3-313* strain, grown at the indicated temperature over 24 hours.








Figure 44. Creating an *rpa3-313* **strain suitable for the SGA and QFA process.** A. A vector diagram of pDL1600 illustrating relevant restriction sites, yeast and bacterial selective markers and the origin of replication. B. A vector diagram of pDL1241 illustrating relevant restriction sites, yeast and bacterial selective markers and the origin of replication. C. A picture of a YEPD plate with spores from dissected tetrads resulting from a cross between *rpa3-313* (marked with *LEU2* and HPH) and DLY7329 placed equidistantly on the plate after 4 days of growth at 23°C. D. Part of a tetrad dissection sheet showing how colonies taken from the first three dissected tetrad on the YEPD plate in figure 2c grew on a range of selective media.



Figure 45. Fitness of *rpa3-313* **strains at 20°C.** *rpa3-313* strains with different gene deletions/knockdowns were grown at 20°C over the course of several days. Coloured points located above the grey line of best fit represent gene deletions or knockdowns that suppress the temperature-sensitivity of *rpa3-313* strains. Coloured points located below the grey line of best fit represent gene deletions or knockdowns that enhance the temperature-sensitivity of *rpa3-313* strains. Out of the highlighted genes *RPA1, 2* and *KRE9* are RNA knockdowns as they are essential genes, while *CPS1* is a gene deletion as it is non-essential.



Figure 46. Variations in fitness between replicates and plates. A. A histogram of pairwise fitness comparisons between all replicates of a single plate grown under the same conditions. Values above 0.5 are considered an acceptable correlation. B. Box plots indicating the distribution of fitnesses across all replicates of a given master plate. Notches in the boxes are an indication of significance of difference, with overlapping notches indicating no significant difference.

6.3 rad17 Δ , rad24 Δ , rad9 Δ , elg1 Δ , rfc5 Δ and pol30 Δ are enhancers of the rpa3-313 temperature-sensitive phenotype

It was decided to investigate the effects of deletion of aspects of the DNA damage response pathway including two components of the 9-1-1 complex (*RAD17* and *DDC1*) the clamp loader (*RAD24*), *RAD9*, an alternative clamp loader (*ELG1*) and finally the 5 components of Replication Factor C (*RFC1-5*). This was decided since deletion of aspects of the DDR pathway in the *cdc13-1* background allows cells to grow more robustly at the semi-permissive temperature through bypass of the G2/M checkpoint ¹⁷⁴. It was therefore surmised that if RPA was performing an analogous role to the putative CST complex similar suppression of *rpa3-313* temperature-sensitivity might be observed. It was also decided to investigate the effect of DAmP PCNA (*POL30*) since overexpression of this gene has been previously shown to suppress the replication defect induced by disruption of the activity of *RFC5* ²⁰⁷.

It is proposed that *RAD9* and *RAD24/RAD17/MEC3* function upstream of *RAD53* and *MEC1*, which signal the presence of DNA damage. It is known that deletion of any of these four genes leads to a reduction in the function of the G1/S and G2/M checkpoints. Furthermore, if both *RAD24* and *RAD9* are deleted together, the G1/S checkpoint is undetectable while the G2/M checkpoint is greatly diminished ²⁰⁸.

Interestingly these results demonstrate that deletion of *RAD9*, *RAD24* or *RAD17* leads to a fitness defect in the *rpa3-313* background at the semi-permissive temperature as evidenced by the positioning of these three points below the line of best fit on the fitness plot (figure 47a). However *rpa3-313 rad24* Δ strains had a much less pronounced fitness defect than the *rpa3-313 rad9* Δ and *rpa3-313 rad17* Δ strains as judged by their positioning on the fitness plot (figure 47a) and the growth curves taken from one of the replicates (figure 47b). (Note- *rpa3-313 bsc5* Δ strains were used as a non-interacting control since *rpa3-313 his3* Δ strains which are normally used for comparison appeared to be suppressive on the temperature-sensitivity of *rpa3-313*. This particular strain was chosen because it was located near the centre of the grey cloud on the fitness plots). Notably, inspection of the plates indicates that *rpa3-313 rad24* Δ strains had mixed phenotypes with some growing nearly as well as wild type,

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while others were very sick (figure 47c). However this could be a consequence of the quadranting effect previously discussed.

Since *rpa3-313* is known to accumulate incomplete replication products at the nonpermissive temperature, which are not adequately recognised by the G2/M checkpoint and are only recognised by the G1/S checkpoint ¹²⁸, one possibility is that this checkpoint is crucial for the viability of *rpa3-313* strains at the semi-permissive temperature.

One possible explanation for this is that compromising the G1/S checkpoint allows cells to continue through the cell cycle in the presence of incomplete replication products leading to genomic instability and cell death or a stronger cell cycle checkpoint at another stage of the cell cycle. Perhaps pausing during G1/S allows time for these incomplete replication products to be repaired. In order to test whether the G1/S checkpoint activation is essential for the viability of these strains at the semi-permissive temperature *RAD9* and *RAD24* could be deleted together which would allow minimal activation of the G2/M checkpoint and no activation of G1/S. It is also intriguing that deletion of *DDC1* did not affect the fitness of the *rpa3-313* strains (figure 47a); however this could be a technical artefact since the deletion library only contains one copy of $ddc1\Delta$.

These results also demonstrated that deletion of *ELG1* which forms an alternative RFC-like complex, and is functionally redundant with *RAD24* ^{209; 210} was an enhancer of the *rpa3-313* temperature-sensitive phenotype as it was located close to *rad9* Δ and *rad17* Δ below the grey cloud of non-interacting genes in the fitness plot (figure 47a). Furthermore, interestingly, of the four subunits of RFC that are proposed to associate with Rad24, only *RFC5* from the DAmP collection significantly affected the fitness of the *rpa3-313* strain. Intriguingly it was the sixth strongest enhancer of the *rpa3-313* temperature-sensitive phenotype of the whole collection and this strong enhancement is evidenced by the positioning of *RFC5* DAmP near the bottom of the fitness plot (figure 47a) and an extremely shallow growth curve (Appendix 4, plate 16).

Notably $elg1\Delta$ mutants show defects in DNA replication in both the absence and presence of DNA damage, leading to the suggestion that *ELG1* plays an important direct role in DNA replication. In particular it is thought that *ELG1* may play an

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important role in lagging strand synthesis, preventing replication fork stalling and encouraging replication fork restart ^{209; 210}. This is particularly interesting in light of the fact that *rpa3-313* mutants accumulate incomplete products of replication. Furthermore *RFC5* temperature-sensitive mutants (*rfc5-1*) develop replication defects and this is proposed to be because *RFC5* plays an important role in sensing incomplete replication and transmitting this signal to the checkpoint machinery ²⁰⁷. One obvious possibility is that incomplete replication products generated as a result of the *rpa3-313* mutation are no longer sensed adequately in the presence of the *RFC5* knockdown mutation allowing cells to continue to divide without repairing the damage and leading to genomic instability. It was also noted that RNA knockdown of *POL30* enhances the temperature-sensitive phenotype of *rpa3-313* (figure 47a). This further supports the notion that *RFC5* could be playing an important role in sensing damage produced as a result of the *rpa3-313* mutation and corresponds well with previous data showing that overexpression of *POL30* suppressed the *rfc5-1* mutation phenotype ²⁰⁷.

Finally our QFA demonstrated that deletion of *EXO1* a key exonuclease involved in resection following telomere uncapping ⁹⁰ had little effect on the fitness of *rpa3-313* strains (figure 47a). Since deletion of both the DDR genes and *EXO1* in the *cdc13-1* background had a strong suppressive effect on fitness (figure 47d, e) these results suggest that it is unlikely that RPA is playing a direct role in protecting the telomere from resection. The interaction profile observed here is more consistent with RPA playing an important role in ensuring complete and efficient DNA replication. However this does not rule out a role for RPA at telomeres, it just suggests that it is more likely to be involved in promoting replication of the telomere and/or is involved in promoting telomerase activity.



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2



cdc13-1 @ 30°C 0.8 Fitness (F) of yfg Δ cdc13-1 at 30°C (MDPxMDR) DDC1 0.6 RAD17 RAD24 EXO1 CHK1 0.4 Ε RAD9 .30 POI 0.2 RFC1 RFC4 RFC 0.0 0.0 0.2 0.4 0.6 0.8 Fitness (F) of yfg Δ ura3 Δ at 30°C (MDPxMDR)

Figure 47. Fitness of rpa3-313 strains with aspects of the DNA damage machinery deleted grown at 30°C. A. rpa3-313 strains with different gene deletions/knockdowns of aspects of the DNA damage response were grown at 30°C over the course of several days and fitness plotted. Coloured points located above the grey line of best fit represent gene deletions or knockdowns that suppress the temperature-sensitivity of rpa3-313 strains. Coloured points located below the grey line of best fit represent gene deletions or knockdowns that enhance the temperature-sensitivity of rpa3-313 strains. Out of the highlighted mutants CHK1, EXO1, RAD17, RAD24, RAD9, DDC1, ELG1 and BSC5 were gene deletions while RFC5, POL30 RFC2, RFC1, RFC4, and RFC3 were RNA knockdowns. B. Representative growth curves taken from plates that had gone through the SGA and QFA procedure in the same batch. BSC5 deletion mutants were used as a non-interacting control for comparison since HIS3 deletion had a suppressive effect on rpa3-313 temperature sensitivity. C. A photograph of plate 15 harbouring CHK1, EXO1, RAD9, RAD24 and RAD17 deletion mutants after growth at 30°C over 4 days. D. cdc13-1 strains with different gene deletions of aspects of the DNA damage response were grown at 27°C over the course of several days and fitness plotted (data taken from Addinall et al 2011). (Note- At the time this screen was carried out there was no DAmP collection available and so these genes are not represented on this plot). E. cdc13-1 strains with different gene deletions/knockdowns of aspects of the DNA damage response were grown at 30°C over the course of several days and fitness plotted (data taken from Addinall et al 2011).

6.4 Ku heterodimer

It was also decided to examine the effects of the Ku heterodimer since this plays an important role not only in non-homologous end joining but also in protecting the telomere end from 5' to 3' resection ⁸⁶. The Ku heterodimer is also involved in the recruitment of telomerase ²¹¹. It was surmised that since both *cdc13-1* and *stn1-13* which are integral "capping" proteins had a synthetic sick interaction with deletion of the two aspects of the Ku heterodimer, then if RPA was involved in capping then *rpa3-313* might also be expected to have similar interactions.

It was found that if either subunit of the Ku heterodimer was deleted in the *rpa3-313* background, there was not a strong effect on fitness. Both *rpa3-313 yku70* Δ and *rpa3-313* yku80 Δ strains were located in the grey cloud of non-interacting genes in the fitness plot (figure 48a) and the sample growth curves were similar to that of the non-interacting control (Appendix 4, plate 10 and 11). However when the same genes are deleted in the *cdc13-1* background (figure 48b) or *stn1-13* background (figure 48c) there is a strong negative effect on fitness. This suggests that the *rpa3-313* mutation does not impact on capping, but since the RPA1/RPA2 complex is proposed to be in tact in this mutant ¹²⁸ it does not rule out a role for RPA in telomere capping. It would be interesting to find out if these results can be recapitulated in the W303 background. Furthermore, to confirm that the *rpa3-313* mutation does not affect classical telomere capping, one possibility would be to measure if ssDNA is generated at the telomere following a shift to the restrictive temperature.





Figure 48. Fitness of *rpa3-313* **strains grown at 30°C with the two subunits of the Ku heterodimer individually deleted.** A. *rpa3-313* strains with the two subunits of the Ku heterodimer individually deleted were grown at 30°C over the course of several days and fitness plotted. B. *cdc13-1* strains with the two subunits of the Ku heterodimer individually deleted were grown at 27°C over the course of several days and fitness plotted (data taken from Addinall et al 2011). C. *stn1-13* strains with the two subunits of the Ku heterodimer individually deleted were grown at 33°C over the course of several days and fitness plotted.

6.5 Deleting any aspect of the MRX complex enhances the temperature sensitivity of *rpa3-313* strains

Next it was decided to investigate what effect deletion of the double strand break processing machinery had on the fitness of *rpa3-313* strains. This is since RPA has previously been implicated as an important component of the long range resection pathways including Sgs1-Top3-Rmi1-Dna2-RPA and Exo1 ¹⁵⁶. Furthermore it was also decided investigate the impact of deletion of aspects of the MRX complex (*MRE11*, *RAD50*, *XRS2*) and *SAE2* since these are involved in short range resection (~300 nucleotides) prior to long range resection ⁴².

Interestingly these results suggest that deletion of aspects of the long range resection pathways had little impact on the fitness of *rpa3-313* strains, in contrast to *cdc13-1* since all of these genes were found in, or close to the grey cloud of non-interacting genes in the fitness plot (figure 49a). However, deletion of the three components of the MRX complex and to a lesser extent *SAE2* enhanced the temperature-sensitive phenotype of *rpa3-313* as judged by their positioning below the grey cloud of non-interacting genes in the fitness plot (figure 49a). Furthermore the growth curves of *MRE11*, *XRS2*, *RAD50* and *SAE2* deletion strains are significantly shallower than the non-interacting control strain (figure 49b, appendix 4- plates 15 and 3), and there is clear inhibition of growth of *rpa3-313 mre11* Δ and *rpa3-313 rad50* Δ strains on visual inspection of the plate (figure 49c).

One possible interpretation of these results is that the *rpa3-313* mutation had inhibited the long-range resection pathways, as this has been shown previously with a temperature-sensitive degron of RPA ²¹². Therefore deletion of their components had little additional effect on fitness. However, it is possible to imagine that in the absence of long-range resection, repair of double strand breaks and stalled replication forks could still be initiated by short range resection by MRX and *SAE2*. Therefore deletion of these genes was much more deleterious to the fitness of *rpa3-313* strains. Notably the interaction profiles of *cdc13-1* (figures 49d and e) were broadly similar to that of *rpa3-313* (figure 49a), except for the positioning of *exo1*\Delta which when deleted was a strong suppressor in the *cdc13-1* screen and had no interaction in the *rpa3-313* screen.

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If, similarly to the temperature sensitive degron of RPA used in the previous study, *rpa3-313* inhibits long range resection, it would be interesting to combine this mutation with *cdc13-1* or *stn1-13*. It might be supposed that the *rpa3-313* mutation may suppress the temperature-sensitivity of these two temperature-sensitive capping mutations, by reducing the amount of resection.







В



Fitness (F) of yfg Δ ura3 Δ at 30°C (MDPxMDR)

Figure 49. Fitness of *rpa3-313* **strains grown at 30°C with aspects of the resection machinery deleted.** A. *rpa3-313* strains with different gene deletions/knockdowns of aspects of the resection machinery were grown at 30°C over the course of several days and fitness plotted. Out of the highlighted mutants *EXO1, RAD50, MRE11, SGS1, XRS2, SAE2, TOP3, RMI1* and *BSC5* were gene deletions while *DNA2* was an RNA knockdown. B. Representative growth curves taken from plates that had gone through the SGA and QFA procedure in the same batch. *BSC5* deletion mutants were used as a non-interacting control for comparison. C. A photograph of plate 15 highlighting *RAD50, MRE11* and *EXO1* deletion mutants after growth at 30°C over 4 days. D. *cdc13-1* strains with different gene deletions of aspects of the resection machinery were grown at 27°C over the course of several days and fitness plotted (data taken from Addinall et al 2011). E. *cdc13-1* strains with different gene deletions/knockdowns of aspects of the resection machinery were grown at 30°C over the course of aspects of the resection machinery were grown at 30°C over the course of aspects of the resection machinery were grown at 27°C over the course of several days and fitness plotted (data taken from Addinall et al 2011). E. *cdc13-1* strains with different gene deletions/knockdowns of aspects of the resection machinery were grown at 30°C over the course of several days and fitness plotted (data taken from Addinall et al 2011).

6.6 Deletion of aspects of the nonsense-mediated mRNA decay (NMD) pathway enhances the *rpa3-313* temperature-sensitive phenotype

Given the role of nonsense-mediated mRNA decay in regulating the RNA levels of various telomere-associated proteins ¹⁷³, and the proposal that it acts in a parallel pathway to Rad24 and Exo1 to suppress *cdc13-1* temperature-sensitivity ¹¹⁶, I wondered whether deletion of aspects of NMD would suppress temperature-sensitivity of rpa3-313 mutants similarly to *cdc13-1*.

It was found that deletion of aspects of the NMD machinery in contrast to its effect in *cdc13*-1 enhanced the temperature-sensitivity of *rpa3-313* strains similarly to its effect in *yku70* Δ strains ¹⁷⁴. This was judged by the positioning of *kem1* Δ , *upf3* Δ , *nmd2* Δ , *nam7* Δ and *ebs1* Δ on the *rpa3-313* fitness plot below the grey cloud of non-interacting genes (figure 50a) similarly to their positioning on the yku70 Δ fitness plot (figure 50b). This was in contrast to their positioning in the *cdc13-1* fitness plot above the grey cloud of non-interacting genes as strong suppressors (figure 50c). Furthermore, the growth curves of *kem1* Δ , *upf3* Δ , *nmd2* Δ , *nam7* Δ and *ebs1* Δ suggested a significantly lower area under the curve than the non-interacting control strain (appendix 4, plates 3, 5, 6, 10, 15). The growth curves indicated that *rpa3-313 kem1* Δ and *rpa3-313 nmd2* Δ strains were particularly sick compared to the non-interacting control (figure 50d) and visual inspection of the plates indicated that both showed strong inhibition of growth, but particularly *rpa3-313 kem1* Δ (figure 50e).

Since the enhancement of the yku70 Δ phenotype by deletion of NMD could be recapitulated by overexpression of *STN1*¹⁷⁴, it would be interesting to determine if a similar genetic intervention could enhance the temperature-sensitivity of *rpa3-313*, as this may suggest a role for RPA in telomere capping.



cdc13-1 @ 27°C Fitness (F) of yfg∆ cdc13-1 at 27°C (MDPxMDR) A₩ C KEM1 Fitness (F) of yfg $\!\!\!\Delta$ ura3 $\!\!\!\Delta$ at 27°C (MDPxMDR)



Figure 50. Fitness of *rpa3-313* **strains grown at 30°C with aspects of the nonsensemediated mRNA decay (NMD) pathway deleted.** A. *rpa3-313* strains with different gene deletions of aspects of the NMD pathway were grown at 30°C over the course of several days and fitness plotted. B. *yku70*Δ strains with different gene deletions of aspects of the NMD pathway were grown at 37°C over the course of several days and fitness plotted (data taken from Addinall et al 2011). C. *cdc13-1* strains with different gene deletions of aspects of the NMD pathway were grown at 27°C over the course of several days and fitness plotted (data taken from Addinall et al 2011). D. Representative growth curves taken from plates that had gone through the SGA and QFA procedure in the same batch. *BSC5* deletion mutants were used as a noninteracting control for comparison. E. A photograph of plate 5 highlighting *KEM1* and *NMD2* deletion mutants after growth at 30°C over 4 days.

6.7 *rpa3-313* does not obviously impact on the telomerase-promotion activity of the RPA heterotrimer

Previous studies have implicated RPA as playing an important role in promoting telomerase activity by forming a transient complex with the Ku heterodrimer, Cdc13 and telomerase ¹²³. Furthermore, recently work has suggested that RPA might contribute to telomere end protection ¹²⁶. Given that previous QFAs identified *est1* Δ , *est3* Δ and *tel1* Δ as enhancers of both the *cdc13-1* and *yku70* Δ strains (figure 51a and b) ¹⁷⁴ we wondered if the same genes might also enhance the temperature-sensitivity of *rpa3-313* strains, given the possible involvement of RPA in telomerase activity.

Interestingly, it was found that deletion of either *EST1* or *EST3* had little effect on the fitness of *rpa3-313* strains as judged by their positioning in, or close to the cloud of non-interacting genes in the *rpa3-313* fitness plot (figure 51c), suggesting that this strain may not have any significant impact on telomerase activity. Furthermore deletion of *TEL1* which is a checkpoint kinase that is also proposed to play an important role in promoting telomerase activity was in contrast to the *cdc13-1* and *yku70* Δ screens a mild suppressor of the *rpa3-313* mutation (figure 51a). Collectively these results suggest that the *rpa3-313* mutation does not strongly impact on the telomerase promotion activity of RPA.







Figure 51. Fitness of *rpa3-313* strains grown at 30°C with the subunits of telomerase and *TEL1* individually deleted. A. *cdc13-1* strains with the three subunits of the telomerase enzyme and *TEL1* individually deleted were grown at 27°C over the course of several days and fitness plotted (data taken from Addinall et al 2011). B. *yku70* Δ strains with the three subunits of the telomerase enzyme and *TEL1* individually deleted were grown at 37°C over the course of several days and fitness plotted (data taken from Addinall et al 2011). C. *rpa3-313* strains with the three subunits of the telomerase enzyme and *TEL1* individually deleted were grown at 30°C over the course of several days and fitness plotted.

6.8 Lagging strand synthesis is compromised in *rpa3-313* mutants

Since the *rpa3-313* mutation seemed to have a weak interaction with aspects of the telomere capping and telomerase machinery, it was asked whether the *rpa3-313* mutation might compromise lagging strand synthesis in these strains, and therefore impact on telomere replication. Lagging strand synthesis is carried out primarily by DNA polymerase α (consisting of the subunits Pol1, Pri1, Pri2 and Pol12) and DNA polymerase δ (consisting of the subunits Pol3, Pol31 and Pol32). It is thought that pol α synthesises the small RNA primers and initiates DNA synthesis, which is then completed by high fidelity pol δ ¹⁷⁵.

It was therefore decided to investigate the effect of 6 of these genes on the fitness of *rpa3-313* strains. Interestingly, this screen only identified deletion of one member of DNA polymerase α as having a strong effect on fitness. Perturbing the levels of mRNA of *PRI1* had a strong negative effect on fitness, but similar interventions with *PRI2* which is proposed to improve the efficiency of *PRI1* priming had no effect on fitness ²¹³. Furthermore perturbing the levels of mRNA for the catalytic subunit of DNA polymerase α (*POL1*) also had no effect on fitness (figure 52a). This suggests that disrupting lagging strand RNA priming has a moderate negative impact on the fitness of *rpa3-313* strains, and suggests that *rpa3-313* mutants may have lagging strand synthesis compromised at the restrictive temperature.

Interestingly, deletion of *POL32* which is required for processive DNA synthesis by DNA polymerase δ^{214} had a very strong negative effect on fitness in the *rpa3-313* background (figure 52a). However RNA knockdown of *POL3* (the catalytic subunit of DNA polymerase δ) had no effect on fitness of the *rpa3-313* strain (figure 52a) and unfortunately *POL31* was not available in the DAmP collection. These results again tentatively suggest that when *rpa3-313* strains are grown at the semi-permissive temperature, lagging strand synthesis may be compromised. It is also interesting to note that deletion of *RAD27* which is involved in cleaving the small flaps generated during okazaki fragment processing ²¹⁵ also had a very strong negative genetic interaction with *rpa3-313* and was located in a similar area of the fitness plot (figure 52a). However deletion of *DNA2* which is proposed to be involved in cleaving longer

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flaps created by the action of *PIF1* ²¹⁶ had no such effect (figure 52a). Inspection of the QFA plot (figure 52a) suggested that growth was most strongly inhibited for DAmP *pri1, pol32* Δ and *rad27* Δ delete strains. Furthermore, interestingly the genetic interactions of *rpa3-313, cdc13-1,* and *stn1-13* with the lagging strand machinery highlighted here are broadly similar (figure 52a-c).

Collectively these results suggest that aspects of lagging strand synthesis are compromised in *rpa3-313* mutants similarly to *cdc13-1* and *stn1-13* strains. It would be interesting to recapitulate these results in the W303 genetic background. Furthermore it would also be interesting to show biochemically that lagging strand synthesis is compromised in this mutant, and determine whether this compromised lagging strand synthesis affects telomere maintenance, such as through causing replication fork stalling and/or telomere shortening.





Figure 52. Fitness of *rpa3-313* strains grown at 30°C with aspects of the lagging strand machinery deleted. A. *rpa3-313* strains with aspects of the lagging strand machinery deleted were grown at 30°C over the course of several days and fitness plotted. B. *cdc13-1* strains with aspects of the lagging strand machinery deleted were grown at 27°C over the course of several days and fitness plotted (data taken from Addinall et al 2011). C. *stn1-13* with aspects of the lagging strand machinery deleted were grown at 33°C over the course of several days and fitness plotted.

6.9 Common suppressors and enhancers in the *cdc13-1* and *rpa3-313* screen

Common suppressors	Common enhancers
kre1∆	elg1∆
rpl34a∆	nup133∆
	mrc1∆
	sit1∆
	pol32Δ

Table 17. Common suppressors and enhancers identified in the rpa3-313 and cdc13-1screens. The top 100 suppressors and enhancers in the rpa3-313 and cdc13-1 screen,as assessed by genetic interaction score were compared and common suppressors andenhancers identified using Venny.

As with *stn1-13* in order to make a more global comparison of the suppressors and enhancers of *rpa3-313* and *cdc13-1* we compared the top 100 GIS scores that suppressed or enhanced the two mutations (Appendix 6). 2 common suppressors were identified and 5 common enhancers (table 17).



Figure 53. Fitness of *rpa3-313* strains grown at 30°C highlighting the common suppressors and enhancers identified in both screens. *rpa3-313*, and *cdc13-1* strains were grown at the indicated temperatures over the course of several days and fitness plotted. A. An *rpa3-313* fitness plot showing the positioning of the common suppressors and enhancers. B. A *cdc13-1* fitness plot showing the positioning of the common suppressors and enhancers when *cdc13-1* was grown at 27°C (data taken from Addinall et al 2011). C. A *cdc13-1* fitness plot showing the positioning of the common suppressors and enhancers when *cdc13-1* was grown at 30°C (data taken from Addinall et al 2011).

6.9.1 KRE1 and RPL34A are likely to be artefacts of the experiment

Rpl34a is a subunit of the 60S ribosome which contains 46 proteins and 3 RNA molecules ²¹⁷. Given that the *rpa3-313* screen only identified *RPL34A* is out of dozens of genes coding for different subunits of the 60S ribosome in the top 100 suppressors, and the position of *RPL34A* on the fitness chart is very close to grey group of non-interacting genes (figure 53a), it would seem unlikely that this result is of biological significance.

Kre1 is involved in the assembly of beta-glucan which is an important component of budding yeast cell walls ²¹⁸. Interestingly the fitness of *kre1* Δ on *rpa3-313* fitness plot is in line with the grey group of non-interacting genes. This gene came up as a strong suppressor in the *rpa3-313* screen because strangely the *RPA3 kre1* Δ mutant was dead (figure 53a), possibly as a result of extended storage or pinning mistakes. Therefore it seems likely that this result is also an artefact.

6.9.2 elg1∆ and pol32∆ are strong enhancers of the rpa3-313 temperature-sensitive phenotype

Both of these genes have already been mentioned in the results above, but briefly Elg1 is proposed to form an alternative RFC-like complex and functionally redundant with Rad24. Both *ELG1* and *RAD24* deletions were identified as enhancers of the *rpa3-313* phenotype, but only *elg1* Δ was identified as an enhancer in the *cdc13-1* screen. This is particularly interesting because *ELG1* is proposed to play important roles in lagging strand synthesis and prevention of replication fork stalling ^{209; 210}. This further supports a role for both Cdc13 and RPA in lagging strand synthesis.

POL32 is required for processive DNA synthesis by DNA polymerase δ , and the strong negative interaction of pol32 Δ with both *cdc13-1* and *rpa3-313* coupled with the fact that rad27 Δ appears close to it in both the *cdc13-1* and *rpa3-313* plots, supports the notion that Cdc13 and RPA play important roles in okazaki fragment processing.

6.9.3 nup133∆ is a strong enhancer of the rpa3-313 temperature-sensitive phenotype

Nup133 is a component of the Nup84 subcomplex of the nuclear pore complex (NPC). It plays an important role in maintaining the architecture of the nuclear envelope ²¹⁹ and is also involved in modulating various processes that require localisation of chromosomes at the nuclear periphery, including double strand break repair and telomere silencing ²²⁰. Interestingly we found that deletion of *NUP133* in both the *rpa3-313* and *cdc13-1* background had strong negative effects on fitness (figure 53a-c). One possible explanation for this result is that errors in telomere replication such as replication fork stalling and accumulation of incomplete replication products are not repaired adequately when *NUP133* is deleted, since nuclear localisation of telomeres is proposed to be important for repair of DNA damage in these regions ²²⁰. The first step in testing this hypothesis would be to delete different aspects of the nuclear pore complex in the W303 background to determine if similar fitness defects were observed.

6.9.4 mrc1∆ is a strong enhancer of the cdc13-1 and rpa3-313 temperature-sensitive phenotypes

Mrc1 is an active component of the replication fork machinery ²²¹ and is thought form a replication pausing complex with Tof1 when replication stress is encountered. This replication pausing complex activates the Rad53 checkpoint pathway and allows DNA repair to take place after which replication is re-initiated ²²². Interestingly it has also been found that Mrc1 directly protects telomeres from resection by Exo1, and so performs a classical "capping" function ²²³. This screen identified the *MRC1* deletion as a strong enhancer of the *rpa3-313* and *cdc13-1* temperature-sensitive phenotype, with the *MRC1* delete strain being located below the grey cloud of non-interacting genes in the *rpa3-313* and *stn1-13* screens (figure 53a-c). The growth curves suggested it had a similar poor growth phenotype to *rpa3-313 pol32* Δ and *rpa3-313 rad27* Δ (appendix 4plates 2, 7, 8). Notably while the replication pausing partner, *tof1* Δ was also identified as a strong enhancer in the *cdc13-1* screen, no interaction was observed with *rpa3-313*. It would be interesting to determine whether the synthetic sick phenotype seen in the *rpa3-313 mrc1* Δ strains was due to accumulation of ssDNA at the telomere or
errors in replication. The former possibility could be tested simply by measuring single stranded DNA at telomeres in *rpa3-313* strains and comparing this to *rpa3-313 mrc1* Δ and *rpa3-313 mrc1* Δ exo1 Δ strains.

6.9.5 SIT1 deletion was synthetic lethal with the rpa3-313 strain at the nonpermissive temperature

Sit1 is involved in iron homeostasis and is transcribed when cells run out of one glucose source and switch to another (diauxic shift) or when iron in the environment is restricted ²²⁴. Deletion of *SIT1* was lethal even at the non-permissive temperature as indicated by both the growth curves (appendix 4- plate 4) and the *rpa3-313* fitness plot (figure 53a) when combined with the *rpa3-313* mutation, so it is difficult to infer biological significance. However it is interesting to note that deletion of the same gene in the *cdc13-1* background did not cause lethality (figure 53b-c).

6.10 Discussion

The biochemical data from Chapter 3 and 4 of this thesis suggest that RPA is able to protect telomere ends from degradation by an Exo1-like exonuclease. This data was particularly interesting in light of the biochemical and structural similarities that have been highlighted between RPA and the putative CST complex ^{108; 117}. Given this structural and functional overlap I wondered if RPA might play a similar capping function to CST. To test this hypothesis I carried out high-throughput genetic screen and quantitative fitness analysis to determine the extent to which the *rpa3-313* and *cdc13-1* genetic interactions overlapped.

6.10.1 The rpa3-313 mutation does not appear to induce a capping defect

The *cdc13-1* screen identified *EXO1* deletion as a strong suppressor of the *cdc13-1* temperature sensitive phenotype, and deletion of the Ku heterodimer as a strong enhancer. My *rpa3-313* screen on the other hand suggested that deletion of *EXO1* or either component of the Ku heterodimer had little impact on the fitness of *rpa3-313* strains. Furthermore while deletion of aspects of the DNA damage response pathway generally induced a suppressive effect on temperature-sensitivity in the *cdc13-1* background, deletion of these same genes in the *rpa3-313* background generally induced an enhancing effect. Taken together this data suggests that it is unlikely that *rpa3-313* induces a defect in telomere capping like *cdc13-1*. A more likely scenario is that since *rpa3-313* is proposed to cause the accumulation of incomplete replication products ¹²⁸, the DDR proteins are necessary to pause the cell cycle and allow time for repair. Deletion of the genes coding for them is likely to lead to genomic instability and cell death.

*6.10.2 T*he double strand break machinery is important for the vitality of rpa3-313 strains

Similarly to *cdc13-1* deletion of *MRE11, RAD50, XRS2 or SAE2* in the *rpa3-313* background led to enhancement of the temperature sensitivity of these strains. However, it would seem unlikely that this is as a result of the proposed protective function of MRX at telomeres ¹⁰⁵, although I cannot rule this out. The reason this

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seems unlikely is because deletion of a major exonuclease involved in telomere resection, *EXO1*, did not suppress the temperature-sensitivity of these strains, and deletion of DDR genes enhanced rather than suppressed this temperature-sensitivity. One hypothesis is that since MRX and Sae2 are involved in short-range resection following double strand breaks ⁴², and other forms of DNA damage, and represent an important initial stage of DNA damage processing, deletion of these genes compromises the ability of *rpa3-313* strains to fix the damage induced when they are grown at the semi-permissive temperature. Interestingly deletion of genes involved in either of the long-range resection pathways i.e. Sgs1-Dna2 and Exo1 had little impact on fitness of these strains. One potential explanation for this may be that *rpa3-313* has inhibited these long-range resection pathways, and similar observations have been reported using another temperature-sensitive degron of RPA ²¹².

6.10.3 The rpa3-313 mutation does not obviously impact on the telomerasepromotion activity of RPA

As previous work has identified RPA as being important in promoting telomerase activity along with Cdc13, the Ku heterodimer and Est1 ¹²³, and previous screens with *cdc13-1* and yku70 Δ demonstrated that deletion of *EST1, EST3* and *TEL1* enhanced their temperature-sensitivity, I wondered if in a similar manner deletion of *EST1, EST3* and *TEL1* in the *rpa3-313* background might cause increased temperature-sensitivity, due to its involvement in telomerase activity. Interestingly, deletion of *EST1* or *EST3* in the *rpa3-313* background had no impact on the fitness of these strains at the semi-permissive temperature. Furthermore deletion of *TEL1* which is proposed to promote telomerase activity ²²⁵ was actually a mild suppressor. These results suggest that the *rpa3-313* mutation does not strongly impact on the ability of RPA to promote telomerase activity.

6.10.4 Lagging-strand synthesis is compromised in rpa3-313 mutants

Interestingly, we found that deletion of *POL32* which is required for processive DNA synthesis by DNA polymerase δ^{214} , and mRNA perturbation of *PRI1* which is sufficient on its own to synthesise the RNA primers required for lagging strand synthesis ²¹³, led to a synthetic sick phenotype in the *rpa3-313* background. This was a similar result to

that seen in both the *cdc13-1* and *stn1-13* backgrounds, suggesting that RPA and CST share a common function in promoting lagging strand synthesis. Furthermore in addition to the interaction observed with *pol32* Δ , in both the *cdc13-1* and *rpa3-313* backgrounds *RAD27* deletion was identified as a strong enhancer of the temperature-sensitive phenotype. This suggests that in both the *rpa3-313* and *cdc13-1* backgrounds okazaki fragment processing, specifically the removal of short flaps ²¹⁵ generated following DNA synthesis by DNA polymerase δ , is compromised. These results suggest a possible mechanism through which RPA might be affecting telomere homeostasis, through promotion efficient lagging strand synthesis of the telomere.

6.11 Further work

The *rpa3-313* quantitative fitness analysis has demonstrated that the *rpa3-313* and *cdc13-1* temperature-sensitive mutations have quite dissimilar roles in genome stability. In particular, I have found that deletion of aspects of the DDR pathway negatively impacts on fitness of *rpa3-313* strains while promoting the growth of *cdc13-1* strains. It would be interesting to determine whether the G1/S checkpoint which I suspect is responsible for allowing growth of *rpa3-313* strains. One potential way of addressing this would be to simultaneously delete both *RAD9* and *RAD24* which would allow minimal activation of G2/M and no activation of G1/S. Inviability at the semi-permissive temperature or below would suggest that the G1/S checkpoint is necessary to repair the incomplete replication products generated by *rpa3-313*.

Furthermore I have also found that deletion of *EXO1* does not suppress the temperature-sensitivity of *rpa3-313* strains, or enhance the temperature-sensitivity of strains deleted for either subunit of the Ku heterodimer. This suggests that *rpa3-313* mutants are not defective for telomere capping. In order to confirm this hypothesis it would be necessary to recapitulate this result in the W303 background and measure single stranded DNA at the telomere using a Q-PCR procedure called quantitative amplification of ssDNA (QAOS) ¹⁴⁵ in wild type, *rpa3-313*, and *rpa3-313 exo1* Δ strains.

These results also suggest that full functionality of the double strand break processing machinery may be necessary for the vitality *rpa3-313* mutants. This is since deletion of *MRE11, RAD50, XRS2* or *SAE2* makes *rpa3-313* mutants sicker at the semi-permissive temperature. However interestingly deletion of genes involved in the long-range resection pathways involving Exo1 and Sgs1-Dna2 had no effect on their fitness. One hypothesis is that *rpa3-313* mutants are defective not only in completing replication properly, but also may have inhibited long-range resection. One way of testing this hypothesis would be measure single stranded DNA generated at a HO-cut site in *rpa3-313, rpa3-313 exo1*Δ, *rpa3-313 dna2*Δ and wild type *S. cerevisiae*. If this hypothesis was correct it might be expected that the single stranded DNA generated would differ very little between the *rpa3-313* strains, and would be significantly reduced compared

to wild type. If this were true it might also be interesting to combine the *rpa3-313* mutation with *cdc13-1* to see if the *rpa3-313* mutation suppresses *cdc13-1* temperature-sensitivity, by reducing resection at the telomere end.

Finally, since *stn1-13*, *cdc13-1* and *rpa3-313* temperature-sensitivity all seem to be enhanced by deletion of aspects of the okazaki fragment processing machinery, it would be interesting to determine to what extent defects in lagging strand synthesis contribute to the generation of single stranded DNA at telomeres. One possible way of doing this would be to generate mutants of *cdc13-1*, *stn1-13* and *rpa3-313* which are deleted for *POL32*, *RAD27* or mRNA perturbed for *PRI1* and determine the levels of single stranded DNA generated at their telomeres, compared to mutants knocked out for *EXO1* or other elements of the resection machinery.

6. Summary

6.1 Achievements

An *in vitro* telomere protection assay has been successfully established and the capacity of Cdc13, Stn1, Ten1 and RPA to protect telomeres has been directly tested. It was found that Cdc13, the DNA binding domain of Cdc13 and the RPA heterotrimer strongly protect telomere substrates but not analogous non-telomere substrates from 5' to 3' resection by lambda exonuclease. It has also been demonstrated that the DNA binding domain of Cdc13 can outcompete the RPA heterotrimer for binding to telomere substrates but not non-telomere controls. Finally it was found that the two smaller subunits of the CST complex, Stn1 and Ten1, were not able to protect telomere or non-telomere substrates from resection. Attempts were also made to purify the CST complex in both *E. coli* and *S. cerevisiae*, however these were unsuccessful, and it is still unclear whether this is because the subunits to not stably associate *in vivo* or because of technical issues relating to expression systems. Furthermore hybrid complexes were not formed following co-expression of the subunits of RPA and CST.

In addition, synthetic genetic array and quantitative fitness analysis have been carried out with temperature-sensitive *rpa3-313* and *stn1-13* and comparisons made with a previous screen carried out with *cdc13-1*. It was found that deletion of *EXO1* in the *stn1-13* background rescued temperature-sensitivity in a similar manner to deletion of *EXO1* in the *cdc13-1* genetic background. Furthermore deletion of aspects of the nonsense-mediated mRNA decay pathway was a strong suppressor of temperature sensitivity in the *cdc13-1* and *stn1-13* backgrounds. These results support the prevailing model that Cdc13 and Stn1 collaborate to cap telomere ends. However, interestingly it was found that deletion of *RAD9*, which rescues temperature-sensitivity in the *cdc13-1* background, enhanced temperature-sensitivity in the *stn1-13* background. This suggests that the G2/M checkpoint is integral for the vitality of *stn1-13* and *cdc13-1* background had no effect on fitness, in contrast to its effects in the *stn1-13* and *cdc13-1* backgrounds. Furthermore deletion of *EXO1* or *RAD9* did not suppress the temperature-sensitivity of *rpa3-313* strains, and deletion of *RAD9* enhanced the

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temperature-sensitivity of *rpa3-313* strains. Collectively these results suggest that the temperature-sensitivity phenotype of *rpa3-313* is not primarily caused by defective telomere capping, in contrast to *cdc13-1* and *stn1-13*.

6.2 Further work

Further investigation of the roles of CST and RPA in telomere end protection will necessitate the purification of the whole CST complex, to compare its activity with that of the subunits in isolation. In order to successfully purify this complex high-throughput expression screening could be carried out to determine the optimum conditions for assembly of the complex and sufficient yields. One such service is the Oxford Protein Purificiation Facility which uses a suite of pOPIN vectors which are compatible with *E. coli*, mammalian and insect expression systems, and cloning is facilitated through the use of recombination-based cloning systems. Furthermore, it would be interesting to compare the ability of CST and RPA to protect telomere substrates in the presence of lambda exonuclease and an exonuclease derived from *S. cerevisiae* such as Exo1.

In order to follow-up the finding that *stn1-13* and *cdc13-1* both have similar interactions with *EXO1* and aspects of the nonsense-mediated mRNA decay pathway but opposite interaction with *RAD9*, it would be interesting to investigate the accumulation of single stranded DNA in these mutants. Use of quantitive amplification of single stranded DNA would facilitate investigation of quantity of single stranded DNA generated. However, it has been hypothesised that the single stranded DNA generated in *stn1-13* mutants is distinct from *cdc13-1* mutants because it is generated internally. To test this hypothesis InGel assays could be carried out following treatment with commercially available Exo1 and Mung Bean endonuclease. Finally, as the results of the *rpa3-313* screen are all in the S288C *S. cerevisiae* genetic background, these could be confirmed by manual recapitulation in the W303 background.

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8. Appendices

Appendix 1: Mass spectroscopy data

(MATRIX) Mascot Search Results

User	: JOeG
Email	: joe.gray@ncl.ac.uk
Search title	: MG_T_0001.dat - SpecView
Database	: NCBInr 20111105 (15916306 sequences; 5467648827 residues)
Timestamp	: 10 Nov 2011 at 16:10:44 GMT
Top Score	: 373 for gi 6319981, Cdc13p [Saccharomyces cerevisiae S288c]

Mascot Score Histogram

Protein score is -10*Log(P), where P is the probability that the observed match is a random event. Protein scores greater than 85 are significant (p<0.05).





Protein Summary Report

Format As	Protein Summary	<u>Help</u>
	Significance threshold p< 0.05	Max. number of hits 50

Re-Search All Search Unmatched

Index

Score Description

	Accession	Mass	Score	Description
1.	g1 6319981	105978	373	Cdc13p [Saccharomyces cerevisiae S288c]
2.	gi 171181	105936	373	cell division cycle protein [Saccharomyces cerevisiae]
3.	g1 349576864	105923	358	K7_Cdcl3p [Saccharomyces cerevisiae Kyokai no. 7]
4.	gi 151941787	106092	298	single-stranded TG1-3 telomere G-tails binding protein [Saccharomy
5.	g1 190405212	105985	281	single-stranded TG1-3 telomere G-tails binding protein [Saccharomy
6.	g1 256271002	106011	281	Cdcl3p [Saccharomyces cerevisiae JAY291]
7.	g1 259145802	105999	281	Cdc13p [Saccharomyces cerevisiae EC1118]
8.	g1 323309920	105970	281	Cdc13p [Saccharomyces cerevisiae Fosters0]
9.	Mixture 1		183	gi 207347163 + gi 207347157
10.	g1 207347163	55460	170	YDL220Cp-like protein [Saccharomyces cerevisiae AWRI1631]
11.	g1 300824148	78218	101	UvrD/REP helicase [Escherichia coli MS 119-7]
12.	g1 345300219	86208	98	Prophage tail length tape measure [Enterobacter asburiae LF7a]
13.	g1 331676752	81863	98	helicase IV (75 kDa helicase) [Escherichia coli H591]
14.	g1 20664217	23812	94	Chain A, Solution Structure Of The Cdc13 Dna-Binding Domain In A C
15.	Mixture 2		92	g1 320196549 + g1 297733825
16.	Mixture 3		92	g1 335576507 + g1 297733825
17.	gi 157155618	78189	91	DNA helicase IV [Escherichia coli E24377A]

(MATRIX) Mascot Search Results

User	: 30eG
Email 1	: joe.gray@mcl.ac.uk
Search title	: M0_B_0001.dat - SpecView
Database	: NCBInr 20111105 (15916306 sequences; 5467640027 residues)
Timestamp	: 10 Nov 2011 at 15:48:09 GMT
Top Score	: 197 for gi 197725000, Chain A, D7 Symmetrized Structure Of Unliganded Groel At 4.2 Angetrom Resolution By Crycem

Mascot Score Histogram

Protein score is -10*Log(P), where P is the probability that the observed match is a random event. Protein scores greater than 85 are significant $(p{<}0.05)$.



B

Protein Summary Report

Format As	Protein Summary	Help
	Significance threshold p= 0.05	Max. number of hits

Re-Search All Search Unmatched

Index

	Accession	Mass	Score	Description
2.	g1 197725088	55406	197	Chain A. D7 Symmetrized Structure Of Unliganded Groel At 4.2 Angetrom Resolution By Crycem
2.	g1 61679903	55224	195	Chain A. Groel
3.	g1 61679937	\$\$3\$2	195	Chain A, Groell4-(Atpgatman)14
4.	g1138492781	\$\$295	194	Chain A. Crystal Structure Of Groel-Gross
5.	g1 197725074	\$\$\$37	194	Chain A. C7 Symmetrized Structure Of Unliganded Groel At 4.7 Angetrom Resolution From Crycem
6.	g111421648	\$7202	185	Chain A. Conformational Variability In The Refined Structure Of The Chaperonin Groel At 2.8 Angetro
7.	01138491454	\$7439	183	GroEL (Recherichia coli)
	g1 18655582	\$7262	183	Chain A. Solution Structure Of Apo Groel By Cryo-Electron Microscopy
9.	g1 2624772	\$7333	182	Chain A. Crystal Structure Of The Asymmetric Chaperonin Complex GroelGROES(ADP)7
10.	g1[18028150	57404	182	GroßL [Escherichia coli]
11.	g1128491478	\$7434	142	GroßL (Recherichia coli)
12.	g11157831241	\$7351	182	Chain A. The Crystal Structure Of The Bacterial Chaperonin Groel At 2.8 Angstrong
13.	g1 333361055	55294	191	Chain A. Crystal Structure Of The Allosteric-Defective Chaperonin Groel E414k Mutant
14.	g1[15034370	\$7464	180	chaperonin GroßL (Escherichia coli 0157:H7 str. Sakai)

(MATRIX) Mascot Search Results

User	: Joed
Email	: joe.gray@ncl.ac.uk
Search title	<pre>MG_0001.dat - SpecView</pre>
Database	: NCBInr 20120519 (18132328 sequences; 6219145704 residues)
Taxonomy	: Saccharomyces Cerevisiae (baker's yeast) (43907 sequences)
Timestamp	: 23 May 2012 at 10:55:31 GMT
Top Score	: 83 for g1 6323038, Tenlp [Saccharomyces cerevisiae S288c]

Mascot Score Histogram

Frotein score is -10*Log(F), where F is the probability that the observed match is a random event. Frotein scores greater than 59 are significant $(p{<}0.05)$.



Index

Accession	Mass	Score	Description
1. g1 6323038	18592	83	Tenlp [Saccharomyces cerevisiae S288c]
2. gi 323303962	18503	70	Tenlp [Saccharomyces cerevisiae FostersB]
3. g1 323308058	18557	70	Tenlp [Saccharomyces cerevisiae Fosters0]
. g1 256272332	18594	69	Tenlp [Saccharomyces cerevisiae JAY291]
5. g1 349579736	18626	68	K7_Tenlp [Saccharomyces cerevisiae Kyokai no. 7]
6. g1 151945562	52672	48	conserved protein (Saccharomyces cerevisiae YJN789

Masco	t	Search Results
User	÷	tf2014
Email	÷	
Search title	÷	Ultraflex_ProteinID
MS data file	÷	13229323905427973.mgf
Database	÷	Uniprot sprot20140219 (542503 sequences; 192888369 residues)
Timestamp	÷	19 Mar 2014 at 12:14:47 GMT
Enzyme	÷	Trypsin
Fixed modifications	÷	Carbamidomethyl (C)
Variable modifications	÷	Deamidated (NQ), Oxidation (M)
Mass values	÷	Monoisotopic
Protein Mass	÷	Unrestricted
Peptide Mass Tolerance	÷	± 100 ppm
Fragment Mass Tolerance		± 0.5 Da
Max Missed Cleavages	÷	1
Instrument type	÷	MALDI-TOF-TOF
Number of queries	ŝ	10
Protein hits	÷	<u>C0Q6A2</u> 60 kDa chaperonin OS=Salmonella paratyphi C (strain RKS4594) GN=groL PE=3 SV=1

Select Summary Report

Format As	Select Summa	ary (protein hits)) 🔻				He	<u>lp</u>	
	Significance th	reshold p< 0.0	5	Max. numb	er of hits	AUTO			
	Standard score	ing 🖲 MudPl'	T scoring 💿	Ions score	or expec	t cut-off 0.05	Sh	ow sub-se	ets 0
	Show pop-up	s 🖲 Suppress	pop-ups 💿				Re	quire bold	red 🔽
	Preferred taxo	nomy All entrie	es			•			
Re-Search	 All queri 	es 🔍 Unass	igned 🔘 B	elow homol	logy three	shold © Bel	low ider	ntity thresh	old
1. <u>C0Q6</u> 60 k	A2 Mass: S Da chaperonin	57421 Sco 1 OS=Salmone	ore: 958 ella paraty	Matches: phi C (st	: 7(7) rain R	Sequences: KS4594) GN=(7(7) groL Pl	E=3 SV=1	
1. <u>C0Q6</u> 60 k Query	A2 Mass: S Da chaperonin 7 Observed	57421 Sco 1 OS=Salmone Mr(expt)	ore: 958 ella paraty Mr(calc)	Matches: phi C (st ppm	: 7(7) rain R Miss Sc	Sequences: KS4594) GN=0 core Expect	7(7) grol P : Rank	E=3 SV=1 Unique	Peptide
1. <u>C0Q6</u> 60 k Query	A2 Mass: 5 Da chaperonin 7 Observed 1 1567.9088	57421 Sco 1 OS=Salmone Mr(expt) 1566.9015	ore: 958 ella paraty Mr(calc) 1566.8730	Matches: phi C (st ppm 1 18.2	: 7(7) rain R Miss Sc 0 1	Sequences: KS4594) GN=q core Expect L35 3.1e-11	7(7) grol P Rank 2	E=3 SV=1 Unique	Peptide R.AAVEEGVVAGGGVALIR.V
1. <u>C0Q6</u> 60 k Query	A2 Mass: 5 Da chaperonin Observed 1567.9088 1711.8007	57421 Sco 1 OS=Salmone Mr(expt) 1566.9015 1710.7934	ore: 958 ella paraty Mr(calc) 1566.8730 1710.7696	Matches: phi C (st ppm 1 18.2 13.9	7(7) rain Ri Miss Sc 0 1 1	Sequences: KS4594) GN=0 core Expect 135 3.1e-11 68 0.00025	7(7) groL P Rank 2 1	E=3 SV=1 Unique U	Peptide R.AAVEEGVVAGGGVALIR.V R.QQIEEATSDYDREK.L
1. <u>C0Q6</u> 60 k Query	A2 Mass: Da chaperonin Observed 1567.9088 1711.8007 1845.9352	57421 Soc 1 OS=Salmone Mr(expt) 1566.9015 1710.7934 1844.9279	Dre: 958 ella paraty Mr(calc) 1566.8730 1710.7696 1844.9116	Matches: phi C (st ppm 1 18.2 13.9 8.84	: 7(7) :rain Ri Miss Sc 0 1 1 0 1	Sequences: KS4594) GN=0 core Expect L35 3.1e-11 68 0.00025 L68 2.5e-14	7(7) groL P Rank 2 1 1	E=3 SV=1 Unique U	Peptide R.AAVEEGVVAGGGVALIR.V R.QQIEEATSDYDREK.L K.DTTTIIDGVGEEAAIQGR.V
1. <u>C0Q6</u> 60 k Query	A2 Mass: Da chaperonin Observed 1567.9088 1711.8007 1845.9352 1957.0035	57421 Soc 1 OS=Salmone Mr(expt) 1566.9015 1710.7934 1844.9279 1955.9963	Dre: 958 Alla paraty Mr(calc) 1566.8730 1710.7696 1844.9116 1955.9986	Matches: phi C (st ppm) 18.2 13.9 8.84 -1.21	: 7(7) rain R Miss Sc 0 1 1 0 1 0 1	Sequences: KS4594) GN=0 core Expect L35 3.1e-11 68 0.00025 L68 2.5e-14 L00 1.3e-07	7(7) groL P Rank 2 1 1	E=3 SV=1 Unique U U U U	Peptide R.AAVEEGVVAGGGVALIR.V R.QQIEEATSDYDREK.L K.DTTTIIDGVGEEAALQGR.V R.QIVINCGEESVVANTVK.G
1. <u>C0Q6</u> 60 k Query	A2 Mass: Da chaperonin Observed 1567.9088 1711.8007 1845.9352 1957.0035 2010.9506	57421 Soc 1 OS=Salmone Mr(expt) 1566.9015 1710.7934 1844.9279 1955.9963 2009.9433	ore: 958 ella paraty Mr(calc) 1566.8730 1710.7696 1844.9116 1955.9986 2009.9438	Matches: phi C (st ppm) 18.2 13.9 8.84 -1.21 -0.24	7(7) Train Ri Miss Sc 0 1 1 0 1 0 1 1 1	Sequences: KS4594) GN=0 core Expect 135 3.1e-11 68 0.00025 168 2.5e-14 100 1.3e-07 103 6.3e-08	7(7) groL P Rank 2 1 1 1 1	E=3 SV=1 Unique U U U U	Peptide R.AAVEEGVVAGGGVALIR.V R.QQIEEATSDYDREK.L K.DTTTIIDGVGEEAAIQGR.V R.QIVLNCGEEPSVVANTVK.G R.EIELEDKFENMAQAVK.E
1. <u>C006</u> 60 k Querr	A2 Mass: Da chaperonin r Observed 1567.9088 1711.8007 1845.9352 1957.0035 2010.9506 2723.4514	57421 Soc 1 OS=Salmone Mr (expt) 1566.9015 1710.7934 1844.9279 1955.9963 2009.9433 2722.4441	Dre: 958 Ella paraty Mr(calc) 1566.8730 1710.7696 1844.9116 1955.9986 2009.9438 2722.4939	Matches: phi C (st ppm 1 18.2 13.9 8.84 -1.21 -0.24 -18.26	7(7) rain Ri Miss Sc 0 1 1 0 1 1 1 0 1	Sequences: KS4594) GN=(bore Expect 135 3.1e-11 68 0.00025 168 2.5e-14 100 1.3e-07 103 6.3e-08 195 3.9e-17	7(7) groL P Rank 2 1 1 1 1 1	E=3 SV=1 Unique U U U U	Peptide R.AAVEEGVVAGGGVALIR.V R.QQIEEATSDYDREK.L K.DTTTIIDGVGEEAAIQGR.V R.QIVLNCGEEPSVVANTVK.G R.EIELEDKFENMGAQMVK.E K.AGKPLLIIAEDVEGEALATLVVNTMR.G

Masc	ot Search Results	
User Email Search title MS data file Database Timestamp Enzyme Fixed modifications Variable modifications Mass values Protein Mass Protein Mass Protein Mass Fragment Mass Tolerance Fragment Mass Tolerance Max Missed Cleavages Instrument type Number of queries	: tf2013 : Utraflex ProteinID : 1322932305422654.mgf : NCBInr 20101130 (12348165 sequences: 4221604734 residues) : 27 Feb 2013 at 12:19:27 GWT : Trypsin : Carbanidomethyl (C) : Oxidation (M) : Monoisotopic : Unrestricted : t 100 ppm : t 0.5 Da : 1 : MALDI-TOF-TOF : 10	
Protein hits	: <u>gi 194445549</u> glucosaminefructose-6-phosphate aminotransferase [Salmonella enterica subsp. enterica serovar Newport str. SL25	4]

Select Summary Report

Forn	nat As	Select Summa	ary (protein hits) 🔻					Hel	n	
		Significance th	reshold p< 0.0	5 1	Max. num	ber of	hits AU	то			
		Standard scori	ing 🖲 MudPI	T scoring 🔘	lons score	or exp	pect cut	off 0.05	Sho	ow sub-se	ets 0
		Show pop-ups	s 💿 Suppress	pop-ups 💿					Rec	quire bold	l red 🗵
Re-S	gi 194 glucos	All quent (445549 M (aminefruct)	dass: 66966 ctose-6-phos	Score: 1	low homo	atche rase	nreshold s: 10(10) Seo	dow iden quences nterica	s: 10(10))), enteric
	Query	Observed	Mr(expt)	Mr(calc)	ppm	Miss	Score	Expect	Rank	Unique	Peptide
	<u>1</u>	1227.7046	1226.6973	1226.6870	8.39	0	85	5e-05	5 1		R.DVAEI
	2	1652.8660	1651.8587	1651.8457	7.85	0	129	1.9e-09	1		R.FIFLE
	3	1848.8552	1847.8479	1847.8472	0.40	0	182	7.9e-15	5 1		R.GYDSAG
	4	2105.0908	2104.0835	2104.0987	-7.19	0	175	3.2e-14	1		K.ELGYLG
	5	2128.1006	2127.0933	2127.1073	-6.56	0	149	1.5e-11	1		K.GLDASI
	6	2169.0906	2168.0833	2168.1014	-8.36	1	70	0.0011	1		R. IEALAE
	7	2178.0835	2177.0762	2177.0998	-10.82	0	162	7.6e-13	8 1		R.NSLMIT
	8	2222.0540	2221.0467	2221.0620	-6.88	0	120	1.2e-08	1	υ	K.VEHIQI
	9	2389.0854	2388.0782	2388.1096	-13.17	0	168	1.5e-13	3 1		R.YWFESL
	10	2855.4915	2854.4842	2854.5263	-14.74	0	231	7e-20	1		R.SGSPLV

Mascot Search Results

User	1	tf2014
Email	4	
Search title	4	Ultraflex_ProteinID
MS data file	4	13229323905428286.mgf
Database	4	Uniprot sprot20140319 (542782 sequences; 193019802 residues)
Timestamp	4	2 Apr 2014 at 14:10:03 GMT
Enzyme	4	Trypsin
Fixed modifications	4	Carbamidomethyl (C)
Variable modifications	4	Deamidated (NQ), Oxidation (M)
Mass values	4	Monoisotopic
Protein Mass	4	Unrestricted
Peptide Mass Tolerance	4	± 100 ppm
Fragment Mass Tolerance	e:	± 0.5 Da
Max Missed Cleavages	4	1
Instrument type	4	MALDI-TOF-TOF
Number of queries	4	10
Protein hits	4	P32797 Cell division control protein 13 OS=Saccharomyces cerevisiae (strain ATCC 204508 / S288c) GN=CDC13 PE=1

SV=2

Select Summary Report

5

	ieip
Significance threshold p< 0.05 Max. number of hits AUTO	
Standard scoring MudPIT scoring Ions score or expect cut-off 0.05 Sh	show sub-sets 0
Show pop-ups 💿 Suppress pop-ups 💿 Re	lequire bold red 🗵
Preferred taxonomy All entries	

1. <u>P32797</u> Mass: 105978 Score: 287 Matches: 4(4) Sequences: 4(4) Cell division control protein 13 OS=Saccharomyces cerevisiae (strain ATCC 204508 / S288c) GN=CDC13 PE=1 SV=2 Query Observed Mr(expt) Mr(calc) ppm Miss Score Expect Rank Unique Peptide <u>5</u> 1411.8245 1410.8172 1410.8082 6.37 0 54 0.00052 1 U K.ISDILLLEKPDR.I 7 1779.0216 1778.0124 1.07 0 63 0.00052 1 U K.IVFOVNCIEIVIPTR.E <u>8</u> 1808.9399 1807.9327 1807.9316 0.61 0 103 7.7e-08 1 U K.AISYEQLSLASVGSVER.L <u>10</u> 2967.5725 2966.5562 2966.5324 11.1 0 70 0.0001 1 U R.IIAIEYHASDLCFHITNELPLLQTR.G

Mascot Search Results

User	۰.	tf2013
Email	4	
Search title	4	Ultraflex ProteinID
MS data file	÷	13229323905424423.mgf
Database	۰.	NCBInr 20101130 (12348165 sequences; 4221604734 residues)
Timestamp	÷	22 Aug 2013 at 11:02:04 GMT
Enzyme	÷	Trypsin
Fixed modifications	۰.	Carbamidomethyl (C)
Variable modifications	۰.	Oxidation (M)
Mass values	÷	Monoisotopic
Protein Mass	÷	Unrestricted
Peptide Mass Tolerance	۰.	± 100 ppm
Fragment Mass Tolerance	::	± 0.5 Da
Max Missed Cleavages	÷	1
Instrument type	τ.	MALDI-TOF-TOF
Number of queries	۰.	10
Protein hits	÷	gi 2493643 RecName: Full=60 kDa chaperonin; AltName: Full=Protein Cpn60; AltName: Full=groEL protein
		gi 43268 unnamed protein product [Escherichia coli]
		gi 15598750 bifunctional UDP-glucuronic acid decarboxylase/UDP-4-amino-4-deoxy-L-arabinose formyltransferase [Pseudomonas aeruginosa PAO1]

Select Summary Report

1.

Format.Aa	Select Summary (protein hits) ·		Help
	Significance threshold p< 0.05	Max. number of hits AUTO	
	Standard scoring @ MudPIT scoring ()	loss score or expect cut-off 0.05	Show sub-sets 0
	Show pop-ups @ Suppress pop-ups ①	Require bold red 🗵	
	Preferred taxonomy All entries	-	

ReSearch
 Al queries
 O Unassigned
 Below homology threshold
 Below identity threshold

 gli2493643
 Mass: 57764
 Score: 803
 Matches: 6(6)
 Sequences: 6(6)

 RecEmen: Full=60 kDs chaperoning AltSame: Full=Protein Opn60: AltSame: Full=groEL protein
 Desty
 Observed Mr(expt)
 Mr(calc)
 ppm Miss Score Expect Rank Unique Peptide

 1 1011.5300
 1610.5325
 1010.5145
 9.94
 0
 67
 0.0035
 1
 R. VERALMATLA

 5 1454.4401
 1453.6329
 1633.6321
 0.53
 0
 100
 1.20-06
 R. QUERATSDIDE.E

 6 1557.0F83
 1566.9710
 -1.23
 0
 153
 6.16-12
 R. AVYERDYNALDATL.E

 8 1865.0F04
 1566.8730
 -1.23
 0
 153
 AltS.N=04
 1.80
 R. TTTIDOWNERALSDER.V

 2 7723.5120
 2722.5047
 2722.4939
 3.97
 0
 155
 2.8-12
 R. AVXEDVERDALATLYNYFMS.0

 10
 2851.3542
 2850.3230
 9.11
 152
 5.9-15
 0
 K. KNYITYEDYTOLOGERLYNYKENARGYNE.0

Mascot Search Results

User	÷	tf2014
Email	:	
Search title	÷	Ultraflex ProteinID
MS data file	÷	13229323905427974.mgf
Database	:	Uniprot sprot20140219 (542503 sequences; 192888369 residues)
Timestamp	÷	19 Mar 2014 at 12:26:07 GMT
Enzyme	÷	Trypsin
Fixed modifications	÷	Carbamidomethyl (C)
Variable modifications	÷	Deamidated (NQ), Oxidation (M)
Mass values	÷	Monoisotopic
Protein Mass	÷	Unrestricted
Peptide Mass Tolerance	÷	± 100 ppm
Fragment Mass Tolerance	÷	± 0.5 Da
Max Missed Cleavages	÷	1
Instrument type	÷	MALDI-TOF-TOF
Number of queries	÷	10
Protein hits	÷	Q07921 Telomere length regulation protein TEN1 OS=Saccharomyces cerevisiae (strain ATCC 204508 / S288c)

Select Summary Report

믔

Format As Select Summary (protein hits)		Help
Significance threshold p< 0.05	Max. number of hits AUTO	
Standard scoring MudPIT scoring 	Ions score or expect cut-off 0.05	Show sub-sets 1
Show pop-ups $\ $ Suppress pop-ups $\ $		Require bold red 🗹
Preferred taxonomy All entries	▼	

Re-Search

All queries
Unassigned
Below homology threshold
Below identity threshold

1. <u>Q07921</u> Mass: 18878 Score: 418 Matches: 6(6) Sequences: 6(6)

Telomere length regulation protein TEN1 OS-Saccharomyces cerevisiae (strain ATCC 204508 / S288c) GN-TEN1 PE-1 SV=1

Query	Observed	Mr(expt)	Mr(calc)	ppm	Miss	Score	Expect	Rank	Unique	Peptide
1	1041.5352	1040.5279	1040.5365	-8.28	0	58	0.0019	1	U	R.LVDFLFCK.S
4	1127.6454	1126.6381	1126.6346	3.10	0	76	2.8e-05	1	υ	R.LINGQVLDVR.I
5	1157.5615	1156.5542	1156.5522	1.80	0	51	0.012	1	υ	K.GFFCQMIVR.N
7	1201.6343	1200.6270	1200.6251	1.58	1	67	0.00032	1	U	R.LFSAHRDDLR.L
8	1746.7451	1745.7378	1745.7461	-4.72	0	54	0.0048	1	U	R.NLHCLNSTNGAEEMR.L
10	2359.1868	2358.1795	2358.2412	-26.18	0	111	9.5e-09	1	U	R.IGVWYGIHQSPPIFEIIDFK.I

Mascot Search Results

User : tf2013 Email : Search title : Ultraflex ProteinID MS data file : 1322932305422652.mgf Database : NCBInr 20101130 (12348165 sequences; 4221604734 residues) Timestamp : 27 Feb 2013 at 12:05:48 GMT Enzyme : Trypsin Fixed modifications : Carbanidomethyl (C) Variable modifications : Oxidation (M) Mass values : Monoisotopic Protein Mass Tolerance : t 100 ppm Fragment Mass Tolerance : t 100 ppm Fragment Mass Tolerance : t 0. Da Max Missed Cleavages : 1 Instrument type : MALDI-TOF-TOF Number of queries : 10 Protein hits : gil1422648 Chain Å, Conformational Variability In The Refined gil13003844 503 ribosomal protein L2 [Escherichia coli 0157:87



GN=TEN1 PE=1 SV=1

: 1 cross Da
: 1
: 1
: MALDI-TOP-TOP
: 0
: gi|1421648 Chain A, Conformational Variability In The Refined Structure Of The Chaperonin Groel At 2.8 Angstrom Resolution
gi|1503644 Structure Structure Of The Chaperonin Groel At 2.8 Angstrom Resolution
gi|1503625 FKBF-type peptidyl-prolyl cis-trans isomerase [Escherichia coli 0157:H7 EDL933]
gi|43268 unnamed protein product [Escherichia coli]

Select Summary Report

Format As	Select Summ	ary (protein hits)	-					He	<u>elp</u>				
	Significance th	reshold p< 0.0	5 1	Max. num	ber of l	uits AUT)						
	Standard scor	ing 🖲 MudPl	F scoring 💿 🛛	lons score	or exp	ect cut-o	ff 0.05	Sh	iow sub-se	ts O			
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		acional vali	ability in	THE REL	incu .	structu:	re or r	ne cn	aperonin	Groel A	At 2.8 A	ngacrom	Resolution
Que	ry Observed	Mr(expt)	Mr(calc)	ppm	Miss	Score	Expect	Rank	Unique	Peptide	at 2.8 A	ngacrom	Resolution
Que	ry Observed <u>4</u> 1567.8878	Mr(expt) 1566.8805	Mr(calc) 1566.8730	ppm 4.84	Miss 0	Score 134 5	Expect	ne Ch Rank 1	Unique	Peptide R.AAVEE	at 2.8 Al SGVVAGGGV	ALIR.V	Resolution
Que	ry Observed <u>4</u> 1567.8878 <u>8</u> 1845.9149	Mr (expt) 1566.8805 1844.9076	Mr(calc) 1566.8730 1844.9116	ppm 4.84 -2.14	Miss 0 0	Score 134 5 148 2	Expect .5e-10	Rank 1 1	Unique Unique	Peptide R.AAVEE K.DTTTI	AU 2.8 A GVVAGGGV IDGVGEEA	ALIR.V	Resolution

Masco	t Search Results
User	tf2013
Search title	Ultraflex ProteinID
MS data file Database	13229323905422655.mgf NCBInr 20101130 (12348165 sequences; 4221604734 residues)
Timestamp Enzyme	27 Feb 2013 at 12:20:58 GMT
Fixed modifications	Carbamidomethyl (C)
Mass values	Monoisotopic
Protein Mass Peptide Mass Tolerance	Unrestricted ± 100 ppm
Fragment Mass Tolerance	± 0.5 Da
Instrument type	MALDI-TOF-TOF
Number of queries Protein hits	10 gil15803765 505 ribosomal protein L13 [Escherichia coli 0157:H7 EDL933]

Select Summary Report

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Form	nat As	Select Summa	ary (protein hits)	-					He	<u>elp</u>	
		Significance the	reshold p< 0.0	5	Max. num	iber of	hits AU	то			
		Standard scori	ng 🖲 MudPl	T scoring 💿	Ions score	e or exp	pect cut	-off 0.05	Sh	ow sub-se	ets 0
		Show pop-ups	Suppress	pop-ups 💿					Re	quire bold	Ired 🗵
Re-Search Mass: 16009 Score: 446 Matches: 5(5) Sequences: 5(5) 											
1.	<u>gi 15</u> 505 r:	<u>803765</u> Ma ibosomal pro	ss: 16009 tein L13 [E	Score: 4 Scherichia	46 Ma coli O1	tches	: 5(5) EDL93	Sequen	ces:	5(5)	
1.	<u>gi 15</u> 505 r: Query	803765 Ma ibosomal pro Observed	ass: 16009 tein L13 [E Mr(expt)	Score: 4 Scherichia Mr(calc)	46 Ma coli O1 ppm	tches 57:H7 Miss	: 5(5) EDL93 Score	Sequen 3] Expect	ces: Rank	5(5) Unique	Peptide
1.	<u>gi 15</u> 50S r: Query <u>2</u>	803765 Ma ibosomal pro Observed 1153.5504	ass: 16009 tein L13 [E Mr(expt) 1152.5431	Score: 4 Scherichia Mr(calc) 1152.5452	46 Ma coli 01 ppm -1.75	tches 57:H7 Miss 0	: 5(5) EDL93 Score 66	Sequen 3] Expect 0.0035	ces: Rank 1	5(5) Unique	Peptide R.DWYVVDATGK.T
1.	gi 15 50S r: Query 2 4	803765 Ma ibosomal pro Observed 1153.5504 1195.5767	uss: 16009 otein L13 [E Mr(expt) 1152.5431 1194.5694	Score: 4 Scherichia Mr(calc) 1152.5452 1194.5703	46 Ma coli 01 ppm -1.75 -0.75	tches 57:H7 Miss 0 0	: 5(5) EDL93 Score 66 62	Sequen 3] Expect 0.0035 0.0092	ces: Rank 1 1	5(5) Unique	Peptide R.DWYVVDATGK.T K.QATFEEMIAR.R
1.	gi 153 505 r: Query 2 4 6	803765 Ma ibosomal pro Observed 1153.5504 1195.5767 1481.7600	ass: 16009 Dtein L13 [E Mr(expt) 1152.5431 1194.5694 1480.7527	Score: 4 Scherichia Mr(calc) 1152.5452 1194.5703 1480.7575	46 Ma coli 01 ppm -1.75 -0.75 -3.24	Atches 57:H7 Miss 0 0 0	: 5(5) EDL93 Score 66 62 98	Sequen [3] Expect 0.0035 0.0092 2.4e-06	ces: Rank 1 1 1	5(5) Unique U	Peptide R.DWYVVDATGK.T K.QATFEEMIAR.R K.VYYHHTGHIGGIK.Q
1.	gi 153 505 r: Query 2 4 6 9	803765 Ma ibosomal pro Observed 1153.5504 1195.5767 1481.7600 2103.9939	ss: 16009 tein L13 [F Mr(expt) 1152.5431 1194.5694 1480.7527 2102.9866	Score: 4 Scherichia Mr(calc) 1152.5452 1194.5703 1480.7575 2103.0134	46 Ma coli 01 ppm -1.75 -0.75 -3.24 -12.71	1tches 57:H7 Miss 0 0 0 0	: 5 (5) EDL93 Score 66 62 98 118	Sequen [3] Expect 0.0035 0.0092 2.4e-06 1.6e-08	ces: Rank 1 1 1	5(5) Unique U	Peptide R.DWYVVDATGK.T K.QATFEEMIAR.R K.VYYHHTGHIGGIK.Q K.VYAGNEHNHAAQQPQVLDI

Masco	ot Search Results	
User Email	: 12013	
Search title	: Ultraflex ProteinID	
Database	NCBInr 20131011 (3022917 seguences: 11501319831 residues)	
Timestamp	: 7 Nov 2013 at 12:29:08 GMT	
Enzyme	: Trypein	
Fixed modifications	: Carbasidomethyl (C)	
Variable modifications	: Oxidation (N)	
Mass values	1 Monolsotopic	
Protein Mass	Unrestricted	
Peptide Mass Tolerance	i s 100 ppm	
Fragment Mass Tolerano	s: s 0.5 Da	
Max Missed Cleavages	11	
Instrument type	: NALDI-TOF-TOF	
Number of queries	: 20	
Protein hits	: gij121697 RedName: Full=Glutathione S-transferase class-mu 26 kDa isoryme; Short=GST 26; AltHame: Full=Sj26	antigen; AltName: Full=5;GST



Select Summary Report

FormatAa	Select Summary (protein hits) ·		Help
	Significance threshold p< 0.05	Max. number of hits 1	
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	Show pop-ups $\ @$ Suppress pop-ups $\ @$		Require bold red 🗵
	Preferred taxonomy All entries	•	

-1. <u>gi|121697</u> Mass: 25710 Score: 744 Matches: 10(10) Sequences: 8(8) RecName: Full=Glutathione S-transferase class-mu 26 kDa isozyme; Short=GST 26; AltName: Full=Sj26 antigen; AltName: Full=SjGST

Query	Observed	Mr (expt)	Mr(calc)	ppm	Miss	Score	Expect	Rank	Unique	Peptide
1	963.5206	962.5133	962.5225	-9.60	0	61	0.05	1	υ	M.SPILGYWK.I
2	1032.5847	1031.5774	1031.5797	-2.22	0	66	0.015	1	υ	K.LTQSMAIIR.Y
6	1182.6963	1181.6890	1181.6768	10.3	1	73	0.0017	1	υ	K.RIEAIPQIDK.Y
<u>9</u>	1516.8296	1515.8223	1515.7966	16.9	0	105	1.4e-06	1	υ	R.AEISMLEGAVLDIR.Y
10	1532.8296	1531.8223	1531.7916	20.1	0	(101)	3.5e-06	1	υ	R.AEISMLEGAVLDIR.Y
13	1801.9624	1800.9551	1800.9403	8.21	1	96	1e-05	1	υ	K.ERAEISMLEGAVLDIR.Y
14	1817.9521	1816.9449	1816.9353	5.29	1	(60)	0.037	1	υ	K.ERAEISMLEGAVLDIR.Y
16	2269.1179	2268.1106	2268.1313	-9.12	1	114	1.3e-07	1	υ	R.LLLEYLEEKYEEHLYER.D
17	2326.1321	2325.1248	2325.1331	-3.57	0	135	8e-10	1	υ	K.YIAWPLQGWQATFGGGDHPPK
20	2357.1794	2356.1722	2356.1991	-11.42	1	95	8.6e-06	1	σ	K. KFELGLEFPNLPYYIDGDVK. L
Mascot Search Results

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	Preferred tanonomy: All entries	7.e	

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 Sequences 223)

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 Mr(sal)
 pm Miss Source Expect Mask Source E t.,

Mascot Search Results

User		tf2014
Email	÷	
Search title	÷	Ultraflex_ProteinID
MS data file	÷	13229323905428029.mgf
Database	÷	Uniprot sprot20140219 (542503 sequences; 192888369 residues)
Timestamp	÷	21 Mar 2014 at 10:18:42 GMT
Enzyme	÷	Trypsin
Fixed modifications	÷	Carbamidomethyl (C)
Variable modifications	\$	Deamidated (NQ), Oxidation (M)
Mass values	4	Monoisotopic
Protein Mass	÷	Unrestricted
Peptide Mass Tolerance	÷	± 100 ppm
Fragment Mass Tolerance	е:	± 0.5 Da
Max Missed Cleavages	÷	1
Instrument type	÷	MALDI-TOF-TOF
Number of queries	4	10
Protein hits	÷	<u>Q9U639</u> Heat shock 70 kDa protein cognate 4 OS=Manduca sexta PE=2 SV=1
		Q05746 Heat shock 70 kDa protein OS=Plasmodium cynomolgi (strain Berok) PE=2 SV=1
		Q4R4T5 Heat shock protein HSP 90-beta OS=Macaca fascicularis GN=Hsp90ab1 PE=2 SV=1

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FormatAs	Select Summa	ary (protein hits) •					1	ieip			
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Re-Search . <u>Q9U63</u> Heat	 All querie Mass: 7 shock 70 kDa Observed 	nomy Allentri s © Unass '1672 Soc protein co Mr (evot)	es signed © B pre: 234 pgnate 4 OS Mr(calc)	elow hom Matche: =Manduca	nology th s: 3(3 a sext Miss 9	hreshold) Sec a PE=2	1 © Be quences: 2 SV=1 Expect	low id 3(3) Bank	entity three	shold		
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Re-Search . <u>Q9U63</u> Heat Query <u>1</u> <u>7</u>	 All querie All querie Mass: 7 shock 70 kDa Observed 1197.6835 1480.7924 	nomy All entri- es Unass '1672 Soci protein co Mr (expt) 1196.6762 1479.7851	es signed © B ore: 234 ognate 4 OS Mr(calc) 1196.6553 1479.7470	elow hom Matches =Manducs ppm 17.4 25.7	nology th s: 3(3 a sext Miss 5 0 1	hreshold) Sec a PE=2 Score 66 45	1 © Be quences: 2 SV=1 Expect 0.00032 0.045	low id 3 (3) Rank 1 1	entity thre: Unique	Peptide K.FELTG R.ARFEE	IPPAPR.G LNADLFR.S	5

·	QU5746	Mass: /	5069 50	ore: 182	Matches	: 2(2) seq	uences:	2(2)		
	Heat s	hock 70 kDa	protein O	5=Plasmodium	cynomo	lgi (strain	Berok)	PE=2	SV=1	
	Query	Observed	Mr(expt)	Mr(calc)	ppm	Miss	Score	Expect	Rank	Unique	Peptide
	<u>9</u>	1487.7349	1486.7276	1486.6940	22.6	0	58	0.0024	1		R.TTPSYVAFTDTER.L
	10	2023.0228	2022.0156	2022.0534	-18.73	1	124	5.8e-10	1	υ	K.SIKNAVITVPAYFNDSQR.Q

Appendix 2: Process of creation and transformation of bacculovirus DNA into insect



Figure 1. Attempted purification of Exo1. Flow diagram illustrating the process of creation and transformation of bacculovirus DNA into insect cells.

E-appendices

Note: Appendices 3-6 contain large datasets that would be inappropriate to display in this thesis. They are therefore available electronically and can be requested from the graduate school or directly from Prof David Lydall.

Appendix 3: *stn1-13* growth curves

The growth curve data which was used to carry out quantitative fitness analysis with *stn1-13* deletion and DAmP mutants is available on request from Prof David Lydall. It has been submitted to the graduate school as a PDF but was omitted due to its large size.

Appendix 4: *rfa3-313* growth curves

The growth curve data which was used to carry out quantitative fitness analysis with *rfa3-313* deletion and DAmP mutants is available on request from Prof David Lydall. It has been submitted to the graduate school as a PDF but was omitted due to its large size.

Appendix 5: stn1-13 QFA genetic interaction strength (GIS) scores

The GIS scores that were used to create the *stn1-13* fitness plots seen in Chapter 5 have been submitted to graduate school as an excel spreadsheet, and are available on request from Prof David Lydall.

Appendix 6: rfa3-313 QFA genetic interaction strength (GIS) scores

The GIS scores that were used to create the *rfa3-313* fitness plots seen in Chapter 5 have been submitted to graduate school as an excel spreadsheet, and are available on request from Prof David Lydall.